**In vivo Administration of Artificial Antigen-Presenting Cells Activates Low-Avidity T Cells for Treatment of Cancer**

Stefano Ugel,1 Alessia Zoso,3 Carmela De Santo,2 Yu Li,3 Iaria Marigo,1 Paola Zanovello,1,2 Elisa Scarselli,1 Barbara Cipriani,4 Mathias Oelke,3 Jonathan P. Schneck,3 and Vincenzo Bronte2

1Department of Oncology and Surgical Sciences, Oncology Section and 2Istituto Oncologico Veneto, Istituto di Ricovero e Cura a Carattere Scientifica, Padua, Italy; 3Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland; and 4Oncology Department, Istituto Ricerca Biologia Molecolare, Pomezia, Rome, Italy

**Abstract**

The development of effective antitumor immune responses is normally constrained by low-avidity, tumor-specific CTLs that are unable to eradicate the tumor. Strategies to rescue antitumor activity of low-avidity melanoma-specific CTLs in vivo may improve immunotherapy efficacy. To boost the in vivo effectiveness of low-avidity CTLs, we immunized mice bearing lung melanoma metastases with artificial antigen-presenting cells (aAPC), made by covalently coupling PP6-MHC-Ig dimers and B7.1-Ig molecules to magnetic beads. aAPC treatment induced a significant tumor reduction in a mouse telomerase antigen system, and complete tumor eradication in a mouse TRP-2 antigen system, when low-avidity CTLs specific for these antigens were adoptively transferred. In addition, in an in vivo treatment model of subcutaneous melanoma, aAPC injection also augmented the activity of adoptively transferred CTLs and significantly delayed tumor growth. In vivo tumor clearance due to aAPC administration correlated with in situ proliferation of the transferred CTL. In vitro studies showed that aAPC effectively stimulated cytokine release, enhanced CTL-mediated lysis, and TCR down-regulation in low-avidity CTLs. Therefore, in vivo aAPC administration represents a potentially novel approach to improve cancer immunotherapy.

**Introduction**

Cancer vaccines have not significantly improved the clinical outcome and patient survival (1, 2), even though it is possible to detect tumor-specific CTL in peptide-vaccinated patients. More encouraging results have been obtained, for both solid and hematologic malignancies, in settings of adoptive immunotherapy by the administration of autologous tumor-specific CD8+ T cells expanded and activated in vitro (3, 4). Still, the clinical success of adoptive immunotherapeutic approaches have been restricted to ~50% of patients, who show mostly partial rather than complete clinical responses (5).

Several factors clearly influence the challenge of an efficient immunotherapy; thymic selection, tumor-released inhibitory cytokines and chemokines, the presence of regulatory T cells, altered macrophage differentiation, and defects in dendritic cells (6–10). Consistent with this idea, we have previously shown that vaccination with mouse TRP-2, a melanoma-associated self-antigen (11), had only a minor protective/therapeutic effect on B16 tumor challenge, due to an abortive immune response (12, 13). In this setting, TRP-2–specific CD8+ T lymphocytes, which proliferated after in vitro stimulation, efficiently recognized peptide-pulsed target cells but recognized B16 melanoma cells poorly (12, 13). This is probably due to reduced antigen class I MHC complex expression on their surface (14).

Different approaches have been proposed to overcome the lack of tumoricidal activity of low-avidity CD8+ T cells, such as the use of synthetic modified peptides to generate high-avidity T cells (15–17), delivery of appropriate local “danger signals” inside the tumor microenvironment (18, 19), and injection of viral vectors expressing the costimulatory molecule B7.1 (CD80) into melanoma lesions (20). Although these approaches have partially augmented the antitumor immune responses, they are not strong enough to result in complete tumor eradication.

