

Inflammasome Reporter Cells

All you have to do is ASC

InvivoGen



This information is current as of July 22, 2018.

CD25⁺ Regulatory T Cell Depletion Augments Immunotherapy of Micrometastases by an IL-21-Secreting Cellular Vaccine

Alberto Comes, Ombretta Rosso, Anna Maria Orenco, Emma Di Carlo, Carlo Sorrentino, Raffaella Meazza, Tiziana Piazza, Barbara Valzasina, Patrizia Nanni, Mario P. Colombo and Silvano Ferrini

J Immunol 2006; 176:1750-1758; ;
doi: 10.4049/jimmunol.176.3.1750
<http://www.jimmunol.org/content/176/3/1750>

References This article **cites 51 articles**, 25 of which you can access for free at:
<http://www.jimmunol.org/content/176/3/1750.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



CD25⁺ Regulatory T Cell Depletion Augments Immunotherapy of Micrometastases by an IL-21-Secreting Cellular Vaccine¹

Alberto Comes,^{2*} Ombretta Rosso,^{2*} Anna Maria Orengo,^{*} Emma Di Carlo,[†] Carlo Sorrentino,[†] Raffaella Meazza,[‡] Tiziana Piazza,^{*} Barbara Valzasina,[§] Patrizia Nanni,^{||} Mario P. Colombo,[§] and Silvano Ferrini^{3*}

IL-21 is an IL-2-like cytokine, signaling through a specific IL-21R and the IL-2R γ -chain. Because the TS/A mammary adenocarcinoma cells genetically modified to secrete IL-21 (TS/A-IL-21) are strongly immunogenic in syngeneic mice, we analyzed their application as vaccine. In mice bearing TS/A-parental cell (pc) micrometastases, vaccination with irradiated TS/A-IL-21 cells significantly increased the animal life span, but cured only 17% of mice. Spleen cells from cured mice developed CTL activity and produced IFN- γ in response to stimulation by the AH1 epitope of the gp70env Ag of TS/A-pc. We tested whether the low therapeutic outcome might be due to CD4⁺CD25⁺ regulatory T cells (Treg) present in TS/A-pc tumors and draining lymph nodes and whether IL-21 had any effect on these cells. Indeed, CD4⁺CD25⁺ cells suppressed IFN- γ production by splenocytes from immune mice in response to stimulation by the AH1 peptide. Low concentrations of IL-21 (10 ng/ml) failed to reverse the inhibitory activity of CD4⁺CD25⁺ cells in an allogeneic MLR, whereas 60 ng/ml rIL-21 partially restored responder T cell proliferation. IL-21R expression on CD25⁺ lymphocytes suggested that IL-21 could be more effective in mice depleted of CD25⁺ cells. Depletion of Treg cells by a single dose of anti-CD25 mAb combined with TS/A-IL-21 cell vaccine cured >70% of mice bearing micrometastases, whereas anti-CD25 mAb treatment alone had no effect. Successful combined immunotherapy required NK cells, CD8⁺ T cells, and IFN- γ . In conclusion, immunotherapy of micrometastases by an IL-21-based cellular vaccine is strongly potentiated by CD25⁺ cell depletion. *The Journal of Immunology*, 2006, 176: 1750–1758.

It is well known that IL-2 exerts therapeutic activity in a fraction of patients with melanoma or renal cancer, with 5 and 9% complete response rates in metastatic patients receiving a high dose i.v. bolus (1, 2). However, the majority of patients failed to respond to IL-2 therapy. The identification of a subset of regulatory T cells (Treg),⁴ which coexpress CD4 and the IL-2R α -chain or CD25 and display potent immune-suppressive activity on T cell function (3–5), suggested that IL-2 may have opposite effects, activating both effector functions and regulatory mechanisms. IL-2-mediated immune regulatory effects involve not only Treg, but also activation-induced cell death of effector cells (6).

Indeed, CD25^{−/−}, CD122 (IL-2R β)^{−/−}, and IL-2^{−/−} mice show lymphoproliferation and develop autoimmune diseases (7–10), indicating that IL-2 has a primary role in immune regulation (11, 12). Natural CD4⁺CD25⁺ Tregs are a distinct lineage of T cells, expressing glucocorticoid-induced TNFR (GITR) (13, 14), CTLA4 (15), IL-10, TGF- β (5), and the transcription factor Forkhead/winged helix transcription factor (Foxp3) (16–18) and physiologically control the immune system homeostasis, preventing autoimmune disorders (3, 4). Several other lines of evidences indicate that not only naturally occurring, but also tumor-induced, Tregs, are involved in tumor-related immune suppression (5). Thus, removal of CD4⁺CD25⁺ cells by depleting Abs (19, 20) or interfering with their functions by CTLA4 blockade (21) or GITR signaling (22) may synergize with different immunotherapy strategies aimed at stimulating immune responses (23).

