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Controlled Local Delivery of CTLA-4 Blocking Antibody Induces CD8$^+$ T-Cell–Dependent Tumor Eradication and Decreases Risk of Toxic Side Effects

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Abstract

Purpose: Blockade of CTLA-4 by antibodies has potentiated antitumor T-cell responses in both preclinical models and clinical trials. However, treatment with CTLA-4 blocking antibodies is associated with autoimmune and inflammatory side effects. In this study, we propose a novel administration method for CTLA-4 blocking antibodies as monotherapy.

Experimental Design: We use different preclinical mouse models of cancer to investigate the local administration of CTLA-4 blocking antibody and its effect on cancer progression and the antitumor T-cell response.

Results: By injecting the antibodies in a subcutaneous slow-release delivery formulation in the tumor area, we show that an eight-fold lower dose of antibody is as effective in inducing tumor eradication as systemic delivery. A lower dose and slow release of the antibody results in thousand-fold decreased levels of antibody in the serum, reducing adverse events and the risk of autoimmunity. The main target and effector cells of the CTLA-4 blockade treatment in the studied tumor models are tumor-specific endogenous CD8$^+$ T cells that are capable of eradicating also distant tumors, whereas CD4$^+$ T cells do not play a prominent role in the antibody-mediated tumor eradication.

Conclusions: Injecting CTLA-4 blocking antibody in a slow-release formulation close to the tumor is an effective way of activating the antitumor T-cell response. This administration method is associated with very low serum levels of antibody, which decreases the risk of treatment-induced side effects. These results call for exploration of a similar delivery principle in clinical settings. *Clin Cancer Res; 19(19); 5381–9. ©2013 AACR.*

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 (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-1, MDM2, HER-2/neu). However, even though spontaneous tumor-specific CD8$^+$ T-cell responses have been found, several factors, such as insufficient dendritic cell activation and antigen availability, and tumor-induced immune suppression limit these responses (3, 4). Therapeutic interventions aimed at enhancing the efficacy of antitumor 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. The main costimulatory molecules, B7.1 (CD80) and B7.2 (CD86), are expressed on antigen-presenting cells (APC). Binding of the B7 molecules to CD28, which is constitutively expressed on T cells, provides essential signals for proliferation, survival, and differentiation (5, 6). Negative feedback is provided by binding of the B7 molecules to CTLA-4 (CD152), a family member of CD28. CTLA-4 expression is inducible on conventional T cells and the molecule is constitutively expressed by regulatory T cells. Several mechanisms of CTLA-4 inhibition have been proposed. CTLA-4 expression by activated T cells outcompetes CD28 for B7 ligation, inhibiting the positive activation effect of CD28. This was established in cells with CTLA-4 molecules containing nonfunctional cytoplasmic tails, which were still able to inhibit T-cell responses (7–9). The constitutive expression by Tregs of CTLA-4, implicated in their suppressive phenotype, leads to downregulation of B7

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Translational Relevance

Systemic delivery of CTLA-4 blocking antibodies induces antitumor immune responses in preclinical models and patients 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 CTLA-4 blocking antibody in the lymphoid drainage area of the tumor, which stimulates local but not systemic T cells. Local antitumor $CD8^+$ T-cell activation and tumor eradication associated with thousand-fold lower serum levels of antibody can be obtained. These results indicate an important novel delivery platform for the use of CTLA-4 blocking antibody and conceivably other immune stimulatory therapies in cancer patients.

molecules on APCs and induction of APC-expressed IDO, a metabolic enzyme that catabolizes tryptophan leading to starvation of T cells (10, 11). CTLA-4 signaling has also 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 (12, 13).

Blocking the interaction of CTLA-4 with B7.1 and B7.2 improves antitumor T-cell responses in preclinical tumor models and in cancer patients (14–18). Recently, important clinical results have been obtained in melanoma patients with Ipilimumab, a human monoclonal antibody that binds to and inhibits the function of CTLA-4 (19). This has led to FDA approval of treatment of advanced melanoma with Ipilimumab (Yervoy). Because Ipilimumab lowers the threshold for T-cell activation, its clinical use can be associated with severe autoimmune and inflammatory effects including colitis, dermatitis, uveïtis, and hypophysitis (20–22).

Recently, 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 (23). We now 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 antitumor $CD8^+$ T-cell response and tumor eradication, whereas levels of systemic antibody in serum remain low. The efficacy of the CTLA-4 blockade treatment was dependent on $CD8^+$ T cells whereas $CD4^+$ T cells did not play a major role. Thus, a low dose of CTLA-4 blocking antibody applied locally induces effective tumor eradication by directly enhancing tumor-specific $CD8^+$ T-cell responses.

