Combined immunotherapy: CTLA-4 blockade potentiates anti-tumor response induced by transcutaneous immunization

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Abstract

Background: The epidermal application of the Toll Like Receptor 7 agonist imiquimod and a T-cell peptide epitope (transcutaneous immunization, TCI) mediates systemic peptide-specific cytotoxic T-cell (CTL) responses and leads to tumor protection in a prophylactic tumor setting. However, it does not accomplish memory formation or permanent defance of tumors in a therapeutic set-up. As a distinct immunologic approach, CTLA-4 blockade augments systemic immune responses and has shown long-lasting effects in preclinical experiments as well as in clinical trials.

Objective: The study investigates the vaccination capacity of TCI in combination with the checkpoint inhibitor CTLA-4 in matters of primary response, memory formation and tumor protection and characterizes the role of regulatory T cells (Tregs).

Methods: After performing TCI with IMI-Sol (containing 5% Imiquimod) and the model epitope SIINFEKL, 6–8 week old C57BL/6 mice received anti-CTLA-4 antibody either s.c or i.p. The CTL responses and frequency of peptide specific CD8+ T-cells were then evaluated on day 8. To determine anti-tumor effects, a therapeutic tumor challenge with B16 OVA melanoma was performed.

Results: The combination of s.c. anti-CTLA-4 antibody and TCI leads to an enhanced systemic cytotoxic response, to memory formation and allows significantly improved survival in a tumor setting with B16 OVA melanoma. Towards the mechanism, we show that in this vaccination protocol the CTLA-4 antibody acts mainly Treg-independent.

Conclusion: We demonstrate that the combination of TCI with IMI-Sol and anti-CTLA-4 can confer potent immune responses and tumor-protection. These results might contribute to the development of advanced vaccination approaches targeting tumors or persistent infectious diseases.

1. Introduction

Immunotherapies have recently developed great importance in defying cancer by utilizing the host’s defense mechanisms. In this context, we have established a transcutaneous immunization (TCI) protocol based on the application of the Toll-like receptor (TLR) 7 agonist imiquimod [1] onto the skin inducing a systemic immune response by targeting the highly developed skin associated lymphatic tissue (SALT) [2,3]. The simultaneous administration of imiquimod (in the commercially available formulation Aldara) and the epitope OVA257-264 leads to a MyD88-dependent activation of dendritic cells resulting in a strong primary cytotoxic T lymphocyte (CTL) response with anti-tumor effects [3–5]. However, it’s impact is limited to a delay in tumor growth in a therapeutic tumor setting and lacks any memory formation [5,6], partly due to a restricting influence of regulatory T-cells and IL-10 [7]. This limitation can be overcome by the combination with other immune stimulants such as UV- light or CD40 ligation [6,8]. Furthermore, we recently developed the imiquimod-containing freeze-dried nanoemulsion IMI-Sol to overcome these drawbacks.

Abbreviations: TCI, transcutaneous immunization; CTL, cytotoxic T-cell; TLR7, toll-like receptor 7; CTLA-4, cytotoxic T-lymphocyte associated antigen 4; s.c, subcutaneous; i.p, intraperitoneal; Treg, regulatory T-cell; IFN, interferon-IL, interleukin; APC, antigen presenting cell.

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and is also able to induce CD4\(^+\) T-cell responses and memory formation [31].

Lately, it has also been increasingly recognized that not only the induction of tumor-specific immune responses is relevant for the successful immunological treatment of tumors, but it is also important to interfere with peripheral as well as tumor-induced mechanisms of tolerance, e.g. mediated by regulatory T (Treg) cells [10] or myeloid derived suppressor cells [11], i.e. with checkpoint inhibitors. Therefore, the combination of TCI with the checkpoint inhibitor cytotoxic T lymphocyte antigen (CTLA) 4 might be a new promising approach. Its clinical significance is evidenced by recent clinical studies using the CTLA-4-specific antibody ipilimumab demonstrating extended survival in metastatic melanoma patients [12,13]. Concerning the mechanisms of a CTLA-4 blockade, the antibody is currently thought to act as a competitive inhibitor of CD28 during T-cell activation by interacting with CD80/86 on antigen presenting cells (APCs) [14], to deliver inhibitory signals during T-cell proliferation [15] and to play a crucial role in immune restricting effects of Treg cells [16]. Comparable to TCI, activated CD8\(^+\) T cells are hereby accounted as a thriving force in tumor regression [17]. Furthermore, the antibody augments initiated immune responses without mediating any effect in monotherapy itself [18], advocating the combination with immunotherapies like TCI.