To overcome issues of low-avidity CTLs in tumor immunotherapy, we designed artificial antigen-presenting cells (aAPC; see Supplementary Fig. S1) for in vivo administration. The aAPC are based on our previous work, in which signal 1 (MHC-Ig) and signal 2 (anti-CD28) were coupled to magnetic beads and used to generate antigen-specific CTLs in vitro (21–23). Here, we show that aAPC can also be used in vivo to augment the activity of adoptively transferred low-avidity melanoma-specific CTLs. This has been shown in both lung metastasis models and in a subcutaneous treatment model. In all models, aAPC administration significantly augmented the in vivo antitumor activity of adoptively transferred CTLs leading to the inhibition of tumor growth in the telomerase antigen–specific lung model and subcutaneous melanoma model, or complete tumor clearance in the TRP-2 antigen–specific lung metastasis model. This novel approach represents the first demonstration of an “off the shelf,” bead-based aAPC for systemic delivery of both antigen-specific and costimulatory signals to tumor-specific CTLs. aAPC administration can thus potentially be used to overcome current problems related to low-avidity antitumor CTLs, therefore increasing the efficiency of the adoptive immunotherapy of cancer.

**Materials and Methods**

**Mice.** Eight-week-old female C57BL/6 (H-2b); B6) mice were purchased from Charles River Laboratories. Procedures involving animals were in conformity with institutional guidelines.
Cell lines and CTL clones. MBL-2 is a leukemia cell line (H-2k) and B16Lu8 (hereafter referred to as B16) is a lung metastases–forming melanoma cell line (H-2b) kindly provided by Dr. James C. Yang (Surgery Branch, National Institutes of Health, Bethesda, MD). B16Lu8 cells stably transfected with mouse B7.1 (B16.F1-mB7.1.32 hereafter referred to as B16-B7.1 cells) were a kind gift from P. Della Bona (Istituto Scientifico San Raffaele, Milan, Italy).

TRP-2–specific CTL clones 8 and 24 were obtained by limiting dilution as previously described (12, 13). The m-TERT immunogenic peptide m-TERT198–VGRNFNL restricted for H2-Kb was previously described (24). B6 mice were immunized against the mouse telomerase antigen and CTL lines were restimulated weekly with irradiated syngeneic splenocytes pulsed with m-TERT198.

Dimer and aAPC preparation. Soluble MHC-Ig fusion protein was derived as previously described (15, 25) and can be purchased under the brand name DimerX from BD. The Kb-Ig molecules were actively loaded either with the TRP-2–180–181, SIY (SIYRYYGL, Commonwealth Biotechnologies), or m-TERT198 peptide (JPT Peptide Technologies; ref. 15). Briefly, Kb-Ig molecules were denatured using an alkaline solution [150 mmol/L NaCl; 15 mmol/L Na2CO3 (pH 11.5)] in the presence of different stimuli: 106 RMA-S cells pulsed with increasing concentration of TRP-2 peptide, different amounts of cognate TRP-2 aAPC ratios. T-cell stimulation reached a plateau at 1:30 ratio. Supernatants were harvested and tested for the IFN-γ release by TRP-2–specific CTLs using anti-mouse CD8+–TriColor mAb. The plates were centrifuged to facilitate conjugate formation and incubated at 37°C. After 5 h, cells were harvested and the amount of TCR downregulation was measured by flow cytometry analysis using anti-mouse TCRαβ–PE mAb (ImmuNoKontact, AMS Biotechnology, Ltd.).

Figure 1. In vitro and in vivo activity of high- and low-avidity TRP-2–specific CTL clones. A, antigen-specific IFN-γ release by TRP-2–specific CTLs was quantified by ELISA 24 h after stimulation with targets and antigens as indicated including MBL-2 cell or TRP-2 peptide–pulsed MBL-2. B, number of pulmonary metastases (columns, mean of two experiments; bars, SEM; n = 10) counted on day 14 after the challenge with B16 tumor cells. Maximum number of resolvable metastases was 300.

Effect of aAPC on subcutaneous tumor growth in vivo. B16 cells (5 × 105) were injected subcutaneously into thighs of 6- to 8-wk-old C57BL/6 female mice. When tumor area was ~10 mm2, mice were sublethally irradiated with 5 Gy to induce lymphopenia before adoptive cell transfer. After 6 h, 5 × 106 hgp10025–33–specific CTLs were injected i.v. to 6 h later, mice were injected i.v. with noncognate ASN-aAPC or cognate hgp100-aAPC (105/mouse/injection) and treated i.p. with 30,000 IU of recombinant IL-2 twice a day. Injections of beads and IL-2 were repeated for 3 consecutive days. Tumor growth was monitored at 2- to 3-d intervals,
using digital calipers, until tumor size was $\sim 100 \text{ mm}^2$ at which point animals were euthanized.