Materials 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.

Tumor experiments

MC-38 tumor cells expressing OVALBUMIN (OVA) (MC38-OVA) and MC-38 tumor cells (24) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) supplemented with 4% fetal calf serum (FCS), 50 μmol/L 2-mercaptoethanol, and 100 IU/mL penicillin/streptomycin. EG7 tumor cells expressing the full-length OVA antigen were cultured in IMDM supplemented with 8% FCS, 50 μmol/L 2-ME (β-mercaptoethanol), 2 mmol/L glutamine, and 100 IU/mL penicillin supplemented with 400 μg/mL G418 (Gibco). The tumor cells (0.5×10^6 for MC-38-OVA, 0.2×10^6 for MC-38, and 0.1×10^6 for EG7) were injected subcutaneously into 8- to 12-week-old female mice in 200 μL of PBS. Treatment was started 6 to 10 days after tumor inoculation, when palpable tumors were present. Mice were sacrificed when tumors reached a size of 1,000 mm^3 because of ethical reasons.

Flow cytometry

Single-cell suspensions of spleens underwent erythrocyte lysis, and were subsequently stained with CD8α (clone 53-6.7), CD4 (clone RM4-5), and CD3ε (clone 145-2C11) monoclonal antibodies (mAb; BD Bioscience) and $OVA_{257-264}$-loaded H-$2K^b$ tetramers. Cells were analyzed on a FACScalibur (Becton Dickinson) and data analysis was conducted with Flowjo (Tree Star).

Blocking CTLA-4 antibody treatment

Hybridoma cells producing CTLA-4 blocking Ab (clone 9H10; ref. 7) 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 intraperitoneally 200 μg mAb (high dose) in PBS on day 0 and day 3 or received 50 μg mAb (low dose) at day 0. Mice treated locally with a low-dose CTLA-4 blocking mAb received subcutaneously 50 μg mAb in Montanide on day 0. Montanide/CTLA-4 antibody emulsions were made by mixing antibody in PBS 1:1 with Montanide (Montanide ISA-51, Seppic), and vortexing for 30 minutes.

T-cell depletion

Hybridoma cells producing either depleting CD8 mAb (clone 2.43) or depleting CD4 mAb (clone GK1.5) were cultured in Protein-Free Hybridoma Medium (Gibco), and mAbs were purified using a Protein G column. To deplete $CD8^+$ or $CD4^+$ T cells mice received an intraperitoneally (i.p.) administration of 100 μg anti-CD4 or anti-CD8 antibodies on day −1, 2, 7, 14, and 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 noncompetitive 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 immunoglobulin G.

Serum analyses

Serum samples were taken from mice at several time points after CTLA-4 treatment. ALT and AST analyses were

conducted by the department of Clinical Chemistry of the LUMC according to standard protocols. Auto antibodies were analyzed in serum with the Anti-Nuclear Antibodies-ELISA kit (US Biological) 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 low-dose treatment with 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; ref. 25) was very effective in inducing systemic antitumor immunity without strong systemic side effects. We hypothesized that this administration principle would also be applicable to other immune-modulating antibodies, such as CTLA-4 blocking antibody. To verify this, mice were inoculated subcutaneously with MC38-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 (2 injections of 200 μg i.p.) of CTLA-4 blocking antibody (hamster-anti-mouse CTLA-4 clone 9H10) or were treated locally by receiving one injection of 50 μg antibody in Montanide subcutaneously close to the tumor. Placing of local treatment was between the tumor and the nearest tumor-draining lymph node (LN), as depicted in Fig. 1A. Both the high-dose systemic and low-dose local treatment with CTLA-4 blocking antibody was able to induce tumor eradication compared to nontreated mice (Fig. 1B and C). Montanide alone injected close to the tumor was not capable of eradicating tumors, as we previously described (23). Mice treated with a systemic administration of the low dose, 50 μg, were not able to clear the tumor, and neither was a low dose, 50 μg in Montanide, injected in the contralateral flank of tumor-bearing mice, indicating that this dose is only effective when delivered into the tumor-draining area (data not shown and Fig. 1D). Weekly repeating the locally administered dose of CTLA-4 blocking antibody slightly enhanced the efficacy of the treatment (Supplementary Fig. S1).

