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ORIGINAL ARTICLE

Ontak reduces the immunosuppressive tumor environment and enhances successful therapeutic vaccination in HER-2/*neu*-tolerant mice

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Abstract Disrupting tumor-mediated mechanisms suppressing host immunity represents a novel approach to tumor immunotherapy. Depletion of regulatory T cells (Tregs) increases endogenous anti-tumor immunity and the efficacy of active immunotherapy in experimental tumor models. HLA-A2.1/HLA-DR1 (A2.1/DR1) × BALB-*neuT*$^+$ (*neuT*$^+$) triple transgenic mice represent an improvement over *neuT*$^+$ mice for evaluating vaccination regimens to overcome tolerance against HER-2/*neu*. We questioned whether depletion of Tregs with Denileukin diftitox (Ontak) enhances the efficacy of a therapeutic vaccine consisting of HER-2(85–94) (p85) CTL and HER-2(776–790) (p776) Th peptides against the growth of TUBO.A2 transplantable tumor in male A2.1/DR1 × *neuT*$^+$ Tg mice. While the therapeutic vaccine primed the tumor-reactive CD8$^+$ CTLs and CD4$^+$ effector T lymphocytes (Teffs) compartment, inducing activation, tumor infiltration, and tumor rejection or delay in tumor growth, treatment with Ontak 1 day prior to vaccination resulted in enhanced CD4$^+$ and CD8$^+$ T-cell-mediated vaccine-specific immune responses in the periphery. This was closely associated with greater infiltration and a striking change in the intratumor balance of Tregs and vaccine-specific CTLs/Teffs that directly correlated with markedly enhanced antitumor activity. The data suggest that Tregs control both CD4$^+$ and CD8$^+$ T-cell activity within the tumor, emphasize the importance of the intratumor ratio of vaccine-specific lymphocytes to Tregs, and demonstrate significant inversion of this ratio and correlation with tumor rejection during Ontak/vaccine immunotherapy.

Keywords Peptide vaccines · Ontak · HER-2/neu · Effector T cells · Fas Ligand

Abbreviations

Tregs	Regulatory T cells
Tg	Transgenic
Teffs	Effector T cell
FasL	Fas ligand
PENT	Pentamer
Tm	Tetramer
TIL	Tumor infiltrating lymphocytes

Introduction

Tregs act to suppress the activation and function of both self-antigen- and foreign-antigen-reactive T cells [1, 2]. The critical physiologic role of Tregs is to control autoimmune diseases [3, 4]. However, because most tumor-associated antigens are self-antigens, Tregs may play a critical role in suppressing an effective antitumor immune response. Indeed, accumulating evidence suggests a significant contribution of Tregs to the impaired host immune response against cancer [5, 6]. Moreover, elevated Tregs levels in the peripheral blood, lymphoid organs, and tumor microenvironment of patients with cancer have been closely correlated with reduced survival [7–10].

Depletion of Tregs in vivo enhances tumor immunity in numerous animal models [11–13] prompting the hypothesis

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that such a strategy would be clinically effective in patients. For example, low-dose cyclophosphamide potentiates the antitumor effects of therapeutic cancer vaccines, with a mechanism appearing to involve selective deletion or inhibition of the cycling population of Tregs [14]. In another strategy, injection of mice with anti-CD25 antibody depletes animals of $CD25^+$ T cells and enhances response to vaccination [15–18].

Denileukin diftitox (DAB389IL-2, Ontak) is a fusion protein of human IL-2 and the enzymatically active and membrane translocating domains of diphtheria toxin [19]. Ontak binds specifically to IL-2 receptor in vitro and is rapidly internalized via receptor mediated endocytosis [19]. Ontak treatment resulted in the selective depletion of Tregs generating an effective endogenous response against breast cancer without eliciting additional cytopenia [20]. Preclinical studies have indicated the advantage of combining Ontak with a vaccine to enhance antigen-specific T-cell immune responses [21, 22]. However, so far, there is no evidence that Ontak by depleting Tregs synergizes with mechanisms breaking tolerance against tumor (self) proteins, resulting in potentiated antitumor responses in vivo. In such a case, Ontak treatment would considerably enhance the potency of therapeutic vaccines targeting tumor antigens. In our recent report [23], we have shown that vaccination of male HER-2/*neu*-tolerant A2.1/DR1 × *neuT*$^+$ Tg animals with a peptide-combination vaccine (consisting of synthetic peptides representing CTL and Th epitopes of HER-2/*neu*) breaks tolerance against HER-2/*neu*, resulting in the development of antitumor immunity against a HER-2/*neu*$^+$ transplantable tumor. Herein, we show that treatment of A2.1/DR1 × *neuT*$^+$ mice with Ontak 1 day before vaccination drastically reduces the number of peripheral Tregs, thereby enhancing the potency of a HER-2/*neu* CTL plus Th peptide-combination vaccine. We also show that Ontak pretreatment of animals vaccinated with the peptide-combination vaccine induces further decreases of intratumor Tregs that ultimately lead to potentiated antitumor response. These studies show for the first time in a murine model that the therapeutic impact of this Ontak/vaccine regimen has a direct as well as an indirect effect on Tregs: administration of Ontak drastically reduces the number of peripheral Tregs (direct effect), thereby allowing an improved priming of both $CD8^+$ and $CD4^+$ T cells against the HER-2/*neu* peptide-combination vaccine. This enhancement of vaccine-specific immune responses in the periphery is closely associated with increased frequencies of vaccine-specific T cells at the tumor site recognizing the tumor (i.e., $CD8^+$ CTLs) and inducing higher levels of apoptosis in Tregs (i.e., $CD4^+$ Teffs) via Fas–FasL interaction (indirect effect), ultimately resulting in potentiated antitumor responses.

Materials and methods

Generation of triple Tg mice

Female A2.1/DR1 mice (provided by Prof. F. A. Lemonnier; Unite d' Immunite Cellulaire Antivirale, Institute Pasteur) were crossed with male BALB/c mice transgenic for the transforming rat Erbb2/*neu* oncogene *neuT*$^+$ mice (provided by Prof. G. Forni; Molecular Biotechnology Center, University of Turin). Approximately 30% of the offsprings expressed the rat *neu* transgene (A2.1/DR1 × *neuT*$^+$ triple Tg) [23]. Experiments for this study were approved by the Saint Savas Institutional Animal Care and Use Committee.