**Statistical analysis.** Wilcoxon Mann-Whitney test was used to examine the null hypothesis of rank identity between two sets of data. All $P$ values presented were two-sided.

**Results**

Low-avidity antigen-specific CTL clones have poor *in vitro* and *in vivo* antitumor effects. Low-avidity effector T cells can recognize peptide-pulsed target cells but often fail to recognize
endogenous antigens on tumor cells (12, 13). We therefore analyzed the in vitro and in vivo activity of a high- and low-avidity CTL clones (clone 24 and clone 8) which are both specific for the cognate antigen, TRP-2_{180-188} presented by H-2K^eta. As expected, clone 8 produced significantly less IFN-γ than clone 24 when stimulated with B16 tumor cells (Fig. 1A). However, when B16 tumor cells transfected with the costimulatory molecule B7.1 (B6-B7.1) were used as targets, clone 8 produced large amounts of IFN-γ (Fig. 1A).

We also found marked differences in vivo between clone 24 and clone 8. When analyzed in the B16 lung metastasis model (see Materials and Methods), only the high-avidity CTL, clone 24, efficiently treated lung metastases, whereas no significant difference was found between clone 8–treated and untreated mice (Fig. 1B). Therefore, clone 8 represents a classic low-avidity antitumor CTL that is inefficient at eradicating tumors.

**aAPC reverse in vivo inefficacy of the low-avidity antitumor CTLs.** To study the effect of aAPC, we tested the ability of aAPC to augment the efficacy of adoptively transferred, low-avidity CTL clone 8. For these studies, we generated aAPC in which signal 1, peptide-loaded K^eta-Ig complexes, and signal 2, B7.1-Ig complexes, are covalently coupled to beads (see Supplementary Fig. S1 for schematics and details of all aAPC used for these studies). In mice previously injected with B16 tumor, we examined whether in vivo aAPC-activated clone 8 could eradicate lung metastases. As schematically shown in Fig. 2A, on day 3 after B16 tumor cell injection, B6 mice received 5 × 10^6 TRP-2–specific CTLs and were injected i.v. on days 4, 5, and 6 with 10^7 aAPC per mouse, and all mice were sacrificed 14 days later. Administration of cognate TRP-2–peptide-loaded aAPC led to complete eradication of the tumor by clone 8, which was otherwise ineffective (Fig. 2B).

To analyze the relevance of signal 1 and signal 2 on the aAPC, we tested the effect of administration of the following aAPC: (a) signal 1 aAPC, (b) signal 2 aAPC, and (c) noncognate SIY aAPC (see Supplementary Figs. S1 and S2). No antitumor effect was seen in mice treated with the signal 1 aAPC, signal 2 aAPC, or noncognate SIY aAPC, demonstrating that both cognate signal 1 and signal 2 were required for effective in vivo activation of low-avidity CTLs (Fig. 2B). Additionally, no antitumor effect was seen in mice that were immunized with cognate TRP-2 aAPC only. These results indicate that aAPC provide more than basic costimulation to increase the efficiency of tumor immunotherapy.

To analyze the requirements for antigen-specific activation, a β-galactosidase–specific (β-gal) CTL clone was adoptively transferred to mice (Fig. 2A) and mice were injected with either β-gal or SIY aAPC (Fig. 2C). No effect was observed on metastasis formation, demonstrating that nonspecific CTLs exert no antitumor activity.