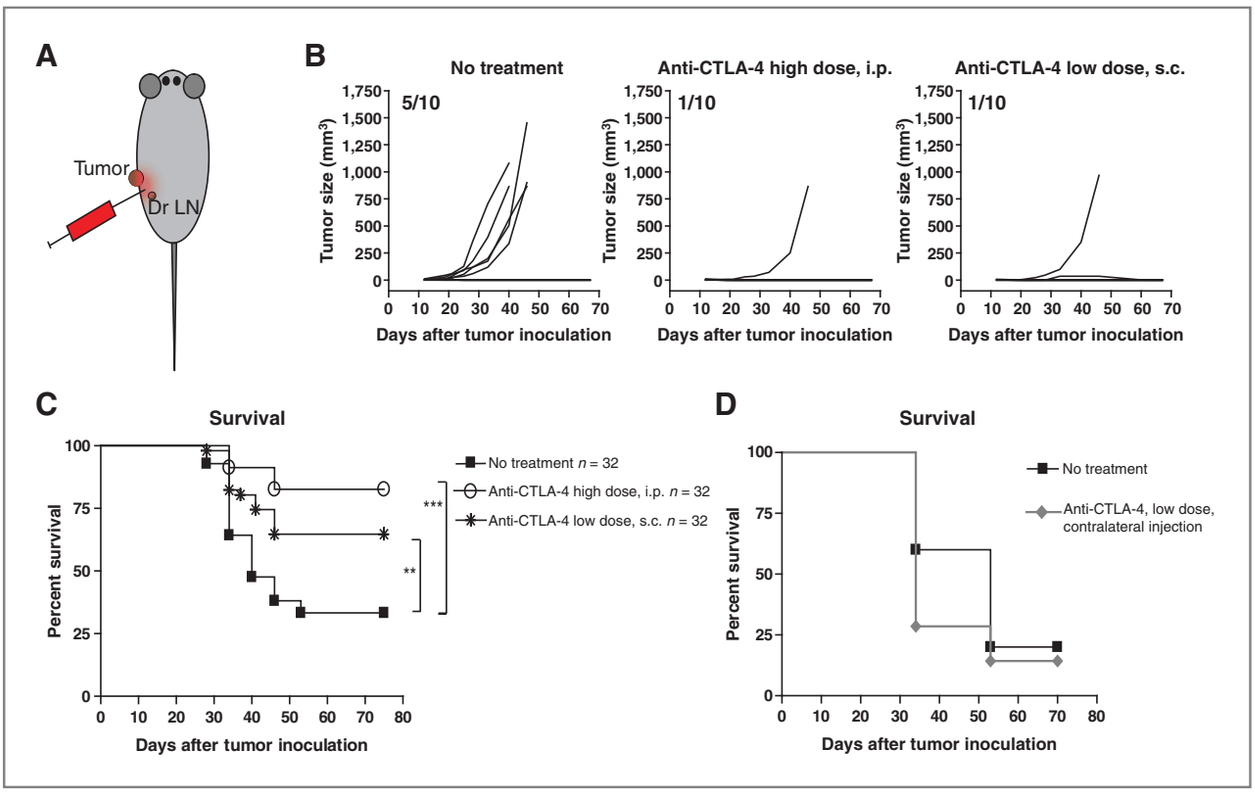

Figure 1. Local treatment with a low dose of CTLA-4 blocking antibody induces effective tumor eradication. Mice bearing palpable MC-38-OVA tumors (0.5–5 mm^3) were treated with 2 intraperitoneal injections with high dose (2 × 200 μg) of CTLA-4 blocking antibody 3 days apart (standard treatment), 1 subcutaneous local injection with low dose (50 μg) CTLA-4 blocking antibody in slow-release agent Montanide ISA-51 or left untreated. Tumor growth was measured twice weekly. A, schematic cartoon of local administration. B, data presented as tumor growth in each mouse, 10 mice per group, indicated in left top corner ratio of mice with tumor per number of mice in the entire group. C, survival curve. Shown are pooled data of 4 independent experiments, 32 mice per group. Kaplan–Meier test revealed significant differences between nontreated group and local treated group or intraperitoneal treated group, $P < 0.002$ (**) and $P < 0.0002$ (***), respectively. D, survival curve. Eight mice per group.

To test whether our administration method would be equally effective in a less immunogenic tumor model without tumor-associated OVA, we treated mice bearing palpable MC-38 tumors with one injection of 50 μg of CTLA-4 blocking antibody in Montanide subcutaneously close to the tumor. As depicted in Fig. 2A and B, mice treated with CTLA-4 blocking antibody were able to eradicate the tumors, whereas untreated mice were not. The local delivery treatment was also effective in mice bearing the more aggressive tumor EG7. Although most mice bearing this tumor could not be cured and eventually succumbed from tumor burden in both groups, local treatment caused significant delay in tumor-outgrowth compared to nontreated mice (Fig. 2C).

Together these results indicate that a local low dose of blocking CTLA-4 treatment has similar tumor-eradicating capacity as high-dose systemic treatment.