IL-21, the last member of the IL-2 cytokine family and sharing functional overlaps with IL-2 and IL-15, may represent a new tool for tumor immunotherapy (24–26). Although IL-2 is required for tolerance, enhanced production of IL-21 has been implicated in the development of autoimmunity (27, 28). The biological activities of IL-21 are mediated by a specific IL-21R (24, 29), which associates with the common γ -chain for signal transduction (30) and is independent from CD25 for its function. IL-21 induces activated NK cell terminal differentiation and functions, thereby limiting NK cell expansion by IL-15 (31). In addition, IL-21 efficiently promotes proliferation, cytotoxic activity, and IFN- γ production by murine and human CD8⁺ effector T cells (32). In view of these findings, it has been proposed that IL-21 drives the transition from NK cell responses to specific CTL responses (31).

Previous studies in different murine models showed that IL-21 gene transfer in tumor cells induces tumor rejection, which depends

*Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; [†]Dipartimento di Oncologia e Neuroscienze, Sezione di Anatomia Patologica, Università G. d'Annunzio and the Aging Research Center, Fondazione Universitaria G. d'Annunzio, Chieti, Italy; [‡]Istituto Giannina Gaslini, Genoa, Italy; [§]Istituto Nazionale Tumori, Milan, Italy; and ^{||}Sezione di Cancrologia, Dipartimento di Patologia Sperimentale, Università di Bologna, Bologna, Italy

Received for publication June 16, 2005. Accepted for publication November 21, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants awarded by the Italian Association for Cancer Research, the Italian Ministry of the University and Research, Ministry of Health, Fondazione Cassa di Risparmio della Provincia di Chieti, and Comitato Interministeriale per la Programmazione Economica.

² A.C. and O.R. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Silvano Ferrini, c/o Centro di Biotecnologie Avanzate, Largo Rosanna Benzi no. 10, 16132 Genova, Italy. E-mail address: silvano.ferrini@istge.it

⁴ Abbreviations used in this paper: Treg, regulatory T cell; Foxp3, Forkhead/winged helix transcription factor; GITR, glucocorticoid-induced TNFR; LN, lymph node; pc, parental cell; TIL, tumor-infiltrating lymphocyte; TS/A-IL-21, TS/A cells transfected with the IL-21 gene; TS/A-pc, TS/A parental cells.

predominantly on NK cells (33) and/or CTLs (34–36). In particular, we reported that TS/A tumor cells genetically modified to secrete IL-21 displayed a reduced tumorigenic potential in syngeneic mice and primed a protective immune response mediated by CD8⁺ CTLs, IFN- γ , and IFN- γ -inducible CXC chemokines (35).

In the present report we show that immunotherapy with irradiated TS/A cells genetically modified to secrete IL-21 (TS/A-IL-21) can inhibit the progression of TS/A-parental cell (pc) micrometastases, although only a fraction of mice are cured over the long term. The presence of CD4⁺CD25⁺ T cells with strong immunosuppressive activity in tumors and draining lymph nodes (LN) suggested that IL-21 only partially overcomes Treg-related immune suppression. However, the effect of the IL-21-engineered cell vaccine was strongly potentiated by the coadministration of a Treg-depleting anti-CD25 mAb and led to the cure of most mice bearing TS/A-pc micrometastases.

Materials and Methods

Cell lines and IL-21-modified tumor cells

TS/A murine breast adenocarcinoma (37), C26 colon carcinoma, and F1F fibrosarcoma cells were cultured in DMEM supplemented with 2 mM L-glutamine, 1% PenStrep (Cambrex), and 10% FCS (Cambrex). The TS/A-IL-21 stable transfectant was previously described (35). F1F cells were transfected with 10 μ g of pIL-21IRES1neo plasmid using the FuGene 6 reagent (Roche) and cloned (35). IL-21 secretion was assessed by ELISA (R&D Systems) (33).