Synthetic peptides

Human HER-2(85–94) (p85; LIAHNQVRQV) and human HER-2(776–790) (p776; GVGSPYVSRL LGICL) peptides were synthesized and analyzed as described in our previous reports [23, 24].

Antibodies

Antibodies used were as follows: anti-CD95-FITC (anti-Fas), anti-CD178-PE (anti-FasL), anti-CD127-PE, anti-mouse IgG2b-FITC and IgG2a-FITC, and Annexin V-PE (BD Biosciences Pharmingen, Franklin Lakes, NJ); anti-CD4-allophycocyanin (APC), anti-CD8-APC, anti-Foxp3-APC, and anti-Foxp3-PerCP-Cy5.5 (eBioscience, San Diego, CA); anti-HLA-A2.1 (a gift from Prof. H. G. Rammensee, Department of Immunology, University of Tuebingen); PE-labeled HLA-A2.1 pentamer presenting the HER-2(85–94) epitope (PENT; Proimmune, Magdelen Center, Oxford); and PE-labeled HLA-DR1 tetramer presenting the HER-2(776–790) epitope (Tm; Beckman Coulter, Fullerton, CA). Anti-CCR7-PE, anti-CD62L-APC, anti-GITR-FITC (eBioscience).

Surface and intracellular staining

For surface staining, cells were incubated with mAb in different combinations on ice for 30 min. For Foxp3, and Annexin V^+ staining, the manufacturers' instructions were followed. $CD8^+PENT^+$ (HLA-A2.1/$p85^+$) and $CD4^+Tm^+$ (HLA-DR1/$p776^+$) cells were detected as recently described [23, 24]. Stained cells were analyzed using a FACSCalibur (BD Biosciences).

Immunization protocol

Mice were immunized thrice every 5 days by s.c. injections at the base of the tail with 100 μg of peptide in 200 μL IFA along with 100 ng of GM-CSF, as described [25]. One day

before immunizations, animals were injected once i.p. with 3 μg Ontak (Ontak™; Eisai Inc., Woodcliff Lake, NJ) in 100 μL PBS (or with PBS alone).

ELISPOT assay

Responder $CD8^+$ and $CD4^+$ T cells were isolated from the spleens of immunized mice by negative selection using the $CD8^+$ or $CD4^+$ T-cell isolation kits, respectively (Miltenyi Biotec, Bergish Gladbach, Germany) [25]. Responders from every single immunized mouse were plated at 250,000 cells per well in quadruplicates in 96-well flat-bottomed plates. Irradiated (3,000 rads) splenocytes from naïve animals pulsed with 500 nmol/L of the indicative peptide were added to the syngeneic responders at a cell ratio of 1:1 in a total volume of 200 mL/well RPMI 1640 containing 10% fetal bovine serum (FBS), 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Invitrogen, Paisley, UK). Control cultures were stimulated with unpulsed syngeneic, irradiated splenocytes. Cultures were incubated for 48 h at 37°C in a CO_2 incubator. IFNγ production was estimated using the IFNγ ELISPOT Assay kit (BD PharMingen) according to the manufacturer's protocol. Spots were enumerated using an ELISPOT analyzer (A.EL.VIS GmbH, Hannover, Germany). Specific spots were calculated by subtracting the mean number of spots obtained from the control cultures.

In vitro cytotoxicity assay

Tumor infiltrating lymphocytes (TIL) were isolated as described [26]. $CD8^+$ TIL were isolated as described in the previous paragraph and then used as effectors against TUBO.A2 targets using a standard cytotoxicity assay [27].

Tregs and $CD4^+CD25^-$ T-cell isolation and cultures

$CD4^+CD25^{high}CD127^-$ cells (Tregs) and $CD4^+CD25^-$ T cells were isolated from spleens of animals as described recently [23]. A total of 50,000 $CD4^+CD25^-$ T cells were plated in round-bottom 96-well plates in the presence of 150,000 T-cell-depleted irradiated (3,000 rads) syngeneic splenocytes and increasing amounts of Tregs plus 10 μg/mL purified anti-CD3 [26]. Cells were incubated at 37°C for 72 h and were pulsed with [^{3}H]thymidine in the last 8 h of culture before being harvested in a β-counter [26].

Therapeutic tumor model

Mice were inoculated s.c. with 5×10^5 transplantable TUBO.A2 cells. Six says later, animals were treated with Ontak in PBS as described above, or with PBS alone. On the next day, mice were immunized with HER-2/*neu* peptides (single or in combination) in IFA along with GM-CSF, as described above. Tumor size was monitored regularly every 4 days and was expressed as the product (in mm^2) of the perpendicular diameters of individual tumors. Mice were killed with euthanasia when tumor size exceeded 200 mm^2.

Statistical analyses

The Student's *t* test was applied with a 95% confidence interval to determine the statistical significance of differences between groups, with $P < 0.05$ being considered significant.

Results

Deletion of Tregs by Ontak in A2.1/DR1 × *neuT*$^+$ triple Tg animals

$CD4^+CD25^+Foxp3^+$ Tregs in the spleen of mice can be systematically deleted without causing lymphopenia upon treatment with Ontak [21]. Studies were conducted here to determine whether and to which extent Ontak depletes Tregs in our triple Tg males after a single i.p. injection. Based on recently published data [22], Ontak was administered at 3 μg, and $CD3^+CD4^+$ cells from the peripheral blood were stained for $CD25^+Foxp3^+$ T cells on various days after the injection. After Ontak administration, the greatest depletion of Tregs relative to naive mice was seen on day 1 after injection (almost 70% reduction) and levels normalized by day 10 after injection (Fig. 1a, b).