We have undertaken this study to assess the effects of a combined immunotherapy with TCI and anti-CTLA-4 antibody on primary immune response, memory formation and tumor deficiency. Furthermore, the participation of Treg cells as underlying mechanism is to be evaluated.

2. Material and methods

2.1. Mice

6-8 week-old C57BL/6 mice were provided by the local animal facility of the University of Mainz and the Harlan Laboratories. All assays involving animals were conducted according to institutional guidelines reviewed and confirmed by an institutional review board/ethics committee headed by the local animal welfare officer (Prof. Dr. O. Kempski) of the University Medical Center (Mainz, Germany). The National Investigation Office Rheinland-Pfalz (Koblenz, Germany) as responsible authority finally approved the animal experiments. The Approval ID assigned by this authority: AZ 23 177–07/G13-1-012.
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2.2. Transcutaneous immunizations (TCI)

Dorsal hair of the mice was removed on day 0 with an electrical hair clipper without compromising the dermal barrier. On day 1, 50 mg of IMI-sol (containing 5% imiquimod) and subsequently 100 μg OVA257-264 (SIINFEKL, peptides& elephants, Potsdam, Germany) dissolved in DMSO and blended in a lipid cream were spread onto the skin of anesthetized animals. The used anesthetic was a dilution of Ketamine and Rompun applied in weight adjusted dosage. In case of an anti-CTLA-4 antibody application, the intervention directly followed the TCI in the foreseen dosage and application form.

2.3. Antibody application

Depending on the experiment either 300 or 500 μg anti-CTLA-4 antibody concentration (clone UC-4F10-11, own production) was suspended in 150 μL PBS and then injected either s.c. into the neck or i.p. in anesthetized mice. To deplete Treg cells 400 μg anti-CD25 (clone PC61, own production) were injected i.p. on day –4.

2.4. Flow cytometric analysis and in vivo cytotoxicity assay

For analysis by flow cytometry, blood samples were collected by tail vein incision and prepared in a hypotonic lysis-step. Afterwards the cells were incubated with mAbs staining CD8 in Pacific Blue or APC, IFN-γ in APC, CD90.2 in PB, CD4 in PE, CD25 in FITC and FoxP3 in APC depending on the experiment to define a specific cell line. To detect the amount of peptide-specific CD8+ cells, blood samples were stained with the OVA257-264-H2-Kβ-tetramer as previously described by Warger et al. [6].

To estimate the cytotoxic activity of the primed CD8+ T-cells, an in vivo cytotoxicity assay explained by Rechtsteiner et al. [3], was conducted.

For an immediate response, the tests were undertaken on day 7. To assess a memory formation, the assays were performed on day 35 post TCI. All analyses were performed with a LSRII Flow Cytometer and FACSdiva Software (Becton Dickinson).

2.5. Intracellular IFN-γ analysis

To estimate the IFN-γ production of peptide-primed T-cells, an in vitro restimulation assay was performed. Blood samples from tail vein incision were divided in peptide- and non-peptide-exposed groups and then restimulated in the presence of brefeldin A over night. Subsequently, the cells were permeabilized and stained for flow cytometric analysis.

2.6. Tumor rejection assay

2 × 10^5 B16 OVA melanoma cells were implanted s.c into the right flank of anesthetized mice. At a size of 25 mm², treatment was initiated with weekly TCI over 3 weeks and anti-CTLA4
antibody injected as indicated. Tumor growth was measured every other day with a caliper in 2 dimensions and death incidents were noted. Mice were sacrificed when tumor size exceeded 400 mm² or when bleeding ulcerations occurred.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.0a for Mac OS X, GraphPad Software). Multiple experimental groups were compared by One Way Analysis of Anova with a Bonferroni Post-Test. Survival analysis was conducted by a Mantel-Cox test. For all analyses, p < 0.05 was considered as statistically significant.

3. Results

3.1. CTLA-4 blockade enhances CTL responses mediated by transcutaneous immunization

To evaluate whether CTLA-4 signalling directly influences TCI induced CTL responses, we treated mice with an anti-CTLA-4 antibody i.p. or s.c. directly after vaccination. 7 days after treatment, we analysed the frequency of peptide-specific CD8⁺ T cells, lytic activity and IFN-γ production. Bypassing the CTLA-4 signalling pathway led to increased levels of induced peptide-specific CD8⁺ T cells (Fig. 1A) permitting enhanced lysis of transferred and peptide-loaded target cells (Fig. 1B). Ex vivo restimulation of blood cells revealed that the combination of anti-CTLA-4 and TCI enhanced production of IFN-γ (Fig. 1C) compared to TCI alone. These effects are more pronounced after s.c. application of anti-CTLA-4.