TS/A-pc micrometastasis induction and immunotherapy

Five- to 7-wk-old female BALB/cAnNCrIBR (BALB/c) mice were purchased from Harlan Italy. IFN- γ knockout mice (38) on a BALB/c background were obtained from The Jackson Laboratory. Homozygous mice were bred and maintained in isolators in-house. The animals were housed in a pathogen-free colony. Experiments were performed according to the National Regulation on Animal Research Resources and were approved by the institutional review board of the Istituto Nazionale per la Ricerca sul Cancro (Genova, Italy). Mice were inspected every day and were killed when they showed weight loss (>15%) or other signs of disease. The presence of metastases was autopsically confirmed.

Animals (five to seven mice per group) were injected i.v. with 5×10^4 TS/A-pc/mouse for induction of micrometastases. Vaccination was performed on days 1, 3, 7, and 10 after micrometastasis induction (day 0) by s.c. injection of 1×10^6 irradiated TS/A-IL-21, TS/A-pc, or F1F-IL-21. In vitro experiments showed that irradiated cells retained the ability to secrete IL-21 for at least 72 h. Cells were mycoplasma-free and were extensively washed in endotoxin-free medium before injection. Statistical analysis was performed by the log-rank or Mann-Whitney tests; a value of $p < 0.05$ was considered significant.

Depletion studies were performed by i.p. injection of anti-CD8 (2.43) or anti-CD4 (GK1.5) rat mAbs, both from American Type Culture Collection, or of anti-asialo GM1 antiserum (Wako Chemicals) as previously reported (39). CD25⁺ cell depletion was performed by a single dose of 100 or 500 μ g of PC61 mAb (American Type Culture Collection) administered i.p. 24 h (or 48 in delayed-onset immunotherapy, as indicated) after micrometastasis induction, followed 6 h later by the first vaccination with irradiated TS/A-IL-21 cells. Control animals received irrelevant rat IgG.

Immunohistochemistry and two-color immunofluorescence

Groups of three BALB/c mice were killed 10 days after s.c. TS/A-pc inoculation or 17 days after induction of lung metastases.

For immunohistochemistry, acetone-fixed cryostat sections were immunostained with anti-CD4 (L3T4; clone YT5.191.1.2; Sera-lab) or anti-CD25 Ab (anti-IL-2R α ; clone 3C7; Santa Cruz Biotechnology). After washing, sections were overlaid with biotinylated goat anti-rat Ig (Vector Laboratories) for 30 min, washed, and incubated with avidin/biotin complex/alkaline phosphatase (DakoCytomation).

For double-immunofluorescent staining, acetone-fixed frozen sections were incubated for 30 min with the primary (rabbit) anti-CD3 (DakoCytomation) or anti-CD4 Ab and washed in PBS for 5 min. Next, sections were incubated for 30 min with biotinylated secondary Ab, washed, and incubated with AlexaFluor 488-conjugated streptavidin (Molecular Probes; 1/800) for 20–30 min. After washing, sections were incubated for 30 min with the second PE-conjugated anti-CD25 Ab (anti-IL-2R α ; clone 3C7;

Santa Cruz, Biotechnology) and then washed. Slides were examined with a Zeiss LSM 510 Meta laser scanning confocal microscope.

Identification and isolation of CD4⁺CD25⁺ cells

Cell suspensions from s.c. tumor draining LN were prepared by mesh grids. Tumor-infiltrating lymphocytes (TIL) from TS/A-pc s.c. tumor nodules were obtained by digestion with proteolytic enzymes and Ficoll density gradient. After washings, cells were stained with anti-CD4-FITC and anti-CD25-PE mAbs (BD Pharmingen) or FITC- or PE-labeled Ig control mAbs in the presence of Fc blocker (BD Pharmingen) and analyzed by flow cytometry using a FACScan analyzer (BD Pharmingen). For the detection of IL-21R, a polyclonal rabbit anti-IL-21R (ab13268; Abcam) and a goat FITC-conjugated goat anti-rabbit Ig (Santa Cruz Biotechnology) were used. Foxp3 expression was detected on permeabilized cell suspensions using an FITC-labeled anti-Foxp3 rat mAb or FITC-labeled rat IgG2a isotype control (all from eBioscience).