Ontak increases the potency of A2.1/DR1 × *neuT*$^+$ triple Tg mice to overcome tolerance to HER-2/neu

We have recently identified p85 and p776 as tumor rejection epitopes capable of eliciting potent antitumor responses in mice and in PBMC from healthy donors and cancer patients [23, 24]. Herein, we sought to determine whether Ontak could enhance vaccine-specific anti-HER-2/*neu* T-cell responses in our HER-2/*neu*-tolerant mice. For this, highly purified $CD8^+$ and $CD4^+$ T cells from the spleens of mice treated with Ontak and vaccinated 1 day later with p85 alone or with a combination of p85 plus p776 were tested as responders in an IFNγ-based ELISPOT assay with stimulatory syngeneic-irradiated splenocytes pulsed with their cognate peptides used for immunizations. Immune assays were performed on day 16 after Ontak treatment (i.e., 5 days post termination of the vaccination protocol). As shown in Fig. 1c, only weak IFNγ responses were mediated by $CD8^+$ T cells from animals vaccinated

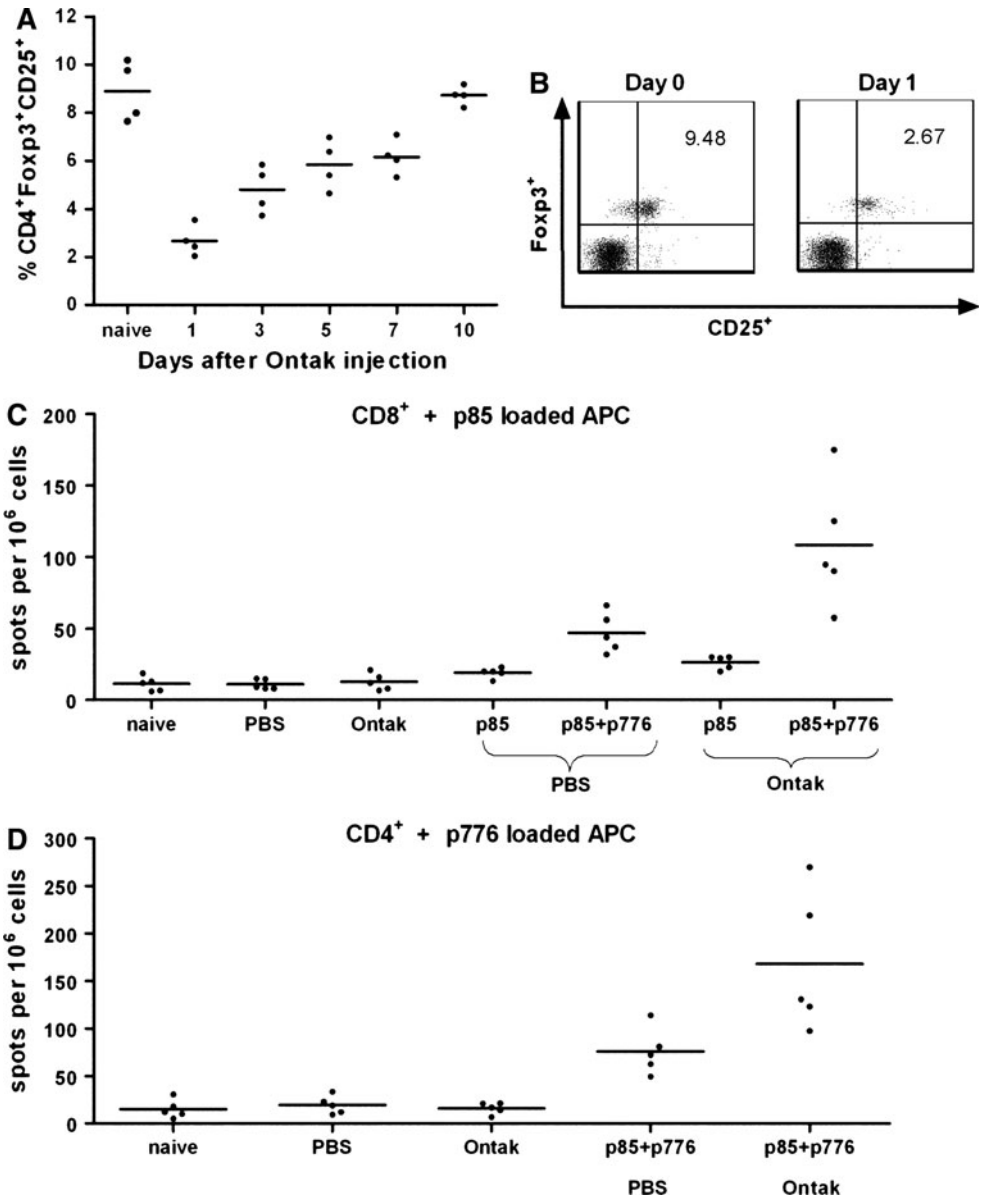

Fig. 1 Deletion of Tregs in vivo enables more efficient priming of $CD8^+$ and $CD4^+$ T cells against the peptide-combination vaccine. **a** A single i.p. injection of 3 μg Ontak was administered to male A2.1/DR1 × *neuT*$^+$ mice. $CD25^+$ $Foxp3^+$ Tregs were gated on $CD3^+CD4^+$ peripheral blood mononuclear cells (PBMCs). Mice ($n = 4$ per group) were analyzed individually (*spots*) at the indicated days after injection. *Horizontal bars*: means for each group. **b** Representative plots for Tregs in untreated PBMCs (day 0) and for Tregs in PBMCs 1 day post in vivo-treatment with Ontak (day 1). **c**, **d** One day after Ontak (or PBS) treatment, mice were immunized as indicated (except for the group of naïve animals in all other groups, vaccinations were performed in IFA plus GM-CSF). Five days after the last injection, $CD8^+$ and $CD4^+$ splenic T cells were tested as responders in the IFNγ-based 48 h ELISPOT assay stimulated by irradiated syngeneic splenocytes as APC from untreated animals loaded with the indicated peptides. *Spots* indicate individual mice ($n = 4$ per group). *Horizontal bars*: means for each group

with p85 upon in vitro stimulation with p85-pulsed syngeneic splenocytes as antigen-presenting cells (APC) ($P = 0.035$ and $P = 0.0369$ compared to control mice injected either with PBS or with Ontak alone, respectively). This finding was expected given that these mice are tolerant for HER-2/*neu*, and, according to our recently published data [23], they develop only weak anti-HER-2/*neu* immunity when vaccinated with single peptides representing HER-2/*neu* epitopes. Treatment of p85-vaccinated animals with Ontak significantly enhanced the $CD8^+$ T-cell-mediated IFNγ response to p85 ($P = 0.0112$ vs. PBS + p85; Fig. 1c). By vaccinating with a combination of p85 and p776 (without Ontak treatment), we also succeeded to significantly increase the IFNγ response to p85 ($P = 0.0012$ vs. PBS + p85; Fig. 1c). However, a remarkable increase of the anti-p85 response was achieved when we combined Ontak with p85 plus p776 vaccination ($P = 0.0092$ vs. PBS + p85 + p776; Fig. 1c). A similar immune response pattern was also observed when analyzing $CD4^+$ T-cell responses: the number of IFNγ producing $CD4^+$ T cells in response to the immunizing peptide p776 was significantly enhanced when animals were treated with Ontak as opposed to PBS treatment ($P = 0.0141$; Fig. 1d).