**aAPC induce in vivo proliferation of adoptively transferred TRP-2–specific CTLs.** To further analyze the effect of aAPC, we evaluated in vivo proliferation of the low-avidity CTLs. Tumor-bearing or tumor-free CD45.1^+ B6 mice were adoptively transfected with 5 × 10^6 CFSE-labeled, CD45.2^+ clone 8 cells. After 3 days, mice were immunized with either cognate TRP-2 aAPC or noncognate SIY aAPC. Seven days later, all mice were sacrificed and organs were collected and analyzed for the presence and expansion of CD45.2^+CD8^+ cells. Transferred CTLs were found only in the lung, which could be related not only to the route of administration but also to the specific homing of the CTLs to the tumor site.
Administration of cognate TRP-2 aAPC induced significantly more proliferation of the transferred clone 8 than the noncognate SIY aAPC in tumor-bearing mice as determined by CTL expansion (Fig. 3A) and CFSE dilution (Fig. 3B). Interestingly, low-avidity TRP-2–specific clone 8 underwent more cell divisions in the presence of cognate TRP-2 aAPC in tumor-bearing mice (Fig. 3B). The majority (~80%) underwent five or more cell divisions and only 2.2% of the transferred CTLs did not proliferate. In contrast, significantly less expansion was observed in mice treated with noncognate SIY aAPC (35.4% of the transferred CTLs remained undivided and only ~20% underwent more than three cell divisions). These data show that aAPC can be used to efficiently expand adoptively transferred CTLs in tumor-bearing mice. The reduced CTL proliferation in mice treated with the noncognate SIY aAPC (Fig. 3A and B) further confirms the finding that the B7.1 alone is not sufficient to induce antitumor activity or proliferation of the low-avidity CTL clone.

Interestingly, TRP-2–specific CTL expansion triggered by cognate TRP-2 aAPC in the lung was more vigorous in tumor-bearing mice, whereas only a limited expansion was observed in tumor-free mice (Fig. 3B, left). This effect might be related to the biodistribution of aAPC. Alternatively, it is also possible that, in the absence of tumor, activated T cells did not localize to the tumor site but distributed throughout the animal and were too dilute to be detected. To investigate whether the differences in proliferation between tumor-free and tumor-bearing mice were dependent on differences in the in vivo distribution of aAPC, we injected tumor-bearing and tumor-free mice with aAPC and analyzed tissue distribution of aAPC in various organs. On day 7, we found ~40% fewer aAPC in the lungs of tumor-free mice than in the lungs of tumor-bearing mice and the absolute number of aAPC was further reduced by day 14 (Fig. 3C). In contrast, no differences were observed in the number of aAPC in other organs. Therefore, the presence of a tumor affects the biodistribution and persistence of aAPC, which may explain the increased T-cell proliferation in lungs of tumor-bearing mice.

TRP-2–specific aAPC enhance in vitro activity of low-avidity CTLs. To further analyze the effect of aAPC, we performed in vitro
assays including effector cytokine secretion and TCR downregulation assays on the TRP2-specific clones. Both low- and high-avidity clones secreted IFN-γ at comparable levels after cognate TRP-2 aAPC stimulation (Fig. 4A). The amount of IFN-γ detected for non-cognate SIY aAPC, signal 1 and signal 2 aAPC were comparable with nonstimulated CTL clones (Fig. 4A). Only cognate TRP-2 aAPC induced significant effector functions in low-avidity CTLs, clone 8. Analogously, anti-β-gal CTLs were only stimulated to release IFN-γ by either β-gal96-103 peptide-pulsed targets or β-gal-aAPC, whereas there was no recognition even of B16-B7.1 melanoma targets (Fig. 4B).

T-cell activation was also evaluated by TCR downregulation after stimulation with either peptide-pulsed cells, plate-bound anti-CD3 mAb or aAPC. TCR downregulation was more sensitive in the high-avidity CTLs, clone 24, than in the low-avidity CTLs, clone 8, when stimulated with peptide-pulsed target cells (Fig. 4C). The limited TCR downregulation for clone 8 CTLs was not due to an overall impairment of the signaling machinery because stimulation with plate-bound anti-CD3 induced strong TCR downregulation in both clone 8 and clone 24 CTLs (Fig. 4C). Cognate TRP-2 aAPC efficiently induced similar TCR downregulation in both CTL clones, whereas signal 1 aAPC did not induce any TCR downregulation (Fig. 4C). In summary, cognate TRP-2 aAPC are effective stimulators of activation of low-avidity CTLs.