Local treatment enhances systemic tumor-specific T-cell responses capable of controlling a distant tumor

To determine whether tumor eradication correlated with enhanced tumor-specific T-cell responses, we analyzed the magnitude of the endogenous $CD8^+$ T-cell response in tumor-bearing mice treated with CTLA-4 blocking antibody. The tumor-specific $CD8^+$ T-cell response, as determined by H-$2K^b$ tetramer staining, in the spleen and blood of mice challenged with MC-38 OVA tumors, was enhanced in mice that underwent either the high systemic dose or the low-dose local treatment as compared to untreated mice (Fig. 3A and data not shown). Similarly, in our EG7 tumor model, we found a significant increase in tumor-specific $CD8^+$ T cells in blood in locally treated tumor-bearing mice, compared to nontreated control mice (Fig. 3B).

The presence of a systemic tumor-specific T-cell response induced by local treatment led us to investigate whether a

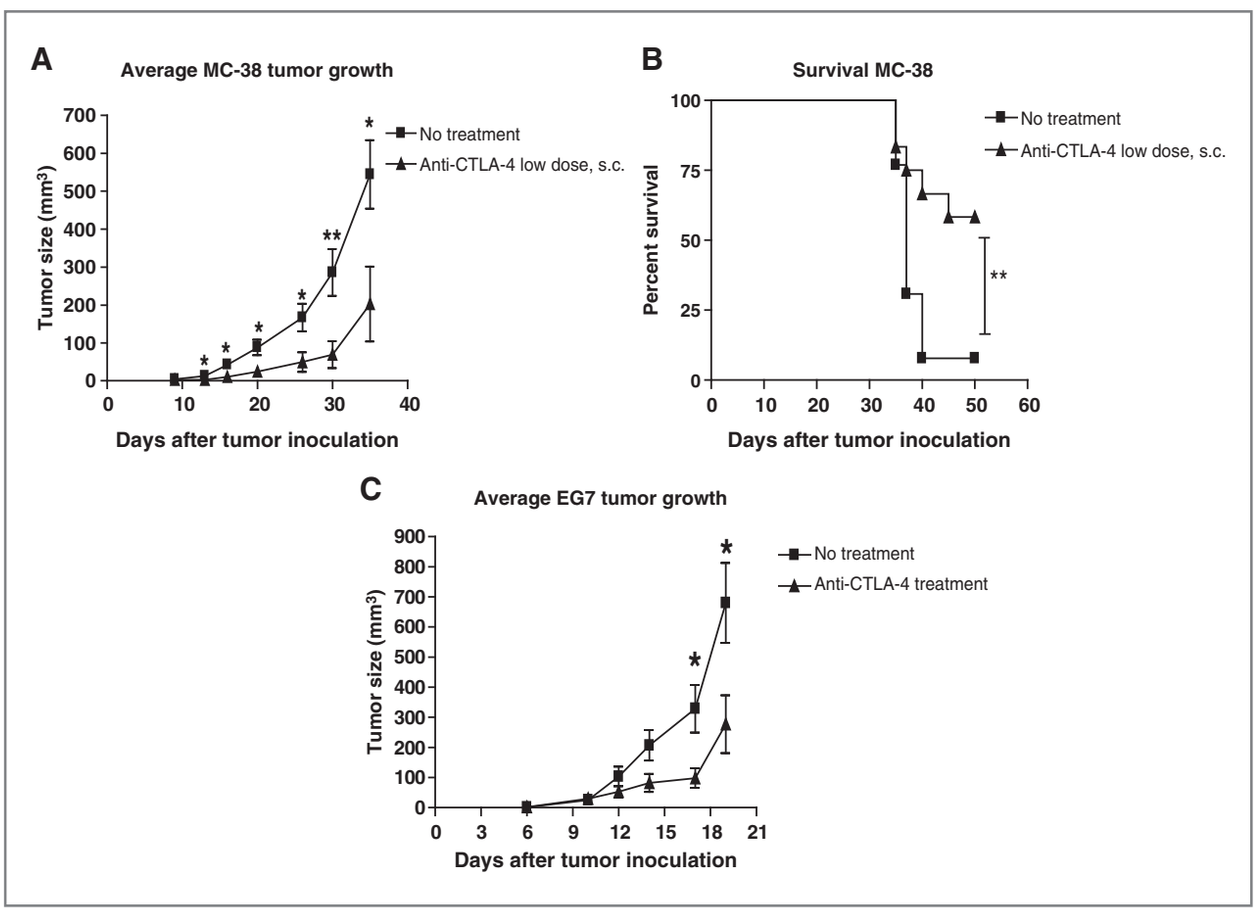

Figure 2. Local treatment with a low dose of CTLA-4 blocking antibody induces effective tumor eradication. Mice bearing palpable MC-38 tumors (0.5–5 mm^3) were treated with 1 subcutaneous local injection with low dose (50 μg) CTLA-4 blocking antibody in slow-release agent Montanide ISA-51 or left untreated. Tumor growth was measured twice weekly. Twelve mice per group, one representative experiment of 2. A, data presented as average tumor growth per group, calculated and depicted until time-point when first mice were sacrificed due to large tumors. At each time-point, Student *T* test was conducted. Significant differences between treated and untreated groups were revealed on all time-points except day 9. $^*P < 0.05$, $^{**}P < 0.01$. B, data of (A) presented as survival curve. Kaplan–Meier test revealed a significant difference between treated and untreated group, $P = 0.008$. Experiment was ended when all surviving mice