Analyses of phenotype and functionality of Tregs recovered after Ontak treatment

To examine whether the Tregs that recovered after Ontak treatment are adversely affected, we examined the effect of Ontak on the expression of homing receptors as well as on functionality of Tregs. We have repeated the same protocol as in Fig. 1a, and mice were killed on day 10 after Ontak

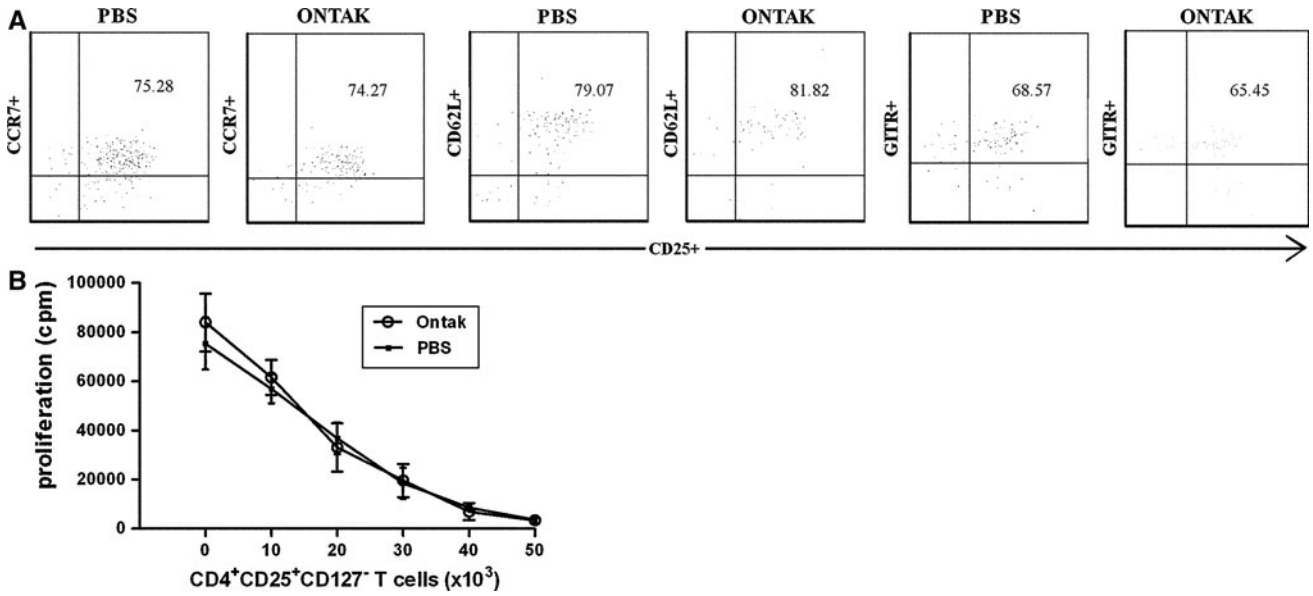

Fig. 2 Phenotype analyses of Tregs 10 days post Ontak or PBS administration. A2.1/DR1 × *neuT*$^+$ mice were treated with Ontak (or PBS) and 10 days later their splenic Tregs were analyzed phenotypically (results from one representative experiment of 3 performed are shown) (**a**), or functionally (**b**). In **b**, Tregs and Teffs were stimulated with irradiated T-cell-depleted syngeneic splenocytes and 10 μg/mL anti-CD3. Mean values ± SD from pooled data (4 animals were tested)

administration at which time point their levels were normalized. Ex vivo analysis of the levels of expression of homing receptors CCR7, CD62L and of activation marker GITR on Tregs showed no differences with those from PBS-treated mice (Fig. 2a). To examine functionality, Tregs and CD4$^+$CD25$^-$ T cells were isolated 10 days after Ontak treatment and were co-cultured at various ratios. As shown in Fig. 2b, Tregs from Ontak-treated mice were as efficient as Tregs from PBS-treated mice in suppressing the proliferation of CD4$^+$CD25$^-$ T cells.

Effect of Ontak on the efficacy of a therapeutic antitumor vaccine

We next sought to assess whether the combination of p85 plus p776 could act as a potent therapeutic vaccine in A2/DR1 × *neuT*$^+$ triple Tg animals and if so, whether Ontak could potentiate the efficacy of this therapeutic vaccine. Ontak was administered 6 days after tumor cell (TUBO.A2) inoculation (at that time-point tumor growth was palpable). The vaccination protocol was applied 1 day post Ontak administration and for the following 10 days (three injections with peptides,or no peptides, in IFA plus GM-CSF every 5 days). Immunizing with p85 plus p776 proved to be efficient acting as a therapeutic vaccine : in 4 of 7 animals (57.14%) tumors regressed and in the remainders tumor growth was greatly delayed (tumors reached a size of >200 mm^2 100–120 days post vaccination) compared to the group of animals that received only the CTL (p85) vaccine (tumor size >200 mm^2 was reached in all 4 animals already 70 days post vaccination, and there were no tumor regressions) (Fig. 3a vs. b; $P = 0.0003$). This therapeutic efficacy of our combination vaccine was further potentiated when mice were treated with a single injection of Ontak: 5 of 7 animals (71.43%) were cured, whereas in the rest of the mice, tumor growth was significantly retarded (tumor size of 100 mm^2 was reached by day 120 post vaccination) compared to the group receiving the combination vaccine and PBS (Fig. 3c vs. b; $P < 0.0001$). Therapeutic efficacy with p85 was also significantly enhanced when mice were treated with Ontak (Fig. 3d vs. a; $P = 0.02$). In control mice that were treated with PBS and injected with GM-CSF and IFA (PBS group), the tumors grew fast and reached the critical size of >200 mm^2 already by days 25–30 (Fig. 3e). Interestingly, mice treated with Ontak and injected with GM-CSF and IFA (Ontak group) showed a trend for increased survival (Fig. 3f). In this group of animals, tumors grew slower compared to animals in the PBS group and reached the size of >200 mm^2 by days 35–50 ($P = 0.0645$). The delay in tumor growth in the group of mice vaccinated with p85 (Fig. 3a) was statistically significant compared to PBS ($P = 0.03$) or to Ontak ($P = 0.04$) groups.