We determined whether low-avidity CTLs stimulated with cognate aAPC in vitro might regain the capacity to recognize B16 tumors not expressing B7.1. Clones 8 and 24 CTL were tested immediately (Fig. 4D, day 0) or 4 days following in vitro stimulation with cognate aAPC at a 1:1 ratio (Fig. 4D, day 4). Although on day 4, we detected a slightly higher reactivity in terms of IFN-γ release in culture upon stimulation, the functional responses of clones 8 and 24 were not affected, i.e., clone 24 recognized efficiently both B16 melanoma and the B7.1-transfected variants, whereas clone 8 only responded to B16-B7.1 cell stimulation (Fig. 4D). As expected, there was also no change in the overall CTL functional avidity as measured by peptide titrations (data not shown).

aAPC injection increases in vivo antitumor responses of low-avidity, telomerase-specific CTLs. We analyzed the efficacy of aAPC in another clinically relevant system, a polyclonal mouse CTL line specific for mouse telomerase (TERT-CTL; ref. 24). TERT-CTLs were >95% CD8+ but only 36% antigen-specific, as determined by tetramer staining (data not shown). TERT-CTLs also did not efficiently recognize B16 melanoma cells in vitro, but they did recognize peptide-pulsed target cells and B16-B7.1 targets (Fig. 5A). Incubation of TERT-CTLs with cognate m-TERT aAPC, but not control aAPC, induced a significant release of IFN-γ, similar to the low-avidity TRP-2–specific clone.
To evaluate the aAPC-based in vivo stimulation of the adoptively transferred TERT-CTLs, we administered TERT peptide–loaded aAPC (see Fig. 2A schematic). Although adoptive transfer of TERT-CTLs alone induced some tumor reduction (Fig. 5B), injection with cognate TERT-aAPC led to a significant decrease in tumor burden ($P = 0.0001$). As expected, noncognate aAPC did not reduce the tumor burden nor did TERT-aAPC alone, without adoptively transferred CTLs (Fig. 5B). Thus, treatment with cognate TERT-specific aAPC also led to a significant reduction in tumor burden in vivo.

One potential reason for incomplete tumor clearance could be epitope loss due to immunoselection by the adoptively transferred CTL. To examine this possibility, we isolated tumors after treatment with mTERT-specific CTL and aAPC and analyzed the ability of the mTERT-specific CTLs to recognize the tumor isolated from various mice. There was no difference in recognition, by TERT-specific CTLs, of the tumors isolated from animals injected with cognate TERT-specific aAPC or noncognate aAPC (Supplementary Fig. S3). Thus, alternative explanations including dosing of aAPC, activity of polyclonal TERT antigen-specific CTLs, and level of TERT antigen on tumor cells may account for residual tumors in the TERT system.

Mechanistically, we tested if aAPC directly stimulated low-avidity CTL activity. For these studies, we used the low-avidity mTERT-specific CTL and clone 8. CTL were incubated with $^{51}$Cr-labeled B16 tumor cells, either alone or in the presence of cognate aAPC or noncognate aAPC. Significant increases in CTL-mediated killing were seen in cognate aAPC–treated CTLs compared with noncognate SIY-aAPC (Fig. 5C). Similar results were obtained for clone 8, whereas no effect was seen on the high-avidity clone 24 response (Supplementary Fig. S4). Thus, aAPC stimulation leads to enhanced killing by low-avidity CTL toward tumors that otherwise would not be efficiently recognized.

**aAPC administration reduces tumor growth in a subcutaneous tumor treatment model.** To evaluate the effect of aAPC in a treatment model of subcutaneous tumors, we injected B16 tumor cells s.c. to induce solid tumors. Once tumors reached a size of $\sim 10 \text{ mm}^2$, hgp10025-33–specific CTLs were injected and mice were treated with either control or cognate aAPC. Administration of cognate aAPC led to a significant reduction in tumor growth ($P = 0.038$) compared with the control groups that were treated with either noncognate aAPC or IL-2 alone (Fig. 6A). Kaplan-Meier survival analysis (Fig. 6B) also revealed statistically significant differences ($P < 0.05$) between the groups B16 plus mTERT-aAPC and B16 plus SIY-aAPC were found at all effector/target ratios.
significant differences between cognate aAPC treatment and non-cognate aAPC (P = 0.0046) and between cognate aAPC and animals receiving just CTLs alone and IL-2 (P < 0.001). In contrast, there was no significant influence of treatment with noncognate aAPC compared with untreated animals. Thus, aAPC treatment is also effective in reducing the growth of an established subcutaneous tumor.