were completely tumor free. Mice bearing EG7 tumors were treated with 1 subcutaneous local injection with low dose (50 μg) CTLA-4 blocking antibody in slow-release agent Montanide ISA-51 or left untreated. Tumor growth was measured 3 times a week. C, data presented as average tumor growth per group, calculated and depicted until time-point when first mice were sacrificed due to large tumors. At each time-point, Student *T* test was conducted. Significant differences between treated and untreated groups were revealed on day 17 and 19; $^*P < 0.05$ (10 mice per group).

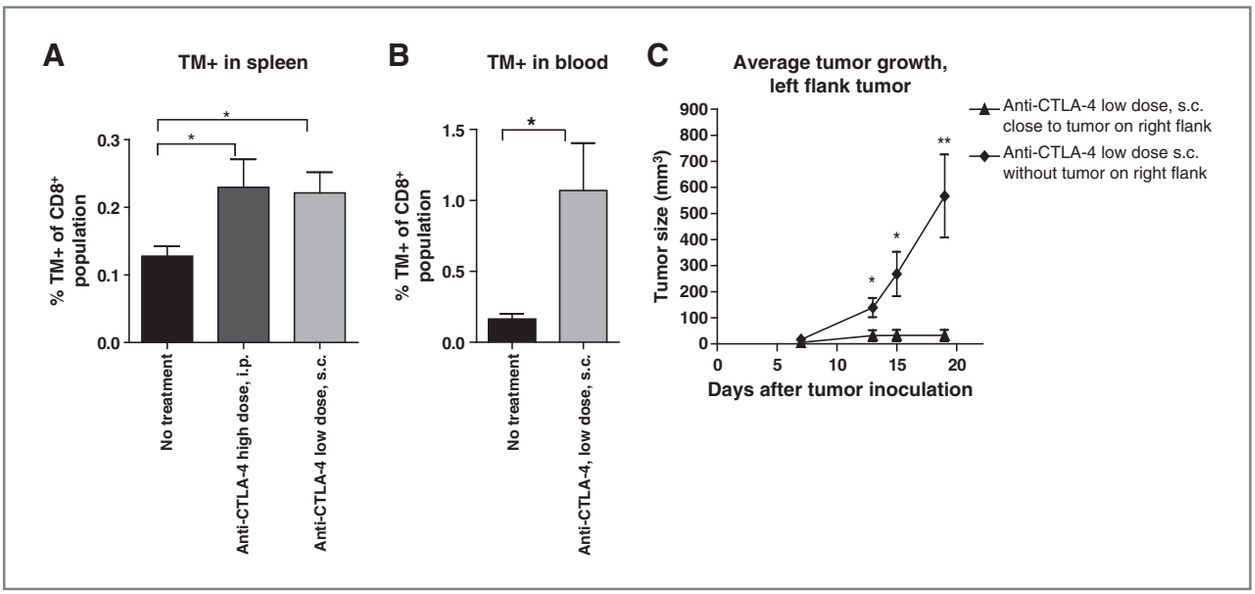

Figure 3. Local treatment with a low dose of CTLA-4 blocking antibody results in an enhanced systemic tumor-specific T-cell response capable of controlling a distant tumor. A, CTL response after low dose, local, treatment with CTLA-4 blocking antibody. Nine days after start of treatment, tetramer$^+$ CD8$^+$ T cells (TM) were analyzed in spleen (mean $\pm$ SE, $n = 10$ mice per group), data pooled of 2 independent experiments. Student *T* test revealed a significant difference between treated groups and nontreated group ($P < 0.05$ for both treated groups). Mice bearing palpable MC-38-OVA tumors on the right flank were treated on the right flank with one injection of 50 μg of CTLA-4 blocking antibody in Montanide, subcutaneously. Control mice were injected with antibody in Montanide in the right flank in the absence of tumor. One day later, MC-38-OVA tumor cells were injected in the left flank of both groups, and tumor size was subsequently measured. B, data presented as average tumor growth per group, calculated and depicted until time-point when first mice were sacrificed due to large tumors. At each time-point, Student *T* test was conducted. Significant differences between treated and untreated groups were revealed on all time-points except day 7. $^*P < 0.05$, $^{**}P < 0.01$ (10 mice per group).