Treatment with Ontak increases the ratio of vaccine-specific intratumor CD4$^+$ Teffs to Tregs

While a few studies have documented the capacity of Tregs to infiltrate tumors [28, 29], little is known with regard to the changes in cellular dynamics in the tumor that take

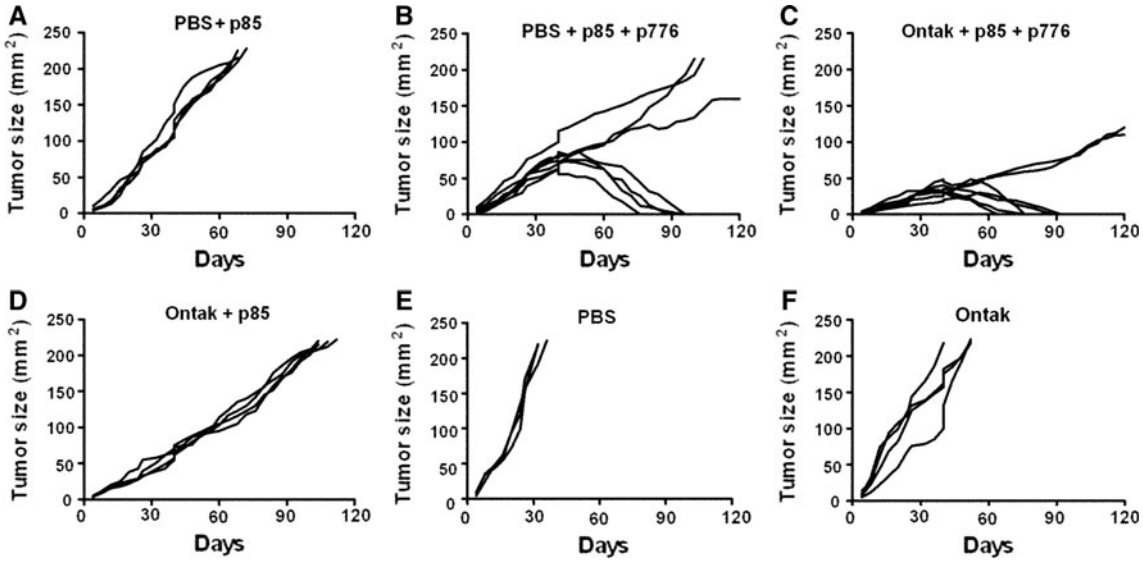

Fig. 3 Ontak improves the therapeutic efficacy of the peptide-combination vaccine. Male A2.1/DR1 × *neuT*$^+$ animals were challenged with TUBO.A2 transplantable tumors, followed by injection 6 days later with Ontak (or PBS). Vaccinations with the indicated peptides in IFA plus GM-CSF were initiated 1 day later. Control mice were treated with PBS (PBS) or Ontak (Ontak) and injected with GM-CSF and IFA. *Curves*: individual mice

place upon antitumor therapeutic intervention. Hence, we studied the impact of Ontak/peptide-combination vaccine on the p776-specific (HLA-DR1/p776$^+$; Tm$^+$) CD4$^+$Foxp3$^-$ Teffs and on Tregs infiltrating the TUBO.A2-induced tumor. Tumors from mice with progressively growing tumors (Fig. 4) were harvested at a time period when these reached a size of approximately 50 mm^2. In mice that finally rejected their tumors, these were removed at an early time point following the beginning of their regression. In the group of animals receiving ONTAK, regression rates were faster, usually starting between days 30–40 and by tumor size of 30–50 mm^2 (Fig. 3c) as compared to the group treated with PBS where tumor regression was noted between days 40–50 when tumors had reached the size of 60–80 mm^2 (Fig. 3b). As shown in Fig. 4a and b, the percentage of Tregs in TILs was significantly reduced upon immunization with p85 plus p776 [$P = 0.0005$ compared to control mice (GM-CSF plus IFA but no vaccine) or mice injected with p85 alone], and this decrease was more intense when mice were additionally treated with Ontak ($P = 0.0089$ compared to PBS + p85 + p776). There was no significant difference in the numbers of Tregs TIL between non-vaccinated control animals and those receiving only p85 (both in PBS and Ontak groups). Ontak/peptide-combination vaccine therapy caused a significant enhancement in the percentages of p776-specific Teffs TIL as compared to PBS plus the peptide-combination vaccine ($P = 0.0136$; Fig. 4c, d). In this way, the ratio of the p776-specific Teffs to Tregs was also increased ($P = 0.005$; Fig. 4e) and correlated well with the kinetics of tumor rejection in these groups of mice (Fig. 3b vs. c). Tregs TIL from both Ontak-treated or non-tretaed animals expressed the GITR activation marker at equal levels (Supplementary Fig. 1). The majority of Tregs TIL were apoptotic (Fas$^+$ Annexin V$^+$) (Fig. 5a, b) as a consequence of their interaction with the FasL$^+$ Teffs TIL (Supplementary Fig. 2 and ref. 23]. The % of Annexin V$^+$ Tregs was higher in the group of p85 + p776-vaccinated animals treated with Ontak as opposed to the vaccinated and PBS-treated group (Fig. 5a, b; $P = 0.0004$). This can be explained by the increased numbers of Teffs TIL in the Ontak-treated group (see previous Fig. 4c, d) given that in both groups, FasL was expressed at equal levels on CD4$^+$Foxp3$^-$ TeffsTIL (Supplementary Fig. 2). In contrast, only low levels of Annexin V$^+$ Fas$^+$ CD4$^+$Foxp3$^-$ Teffs could be detected in both Ontak- and PBS-treated mice (Fig. 5c, d).