We further determined the influence of CTLA-4 analysing memory formation after TCI with or without additional treatment with the anti-CTLA-4 antibody (s.c.). Therefore, lysis of peptide-loaded target cells was assessed 35 days after treatments. As depicted in Fig. 1D, 30.5% of targets were depleted after TCI alone whereas 57.9% of specific lysis was detected after combined treatment.

These results clearly demonstrate a major participation of the CTLA-4 signalling in diminishing TCI-induced immune responses and that blockage of this checkpoint inhibitor leads to enhanced primary as well as memory responses.

3.2. Suppression by CTLA-4 is not exclusively Treg-dependent

Recently, we have demonstrated that CTL responses induced by transcutaneous immunization are restricted by the presence of regulatory T cells [7], therefore a possible relation between suppression mediated via Treg cells and the expression of CTLA-4 has to be elucidated. To this end, mice were treated as before (TCI alone or anti-CTLA-4 and TCI) in the presence or absence of Treg cells. To deplete Treg cells, mice received an injection of an anti-CD25 mAb (clone PC61) 4 days prior to vaccination. We assessed the peptide-specific CTL for frequency and in vivo cytolytic activity one week after treatments and during the memory phase (D35). Depleting Treg cells before vaccination increased the population of CD8⁺ peptide-specific T cells (Fig. 2A) leading to enhanced cytolytic activity compared to the undepleted, but vaccinated group (Fig. 2B, C). However, these effects are not as pronounced as the augmentation mediated via anti-CTLA-4 application and TCI. Interestingly, analysing the presence of Treg cells on day 7, we observed an increase in Treg cells in the blood of mice treated with anti-CTLA-4 antibody, in contrast to untreated or immunized animals (Fig. 2D).

During the memory phase the influence of CTLA-4 and Treg cells changes compared to the primary immune response. Depleting Treg cells before vaccination leads to a significant improvement in the memory phase compared to TCI alone, enhancing the effects induced by anti-CTLA-4 application. The synergistic activation seen in the primary response, utilizing both antibodies before TCI, can be verified during the memory response anyway (Fig. 3).

All together these data clearly indicate that in this vaccination protocol Tregs mediate their suppressive capacity only partially via the coreceptor CTLA-4 and that CTLA-4-dependent inhibition is mainly Treg-independent.

3.3. CTLA-4 blockade enhances antitumor effects of TCI in B16 OVA melanoma

After showing the influence on the primary and memory immune response, we next aimed to detect the potency of the combinatorial treatment of TCI and antibody in a therapeutic tumor setting. We implanted B16 OVA melanoma cells expressing chicken ovalbumin (OVA) as surrogate tumor antigen into the flank of 6–8 week old C57BL/6 mice and began treatment when tumor dimensions exceeded a size of 25 mm². The animals were immunized with 50 mg IMI-Sol and 100 μg OVA257-264 once a week for three weeks and received 300 μg anti-CTLA-4 antibody s.c. either once with the first or three times with every vaccination. Untreated animals and animals treated only with TCI or only receiving the antibody and without any further treatment served as controls. Tumor growth was measured every other day and survival was documented.

The results display a delay in tumor growth in TCI treated (Fig. 4, upper panel), but not in untreated or anti-CTLA4 treated animals (Fig. 4, lower panel). The combination of anti-CTLA4 and TCI significantly further delayed tumor growth (Fig. 4 middle and right panel) converting to an improved survival time compared to a mere TCI (24 vs. 31 days) (Fig. 5). Regarding the frequency of the antibody treatment, a delay in tumor growth (Fig. 4, until day 20 vs. day 25) was observed, however growth eventually accelerated in all groups and not leading to a benefit in overall survival (Fig. 5AB, red lines). 44.4% of the animals receiving TCI and anti-CTLA4 (3 ×) survived (Fig. 5B), while only 5.8% of the merely immunized mice were still alive (Fig. 5). Concerning the single anti-CTLA4 application (Fig. 5A), the proportions were 60% survival (anti-CTLA-4) versus 20% (TCI) (Fig. 5). There was no difference between an isolated antibody treatment and untreated animals with 0% survival and a median survival time of 19 days (Fig. 5).