CD4⁺CD25⁺ or CD4⁺CD25[−] cell fractions were isolated by a two-step immunomagnetic procedure (Miltenyi Biotec). Briefly, negative selection was performed with a mixture of biotin-conjugated anti-CD8, -CD11b, -CD45R, -CD49b, and -Ter-119 mAbs and anti-biotin Ab-conjugated magnetic beads, followed by a second step of positive selection by an anti-CD25-PE mAb and anti-PE Ab-conjugated microbeads. Purity was >90%, as assessed by immunofluorescence.

Inhibition of T cell responses by Tregs

CD4⁺CD25[−] (10^5) responder splenocytes from naive BALB/c mice (H2^d) were plated in the presence of various numbers of CD4⁺CD25⁺ or CD4⁺CD25[−] cells and 10^5 lethally irradiated stimulator splenocytes from C57BL/6j mice (H2^b) in triplicate wells of 96-well plates. Experimental controls were the responder and stimulator combination, Treg and stimulator, only stimulator, or only responder cells. Proliferation was measured after 5 days of culture by an additional pulse with 0.5 μ Ci/well [³H]thymidine (Amersham Biosciences). Cultures were then harvested onto glass-fiber filters and counted in a beta counter.

RT-PCR analysis of Foxp-3, GITR, IL-21R, and IL-10 expression

Total RNA was extracted from CD4⁺CD25⁺ and CD4⁺CD25[−] cell fractions using the RNeasy kit (Qiagen). RT-PCR was performed as previously described, using primers specific for the housekeeping gene β -actin (35) or for Foxp-3 (5'-CAG CTG CCT ACA GTG CCC CTA-3' and 5'-CAT TTG CCA GCA GTG GGT AG-3'), GITR (5'-TCT CGA TGC TCT GTG TGC TG-3' and CGT GGC ACA GGC AAC ACA C-3'), IL-21R (5'-CCAC CTCAAACCTTCACCTC and 5'-TGCTCTCAGCCAGGACAAAG), or IL-10 (5'-GCT ATG CTG CCT GCT CTT AC-3' and 5'-ACT CTT CAC CTG CTC CAC TG-3') genes. The amplifications were conducted for 32 or 27 cycles (for β -actin).

In vitro restimulation and CTL assay

Spleen cells from cured mice (10^6 cells/ml) were restimulated in vitro for 5 days at 37°C in the presence of the gp70-derived L^d-restricted AH1 peptide (40, 41) (SPSYVYHQF; Primm) at 1 μ g/ml. Cytolytic activity was evaluated by a standard ⁵¹Cr release assay using F1F, C26, and TS/A-pc as target cells.

ELISPOT assay was performed on multiscreen Immobilon-P plates (Millipore) coated with anti-IFN- γ Ab (Endogen). Splenocytes were seeded at 2×10^5 /well in duplicate in the presence of relevant or irrelevant peptides. After 36 h, plates were washed and incubated with biotinylated second mAb to IFN- γ (Endogen). Then, HRP-conjugated streptavidin (1/5000) was added for another 2 h. After washings, the plates were stained with a 3-amino-9-ethyl-carbazole kit (Sigma-Aldrich), and spots were counted using a stereomicroscope.

The evaluation of Treg inhibitory activity on AH1-induced IFN- γ production was assessed by ELISPOT on spleen cells from naive or cured mice restimulated in vitro for 5 days with either AH1 peptide or an irrelevant peptide. Tumor draining LN CD4⁺CD25⁺ Tregs were added at a 10:1 responder/Treg ratio in the presence or the absence of 60 ng/ml IL-21.

Results

IL-21 gene-modified tumor cells display therapeutic activity against TS/A-pc micrometastases

The therapeutic potential of TS/A-IL-21 cells was assessed in a micrometastatic TS/A-pc tumor model. Mice were injected i.v. on day 0 with 5×10^4 viable TS/A-pc and were randomized into three