Ontak/peptide-combination vaccine therapy markedly increases intratumor p85-specific CD8$^+$ CTLs

Analysis of TIL in the group of p85 plus p776-vaccinated animals, which were treated with PBS, showed more than 2.5% CD8$^+$HLA-A2.1/p85$^+$ cells compared to only 0.6% when mice were vaccinated with p85 alone ($P < 0.0001$; Fig. 6a). Upon Ontak treatment, the percentage of p85-specific CD8$^+$ TIL in p85-vaccinated animals reached 2.5% ($P < 0.0001$ compared to animals receiving PBS + p85), whereas in p85 plus p776-vaccinated animals, we scored almost 4% of p85-specific TIL ($P = 0.0234$ compared to PBS + p85 + p776; Fig. 6a, b). As a consequence, the ratio of p85-specific CD8$^+$ TIL to Tregs increased fivefold, from an average of 0.04 in the PBS + p85 group to 0.2 in the

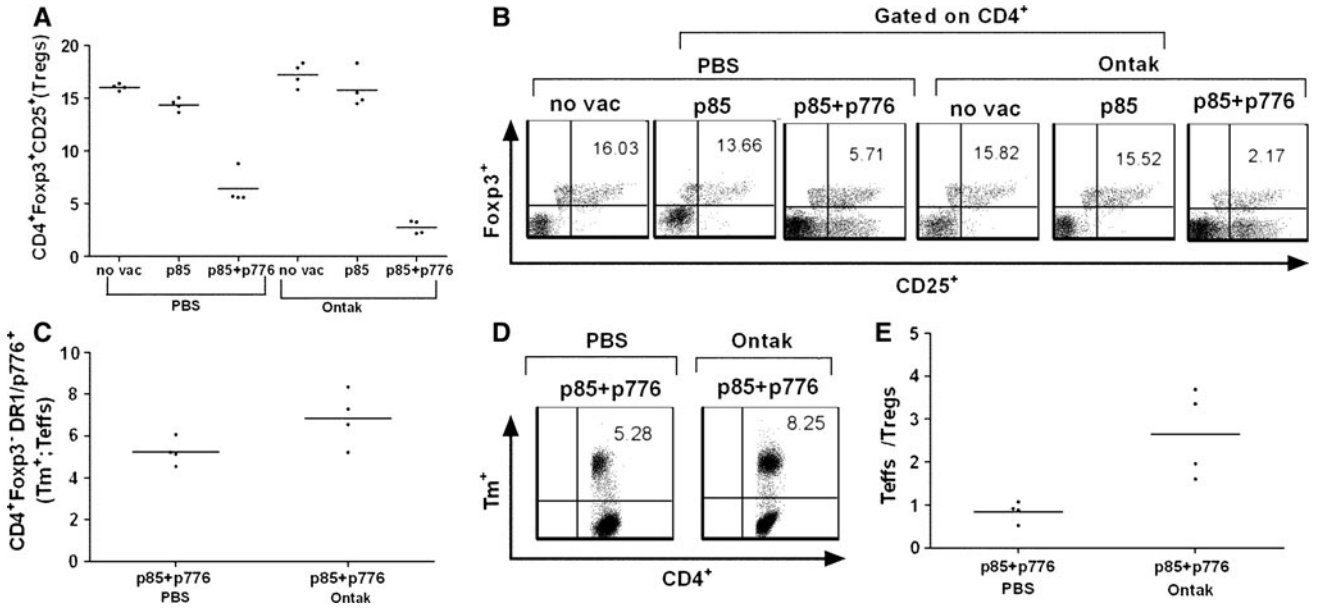

Fig. 4 Ontak/peptide-combination vaccine therapy increases the Teffs/Tregs ratio among TIL. **a** A2.1/DR1 × *neuT*$^+$ mice were injected as indicated. Percentages of Tregs TIL in individually ($n = 4$) analyzed mice (*spots*). **b** Representative dot plots for Tregs from the groups shown in (**a**). **c** Percentages of HLA-DR1-restricted and HER-2 (776–790)-specific (DR1/p776$^+$) (tetramer-positive; Tm$^+$) CD4$^+$ Foxp3$^-$ Teffs TIL in individually analyzed mice. **d** Representative dot plots for Teffs from the groups shown in (**c**). **e** Teffs to Tregs ratios in the group of animals vaccinated with the peptide-combination vaccine after treatment with Ontak or PBS

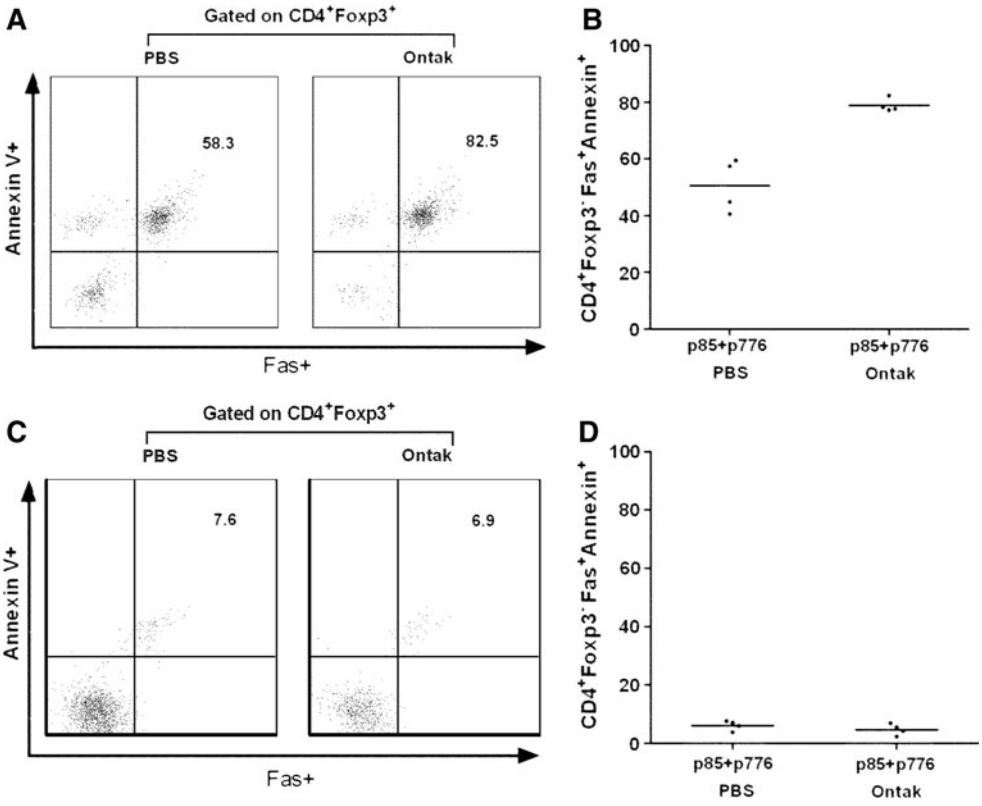

Fig. 5 Apoptotic CD4$^+$ TIL in *neuT*$^+$ mice immunized with the peptide-combination vaccine after treatment with PBS or with Ontak. Representative plots and values from individually analyzed mice ($n = 4$) are shown for Annexin V$^+$ Fas$^+$ cells gated on CD4$^+$Foxp3$^+$ Tregs (**a**, **b**) or on CD4$^+$Foxp3$^-$ Teffs (**c**, **d**)