subsequent distant tumor could be eradicated. Mice bearing a tumor on the right flank were treated locally, after which they received a second tumor on the left flank. Control mice received a tumor on the left flank and local treatment on the right flank, where no tumor was present. As is shown in Fig. 3B, the left flank tumor displays a significantly delayed outgrowth in mice treated locally near a tumor compared to control-treated mice, indicating that the T-cell response induced by the local treatment can eradicate distant tumors.

The low-dose treatment did not result in a different effect on local CD8$^+$ T cells or systemic T regulatory cells compared to systemic treatment. Both administration methods resulted in similar numbers of activated tumor-specific T cells in tumor and tumor-draining LNs as analyzed by tetramer- and phenotypic staining of LN and tumor tissues (Supplementary Figs. S2 and S3).

These data show that local treatment with CTLA-4 blocking antibody is proficient in inducing tumor-specific CTL responses and capable of controlling distant tumors.

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

To determine the CTLA-4 blocking antibody levels in the serum, we conducted a hamster antibody-specific ELISA on serum samples, taken at different intervals after start of treatment. As depicted in Fig. 4A, antibody concentrations in the high-dose systemically treated mice were more than 1,000-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 levels 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 considerably lower concentration of antibody in the serum in locally treated mice, we hypothesized that this treatment would be associated with less severe adverse side effects than systemic administration. To determine this, we analyzed the liver enzymes ALT and AST, known to be indicative for tissue damage (26), in serum samples of treated mice at several time-points after administration of the antibodies. As indicated in Fig. 4B 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. Because systemic CTLA-4 blocking treatment in cancer patients can induce serious autoimmune and inflammatory side effects (15), we analyzed the serum levels of antinuclear 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 (27). Liver and kidney from treated mice showed only mild inflammation and no differences between the different treatments were detected. 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).