![Fig. 3. Treg depletion leads to significant improvement in the memory phase.](image-url)
In conclusion, the combination of TCI and anti-CTLA-4 significantly improves survival and limits tumor growth, however it does not lead to a complete tumor rejection.

4. Discussion

Finding solutions in the battle against cancer has become one of the main challenges of our time. While chemotherapy and radiation have dominated clinical routine over the last decades, immunotherapy has recently opened new treatment options, especially in the cure of radiation-resistant malignant melanoma.

The discovery of tumor-specific antigens [19] established vaccination as a mean against malignancies and supports Transcutaneous Immunization as a non-invasive, simple to use strategy [2,3]. However, the vaccination protocol is limited in both potency and long-term effect [5,6] and is therefore better to be combined with other immunotherapies [6,8].

The combination of TCI and anti-CTLA-4 is hereby a promising approach mediating an increased primary CTL response and an enhanced priming of peptide specific CD8+ T cells (Fig. 1). Most likely, the vaccination triggers a CD8+ T cell-dependent immune reaction, which is modified and prolonged by the anti-CTLA-4
antibody [20,21]. Despite the limiting effect of Treg cells on TCI (Fig. 2, [7]) and the constitutive, function-determining expression of CTLA-4 on their surface [10,16] the effect of anti-CTLA-4 treatment revealed to be independent of Treg inhibition. The antibody predominantly augments the number of peptide-specific CD8+ T cells, while Treg depletion enhanced the cytotoxic activity of the effector cells (Fig. 2). Furthermore, the effects of antibody treatment and Treg depletion added up (Fig. 2), suggesting that the antibody neither fully inhibited Treg cells nor its effects to be exclusively Treg dependent. This assumption is supported by experiments in a tumor setting that suggest the anti-tumor response to be mainly effector-cell dependent [10,22,23]..

It has been assumed that a higher antibody dosage increases the amount of Treg cells in the tumor environment, thus being therapy-limiting [23,24]. This might explain why the local, subcutaneous injection of the antibody presented superior to an intravenous administration, corresponding to a previous study [17]. This lower dosage furthermore bears an advantage for clinical application, since an increasing incidence coincides with more adverse effects [13,17,25]. Therefore, the combination of antibody and TCI might reduce dosage and subsequently minimize adverse effects.

A limitation of our previous TCI protocol using Aldara [6] has its incapacity to establish long-term effects. This obstacle is in part overcome by the novel imiquimod formulation IMI-Sol that is capable of additionally passing MHC class II restricted T cell epitopes via the skin thus eliciting helper T-cell responses in addition [31]. However, the combination of TCI and anti-CTLA-4 antibody can further settle this obstacle (Fig 1D) showing increased memory information. This long-term immunity after CTLA-4 blockade is ascribed to an accumulation of CD8+ memory T cells [26]. Regarding the effect on Treg cells, the CTLA-4 inhibition seems to limit the memory formation compared to a mere Treg depletion (Fig. 3). An explanation might be the augmented, therapy-limiting percentage of regulatory T-cells on day 7 after anti-CTLA-4 antibody treatment compared to the other groups (Fig 2D).

In our therapeutic tumor setting, the double treatment significantly augmented survival rate of mice and delayed tumor growth. While the antibody failed to achieve any improvement in monotherapy, it fortified effects mediated by TCI (Fig. 3), an observation already described by van Elsas et al. in the context of combined immunotherapy [18]. A review of Grosso et al. [27] offered the provision of antigens and activation of dendritic cells as underlying explanation.

Despite the initial success, the effect of the double treatment is still limited since the growth rate eventually reaccelerates. For the aggressive B16 OVA melanoma an earlier begin of treatment might offer a simple solution. Just as well should the concept of “immunoediting” and “tumor escape mechanisms” be reconsidered, encompassing a loss of death ligands (Fasl, TRAIL), an induction of inhibitory cytokines (IL-10, CTLA-4, PD1 TGF-B) [28,29] and the loss of the OVA-epitope or MHC-molecules [30]. Analysing these mechanisms might be an approach to clarify tumor dynamics and achieve long-term effects.

In conclusion, TCI combined with anti-CTLA-4 antibody is a prospective option in the treatment of malignant melanoma. Even though the antibody alone already shows promising results in clinical routine [12,13], the responder rate remains low with only 10%. Regarding the preclinical results, a combination with the topical, non-invasive but immune stimulating TCI might set the necessary stimulus to trigger an optimized immune response and build hope for patients that are out of options.

Conflicts of interest
No conflict of interest discloses.

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References