Ontak + p85 group ($P = 0.0002$) and more than threefold, from 0.43 to 1.47, upon vaccination with both peptides without or with Ontak treatment, respectively ($P = 0.0007$; Fig. 6c). The result of this was more efficient tumor rejection (Fig. 3a–d). Moreover, CD8$^+$ TIL from animals receiving Ontak plus the peptide-combination vaccine mediated significantly higher cytotoxic responses against TUBO.A2 targets in vitro as compared to their counterparts from animals

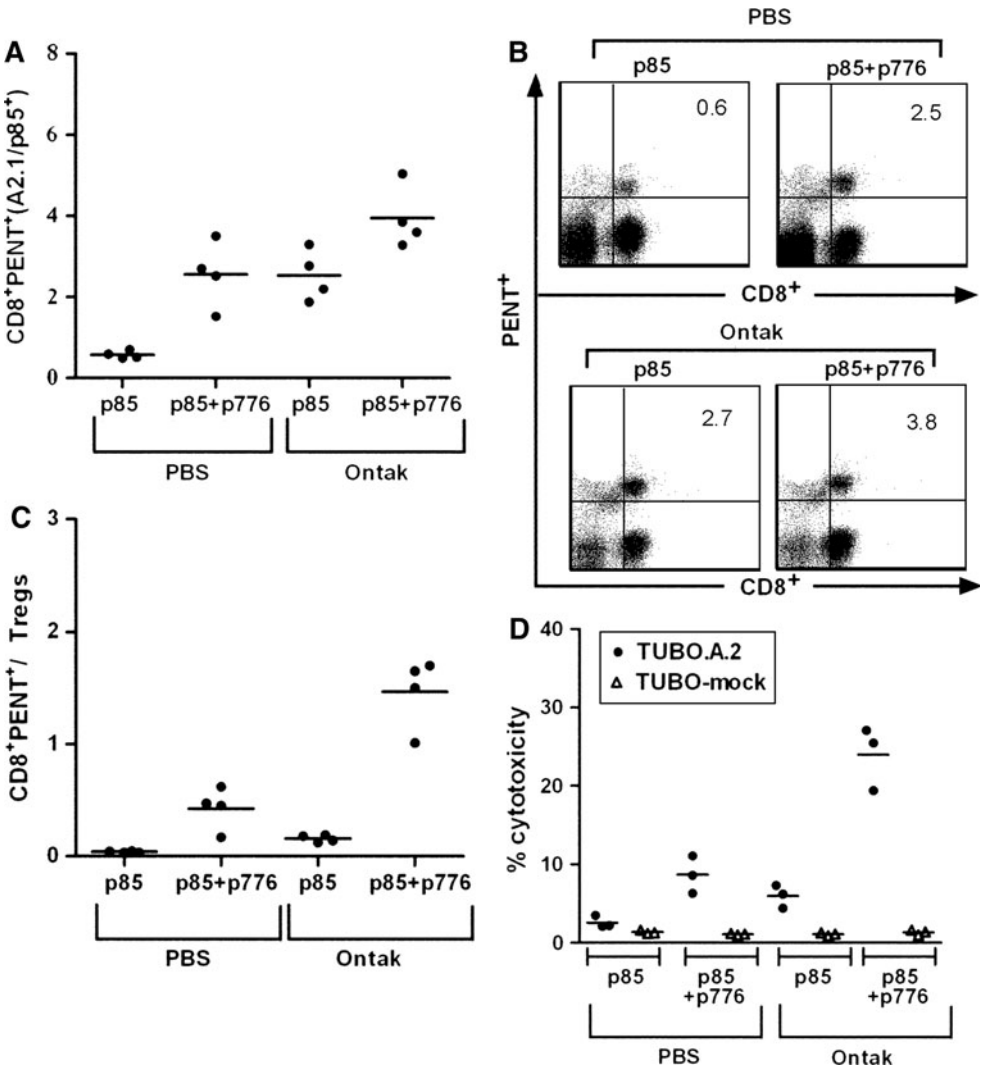

Fig. 6 Ontak/peptide-combination vaccine therapy increases the percentage of HLA-A2.1-restricted and HER-2 (85–94) specific (A2.1/p85$^+$) (pentamer-positive; PENT$^+$) CD8$^+$ TIL. **a** Individual and mean values from mice ($n = 4$) treated as indicated (analyses were performed in the same mice as in Fig. 4) and **b** representative *dot* plots for the groups shown in (**a**). **c** Ratios among CD8$^+$ PENT$^+$ to Tregs TIL in individually analyzed mice treated as indicated and mean values from these groups. Values for Tregs were taken from Fig. 4. **d** CD8$^+$ TIL from the indicated groups of mice were tested as cytotoxic effectors against TUBO.A2 and TUBO-mock transfectants

without Ontak treatment (24% vs. 8.67%; $P < 0.0001$) (Fig. 6d). A much lower cytotoxic effect was detected in CD8$^+$ TIL from Ontak-treated animals vaccinated with p85 (average 6%; $P = 0.0005$, compared to Ontak/p85 + p776 treatment) which, however, was significantly higher compared to PBS-treated animals vaccinated with p85 alone (2.6%; $P = 0.0125$; Fig. 6d).

Discussion

We have used the HER-2/*neu*-tolerant A2.1/DR1 × *neuT*$^+$ males and the HER-2/*neu*$^+$ HLA-A2.1$^+$ TUBO mammary adenocarcinoma as an in vivo model to analyze the impact of Ontak/peptide-combination vaccine therapy on the priming of peripheral vaccine-specific T cells as well as on the dynamics of vaccine-specific CTLs, Teffs and Tregs in the tumor. Our results have a significant relevance for our understanding of the mechanisms by which Ontak-mediated depletion of Tregs regulates responses of Teffs and CTLs to the vaccine and provide insights in the way in which this immunotherapeutic approach affects the interplay of these cells in enhancing tumor rejection. First, we show that treatment with Ontak decreases to a great extent the number of circulating Tregs. Second, Ontak treatment has a positive impact on the frequencies of peripheral vaccine peptide-specific CD4$^+$ and CD8$^+$ T cells. Third, a significantly increased percentage of Ontak-treated and vaccinated animals rejects their tumors compared to vaccinated animals only. Finally, TIL of mice that received Ontak/combination-peptide vaccine therapy have increased Teffs/Tregs and CTLs/Tregs ratios.