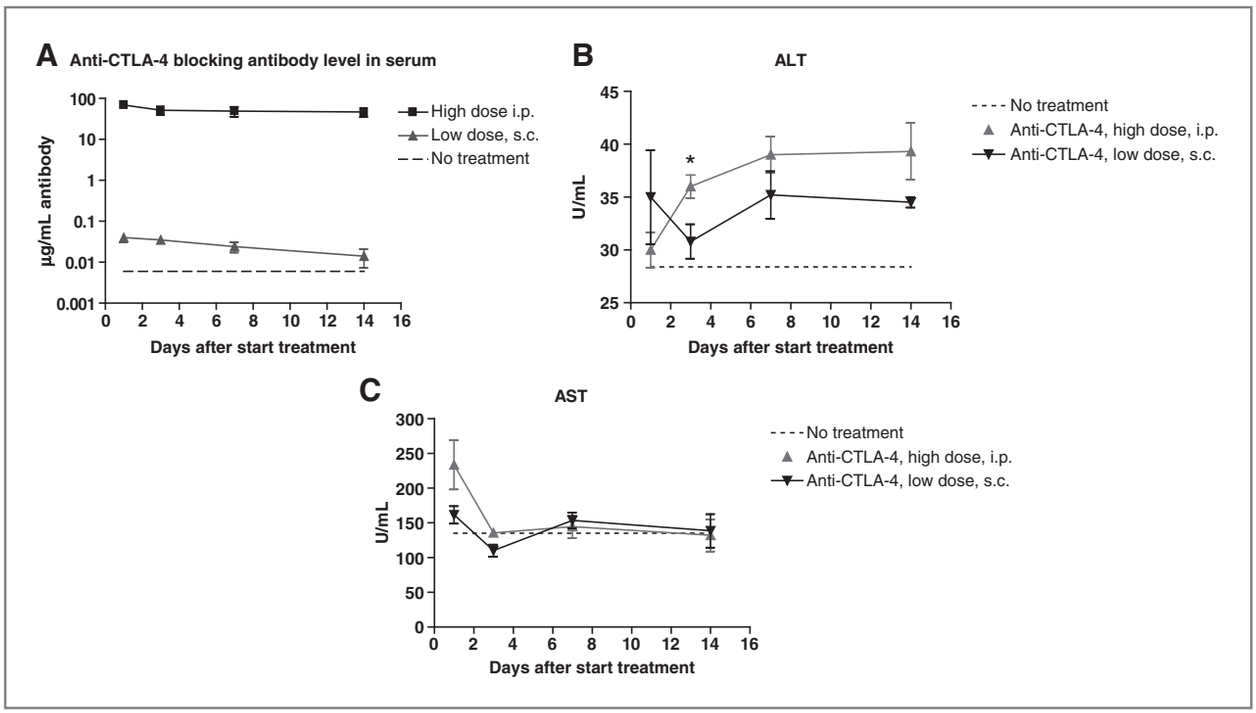

Figure 4. Local treatment with a low dose of CTLA-4 blocking antibody results in decreased treatment induced toxicity as compared to high dose, systemic treatment. Shown are CTLA-4 antibody concentrations and liver enzyme levels in serum in time after treatment with low dose (50 µg) local treatment or high dose (2×200 µg), intraperitoneal treatment. A, antibody concentrations in serum. B, ALT levels in serum. Student *T* test revealed significant difference on day 3 between high-dose intraperitoneal treatment and low-dose local treatment groups, $P = 0.029$. C, AST levels in serum (mean ± SE, $n = 5$ mice per group).

Local treatment depends strictly on induction of tumor-specific CD8$^+$ T-cell responses

Because CD8$^+$ T cells responses were increased after local treatment with CTLA-4 blocking antibody, we assessed whether only CD8$^+$ or also CD4$^+$ T cell populations were important for the efficacy of local CTLA-4 treatment. We injected tumor-bearing mice for 3 weeks with CD8$^+$ or CD4$^+$ T-cell depleting antibodies, starting 1 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 identically to CTLA-4 treatment as the control group, indicating that CD4$^+$ T-cell populations were not involved in tumor eradication in this model (Fig. 5A and B). Together these data show that in our tumor model the main cells responsible for tumor eradication are CD8$^+$ T cells and indicate that CD8$^+$ T cells are the main targets of the CTLA-4 blockade treatment.

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 an endogenous tumor-specific CD8$^+$ T-cell response, capable of tumor eradication. We further show that CTLA-4 treatment can operate directly on CD8$^+$ T cells. 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 autoimmune 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 (28, 29). In these studies, the CTLA-4 blocking antibody treatment was given in combination with CpG, GM-CSF secreting vaccines, or as CTLA-4–secreting cellular vaccines. Here, we show that the local administration is also applicable for monotherapy with CTLA-4 blocking antibody, and that simultaneous use of a slow-release delivery system further decreases the systemic levels of CTLA-4 antibody, thereby reducing risk of adverse side effects. In our study we use Montanide ISA-51, because it has proven to be effective in previous studies with CD40 agonistic antibody and it is safe to use in human subjects. We have not studied the use of other slow-release mechanisms, such as liposomes or PLGA microparticles and cannot rule out that these might be equally effective (30, 31). However, we are convinced that our studied method is more readily applicable to the clinic.

Contrary to other studies that examined the effects of CTLA-4 blocking antibody (16, 28, 32), CD4$^+$ T cells do not play an essential role in our tumor model, as evidenced by