groups: the first received no therapy, the second received four s.c. injections of 10^6 irradiated TS/A-IL-21 cells starting from day 1, and the third received the same schedule, but using irradiated TS/A-pc as a control vaccine. Low, albeit detectable, levels of IL-21 (2.4 ± 1 ng/ml) were present in the serum of mice 48 h after the last TS/A-IL-21 injection, whereas IL-21 was below the detection limit in the serum of mice receiving TS/A-pc. All mice receiving no therapy developed signs of metastases within 27 days and showed massive metastatic dissemination in their lungs at necropsy (Fig. 1, A and B). Mice receiving immunotherapy with irradiated TS/A-IL-21 cells showed significantly increased tumor-free survival ($p < 0.0001$) and a sharp reduction in the number of lung metastases, especially those showing a late (>35 days) onset of symptoms (Fig. 1B). More importantly, 17% of TS/A-IL-21-treated mice achieved long-term survival (>150 days; Fig. 1A) and were resistant to subsequent s.c. rechallenge with a fully tumorigenic dose of TS/A-pc. In the group of mice receiving irradiated TS/A-pc as therapy, only a slight increase in mean survival time was observed ($p = \text{NS}$), and no mouse showed a long-lasting cure (Fig. 1A). Similarly, in a parallel experiment with a group of 10 mice, vaccination by TS/A cells transfected with the empty vector failed to cure any mice (data not shown).

Because the gp70env endogenous retroviral Ag is an immunodominant Ag of the TS/A tumor (40, 41), we analyzed whether mice cured by TS/A-IL-21 vaccine developed gp70-specific CTLs. Indeed, splenocytes from cured mice developed CTL activity against the gp70-expressing TS/A-pc and C26 carcinoma syngeneic cells upon restimulation in culture with the AH1 CTL epitope of gp70 (Fig. 1C), whereas syngeneic F1F cells lacking gp70env were not lysed. The higher sensitivity of C26 to CTL-mediated

lysis may be related to higher the MHC class I and gp70 expression levels in this cell line compared with TS/A-pc (40). Unstimulated splenocytes from cured mice or AH1-stimulated splenocytes from naive mice failed to lyse TS/A-pc or C26 cells (data not shown). The induction of an Ag-specific immune response in cured mice was confirmed by ELISPOT assay. Splenocytes from cured mice showed higher numbers of IFN- γ -producing cells upon AH1 peptide stimulation compared with splenocytes from naive mice ($p < 0.01$; Fig. 1D).

These data indicated the induction of an Ag-specific immune response by TS/A-IL-21 vaccination. Indeed, if IL-21-transduced F1F (F1F-IL-21) cells were used as a vaccine, only an increase in the mean survival time was observed ($p < 0.03$), but no animals were cured (Fig. 2B). In addition, no induction of AH1-specific CTLs was observed in splenocytes from these mice (data not shown). Because F1F-IL-21 secreted ~ 2 -fold more IL-21 than TS/A-IL-21 (Fig. 2A), these data indicate that both the relevant Ag (gp70) and IL-21 should be expressed by the cell vaccine to induce a therapeutic effect.

CD4⁺CD25⁺ T cells with Treg activity are present in TS/A-pc tumors and draining lymph nodes

The limited therapeutic activity of TS/A-IL-21 vaccine prompted us to investigate whether CD4⁺CD25⁺ Tregs play a role in this process. Indeed, CD25⁺ cells were detected on frozen sections of TS/A-pc s.c. tumors (Fig. 3) or of lung metastases (Fig. 3g) by immunofluorescence or immunohistochemistry. At early stages, CD25⁺ cells were found predominantly at the boundaries of tumor