Discussion

Many tumor antigens are self-antigens. As a result, it is not surprising that T-cell antitumor immune responses are often of low avidity and fail to recognize endogenous antigens on tumor cells. Therefore, strategies are being developed to rescue the functional antitumor activity of low-avidity CTL.

Using an adoptive transfer model of low-avidity TRP-2-specific CTLs in a B16 lung metastases melanoma model, we show that aAPC administration specifically induced in vivo CTL proliferation and CTL-mediated antitumor responses. Interestingly, CTL proliferation in the lung was more vigorous in the lungs of tumor-bearing mice, which might be related to aAPC biodistribution, as the lungs of tumor-bearing mice retained more aAPC than tumor-free mice. Our results further show that only the cognate aAPC activate the melanoma-specific CTLs and lead to tumor clearance. Whereas aAPC activation of non–tumor-specific β-gal-aAPC did not affect the metastatic tumor growth.

To further explore the potential of aAPC treatment, we studied aAPC-mediated in vivo activation of low-activity, m-TERT–specific CTLs. aAPC administration also elicited a robust antitumor response confirming the value of the system in enhancing the therapeutic effectiveness of other low-activity tumor-specific T cells. These results highlight the fact that the in vivo use of aAPC is efficient in enhancing an antigen-specific CTL response to another entirely different, yet clinically relevant antigen.

We also explored the generality of the effect of aAPC injection in a subcutaneous tumor treatment model. These studies show that aAPC treatment enhanced the activity of adoptively transferred CTLs and significantly delayed the tumor growth of established subcutaneous tumors. In this system, there are additional variables that might affect the in vivo activity of aAPC. One variable to consider is the aAPC tissue distribution and the sites of CTL activation by the aAPC. The lungs are the first tissue bed where aAPC can lodge and activate CTLs, whereas distribution in other tissues may be less efficient. Alternatively, this finding may highlight the interest in adding targeting molecules that could help the aAPC home to either lymphoid organs or specific tissues.

Approaches to targeting antitumor activity T cells are very broad. One previous approach has been to target antigen-specific CTLs using soluble MHC complexes, including soluble dimers and tetramers. Under some regimens, soluble MHC appears to delete antigen-specific CTLs (26), whereas in others, soluble dimers and/or tetramers alone activate CTL in vivo (27–29). In these previous reports, only high-avidity CTLs were targeted, with no attention to antitumor activity of low-avidity CTLs as we report here. Indeed, in our studies, aAPC made with only MHC-Ig dimers, signal 1 alone, were not effective. Although there are many differences between the systems, one possible reason for the requirement of both signal 1 and signal 2 could relate to the overall dose of antigen being delivered. To prepare aAPC, we used only 1.5 μg per mouse (for 3 × 10^7 aAPC total dose), whereas much larger doses, up to 90 μg of tetramer per mouse, were used previously (28). Another possibility is the role of endogenous antigen-presenting cells, which might be different between the use of soluble MHC complexes and administration of aAPC beads. Overall, either aAPC and/or soluble MHC complexes represent new approaches to antigen delivery that may each have valuable clinical applications in immunotherapy.

In recent trials, combination of myeloablation and adoptive immunotherapy with ex vivo expanded tumor-infiltrating lymphocytes (TIL) achieved objective clinical responses in ~50% of patients with metastatic melanomas (3, 5). Unfortunately, TILs can only be isolated and grown for a limited number of patients. To circumvent these limitations, high-avidity TCR, derived from TIL, have been identified, cloned, and transduced into the peripheral blood mononuclear cells of patients with cancer (30). However, there are still many problems that have thus far limited a widespread clinical application. In this article, we show the feasibility of an alternative strategy aimed at rescuing...
the functional antitumor activity of low-avidity T cells. This is the first report exploiting the use of an "off the shelf" aAPC injection as a novel approach for antigen-specific in vivo activation of tumor-specific CTL.

Disclosure of Potential Conflicts of Interest

M. Oelke and J.P. Schneck are inventors on a pending patent, owned by the Johns Hopkins School of Medicine, for the development of aAPC and its use in manipulation of CTL responses. The other authors disclosed no potential conflicts of interest.

References

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