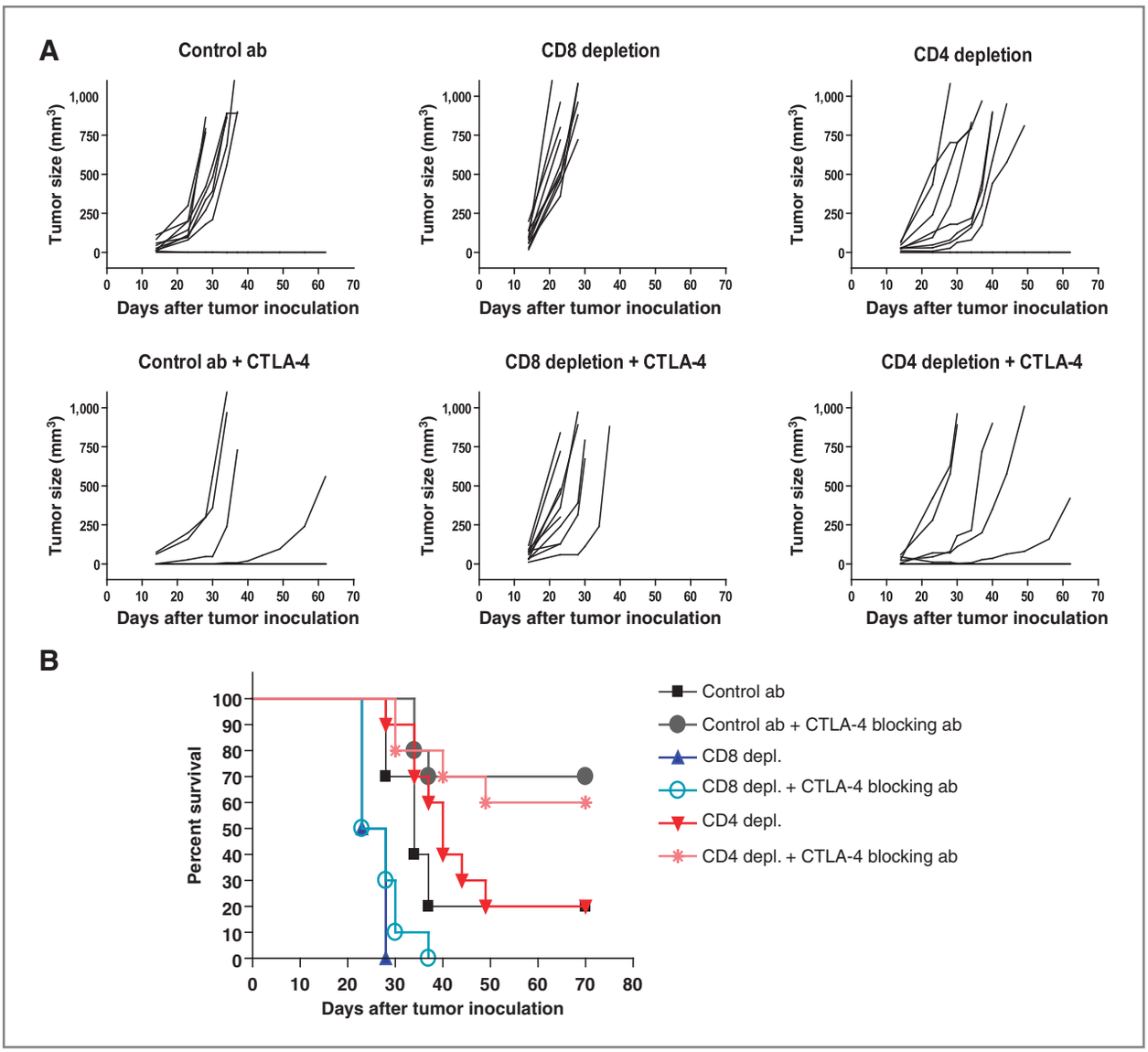

Figure 5. CD8$^+$ T cells are the main effector cells involved in tumor eradication and the main target of local treatment with CTLA-4 blocking antibodies. Mice were depleted of CD8$^+$ or CD4$^+$ T-cell populations, starting 1 day before tumor inoculation, for 3 weeks. 8 days after tumor inoculations, when palpable tumors had formed, treatment was started. Mice bearing palpable MC-38-OVA tumors (0.5–5 mm^3) were treated with 2 intraperitoneal injections with high dose (2 $\times$ 200 μg) of CTLA-4 blocking antibody 3 days apart (standard treatment), 1 subcutaneous local injection with low dose (50 μg) CTLA-4 blocking antibody in slow-release agent Montanide ISA-51 or left untreated. Tumor growth was measured twice weekly. A, data represents tumor growth in each mouse, 10 mice per group. B, survival curve. Data are representative of 2 independent experiments.

the fact that CD4$^+$ T-cell depleted mice showed similar antitumor activity as nondepleted control mice (with and without CTLA-4 blockade). However, we cannot 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. As we detected tumor-specific CD8$^+$ T cells in both tumor-draining lymph nodes and in the tumor itself (Supplementary Fig. S1), we are convinced that our locally administered treatment activates CD8$^+$ T cells in both these locations. We conclude that CD8$^+$ T-cell responses can be augmented directly by CTLA-4 blockade without necessary participation of CD4$^+$ T cells.

CTLA-4 blocking antibody treatment did not lead to an 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 can 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. In addition, the antibody used in mouse studies (hamster-anti-mouse CTLA-4 clone 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, which leads to a decrease in risk of treatment-induced toxicity. The main target cells of CTLA-4 treatment in this model are endogenous tumor-specific $CD8^+$ T cells, which are increased in numbers after treatment and found to be essential for local and distant tumor eradication. This approach lends itself without difficulty to clinical trials, because slow-release methods such as Montanide ISA-51 are safe in human individuals (33), and appropriate FDA-approved human CTLA-4 blocking antibodies are available (19).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.F. Fransen, F. Ossendorp, R. Arens, C.J.M. Melief
Development of methodology: M.F. Fransen, R. Arens
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.F. Fransen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.F. Fransen, Tetje C van der Sluis, R. Arens
Writing, review, and/or revision of the manuscript: M.F. Fransen, T.C. van der Sluis, F. Ossendorp, R. Arens, C.J.M. Melief
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.C. van der Sluis
Study supervision: F. Ossendorp, R. Arens, C.J.M. Melief

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