There are contradictory data regarding the prognostic value of Tregs presence in tumors. Several studies have reported a correlation between the number of Tregs and favorable clinical outcome [30–34], whereas in others, Tregs were associated with poor prognosis [28, 35–38]. A better understanding of both phenotype and function of Tregs in the tumor microenvironment could help establish a more reliable prognostic role of Tregs TIL in patients with

solid tumors. Furthermore, such an understanding would be essential for the development of targeted approaches eliminating or functionally modulating Tregs, thereby specifically promoting antitumor immune responses. In the studies reported here, we first investigated the kinetics of Tregs depletion in our triple transgenic mice. We have shown that a single intraperitoneal injection of Ontak was sufficient to considerably decrease the frequency of Tregs in peripheral blood of treated mice. The reduction was evident within 24 h of Ontak injection and lasted approximately 10 days, with gradual recovery of the Tregs. Our results are in line with recent reports showing similar kinetics of Tregs frequencies in the periphery and spleen with a single dose of Ontak [21, 22]. We have not found any functional differences among Tregs from Ontak-treated (10 days post Ontak treatment) and non-treated animals since both could highly suppress proliferative responses mediated by autologous anti-CD3-stimulated $CD4^+CD25^-$ T cells. Most important, our data show that this transient decrease in peripheral Tregs after Ontak treatment was enough to allow a better priming against the vaccine which was shown by the increased frequencies of circulating $CD8^+$ and $CD4^+$ T cells producing IFNγ upon stimulation with p85 or p776, respectively. Moreover, based on our recent observations [23] and the data from this report, we would like to suggest that the decreased frequencies of Tregs within the tumor microenvironment in the Ontak-treated animals could be explained on a quantitative basis due to the higher numbers of vaccine-specific $FasL^+$ $CD4^+$Teffs TIL which more effectively induce apoptosis in Fas^+Tregs via FasL-Fas interaction. Thus, the increased frequencies of vaccine-specific and functionally active $CD8^+$ CTL at the tumor site may be a result of the increased Teffs TIL or the decreased Tregs or a combination of both.

Tregs may be recruited from the periphery and accumulate at the tumor. Most peripheral blood Tregs express the lymphocyte homing markers CD62L and CCR7 [39]. This enables them to migrate from the periphery to lymphoid organs as well as to the tumor. We have shown that splenic Tregs recovered after Ontak treatment express similar levels of CD62L and CCR7 as compared to the levels in Tregs from animals non-treated with Ontak, demonstrating that Ontak treatment does not negatively affect the migratory capacity of Tregs. Notably, we found that the vast majority of Tregs in TIL are $GITR^+$ (supplementary Fig. 1B), although significantly less peripheral Tregs expressed GITR (Fig. 2a), suggesting that Tregs at the tumor site acquire GITR expression perhaps as consequence of local activation. Whether this activation involves conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ Tregs in situ remains at present unknown. Thus, our data suggest that there is a considerable change in the dynamic balance of infiltrating lymphocytes (i.e., increased ratios of CTLs and Teffs to Tregs) that seems to be responsible for the improved therapeutic efficacy mediated by the Ontak/peptide-combination vaccine treatment.

ONTAK has been successfully applied (objective response rate 44%) in patients with cutaneous T-cell lymphoma [40] by a mechanism involving elimination of neoplastic targets cells and Tregs expressing high levels of CD25 [41]. In patients with stage IV melanoma, one cycle of ONTAK administration caused a transient depletion of Tregs and regression of hepatic and pulmonary metastases [42, 43]. Interestingly, patients who responded to ONTAK treatment had increased percentages of melanoma antigen (MART-1)-specific peripheral $CD8^+$ T lymphocytes [42]. ONTA-mediated elimination of Tregs followed by vaccination with RNA-tranfected dendritic cells significantly improved the stimulation of tumor-specific T-cell responses in patients with renal cancer when compared to vaccination alone [44]. For Tregs depletion in humans, besides Ontak, several reagents are employed. For example, low-dose cyclophosphamide potentiates the antitumor effects of therapeutic cancer vaccines, with a mechanism appearing to involve population of Tregs [45]. Recent studies with a limited number of cancer patients indicate that daclizumab can reduce the frequency of $Foxp3^+$ Tregs in peripheral blood, but the efficiency of treatment with this antibody remains to be determined [46]. However, independently of the modalities applied, for an effective clinical immunotherapy of cancer, it would be desirable to employ methods that allow high-level depletion of Tregs, which, in conjunction with treatment strategies such as vaccination, may concomitantly increase T-cell activation and infiltration in the tumor. In the current work, we provide a two-stage elimination of Tregs that allow the development of robust immunity in HER-2/*neu*-tolerant animals against the growth of HER-2/neu^+ TUBO.A2 tumor cells. In the first stage (priming phase), Tregs are eliminated upon Ontak treatment : the first vaccination is given one day post Ontak treatment when Tregs number is reduced by almost 70%, which results in a better priming during the following two vaccinations thus allowing the generation of higher frequencies of vaccine-specific $CD8^+$ and $CD4^+$ T cells. The gradually increasing numbers of Tregs beyond day 1 post Ontak treatment and up to day 10 when they reach normal levels (i.e., day 9 post begin of immunizations) should not greatly interfere with the process of priming given that 1 week later the frequencies of vaccine-specific $CD4^+$ and $CD8^+$ T cells are considerably higher compared to PBS-treated mice (Fig. 1b). In the second stage (effector phase; begin of tumor regression), Tregs are eliminated within the tumor microenvironment through Fas–FasL interactions with the $FasL^+$ $CD4^+$ Teffs that have infiltrated the tumor, allowing for a more effective collaboration with the vaccine-specific $CD8^+$ TIL resulting in enhanced tumor killing. Thus, one

single injection with Ontak is enough to generate sufficient help for eliminating Tregs at later stages of tumor development without the need of repeated injections which may also cause a reduction in the numbers of $CD4^+$ Teffs. The data presented herein do not address a direct involvement of Teffs in tumor cell killing although the possibility exists that this could be feasible given the capacity of $CD4^+$ to also express perforin and granzymes [47].

We believe that the data presented in this article shed light on the mechanisms by which Ontak/vaccine therapy induces antitumor activity. Our data are consistent with a two-stage depletion of Tregs resulting in the expansion of CTLs and Teffs in response to the peptide-combination vaccine. The studies presented herein thus provide the proof of concept for clinical studies in the use of Ontak combined with vaccination therapy.

Conflict of interest The authors declare that they have no conflict of interest.

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