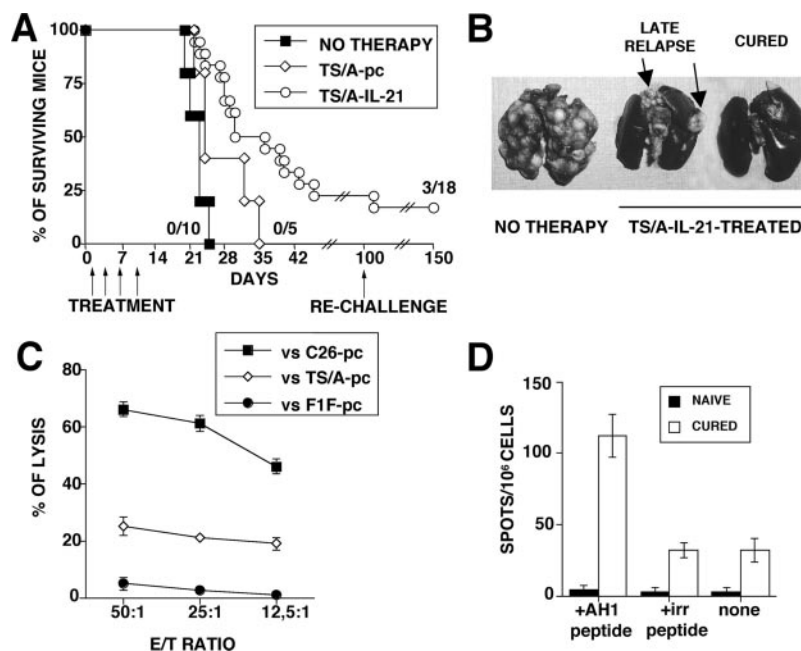


FIGURE 1. Vaccination with TS/A-IL-21 cells has a therapeutic effect on the development of TS/A-pc micrometastases in syngeneic mice. **A**, One day after induction of micrometastases by i.v. injection of 5×10^4 TS/A-pc, mice were randomized into three groups: the first was left untreated; the second was vaccinated with irradiated TS/A-IL-21 (1×10^6 TS/A-IL-21 s.c. on days 1, 3, 7, and 10 after challenge), and the third was given irradiated TS/A-pc, using the same doses and schedule described above. Data are expressed as tumor-free survival. TS/A-IL-21-vaccinated mice showed a statistically significant increase in survival rate ($p < 0.0001$ vs untreated; $p < 0.04$ vs TS/A-pc-vaccinated). **B**, Lungs from representative mice receiving no therapy or showing late (>35 days) or no signs of metastasis (>150 days survival). **C**, Cytolytic activity of splenocytes from mice cured by therapeutic vaccination performed after 6-day coculture with $1 \mu\text{g/ml}$ of the AH1 epitope of gp70env. Data are expressed as the percent lysis of ^{51}Cr -labeled TS/A-pc, C26 or F1F cells in a 4-h assay. Lysis of target cells by splenocytes stimulated with no peptide or with an irrelevant peptide showed CTL activity $<10\%$ (not shown). **D**, IFN- γ production assessed by an ELISPOT assay in the presence or the absence of AH1 gp70env epitope or with an irrelevant peptide. Splenocytes from naive mice (■) or from mice cured by immunotherapy (□) were restimulated for 36 h in the presence of $1 \mu\text{g/ml}$ of either peptide. Spots were counted using a stereomicroscope.

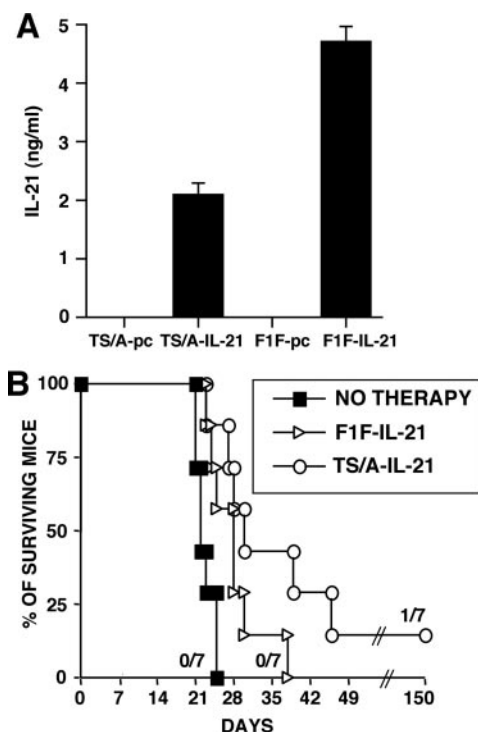


FIGURE 2. Vaccination with antigenically unrelated F1F-IL-21 cells has a limited effect on the development of TS/A-pc micrometastases in syngeneic mice. *A*, Production of IL-21 by TS/A-IL-21 and F1F-IL-21 cells detected by ELISA in 48-h cultures. Data are normalized for 10^6 cells/ml. *B*, One day after induction of micrometastases by i.v. injection of 5×10^4 TS/A-pc, mice were randomized into three groups: the first was left untreated; the second was vaccinated with TS/A-IL-21, and the third was vaccinated with F1F-IL-21 cells, using the same doses and schedule as described in Fig. 1.

nodules, whereas at later stages they were found scattered throughout the tumor tissue. Two-color immunofluorescence analysis revealed that a substantial fraction of these CD25⁺ cells were CD4⁺CD25⁺ (Fig. 3, *a–c*). In addition, most CD25⁺ cells were CD3⁺ (Fig. 3, *e–g*).

These data were confirmed by two-color immunofluorescence and FACS analysis of s.c. tumors and their draining LN cell suspensions (data not shown) demonstrating the existence of a CD4⁺CD25⁺ population (6.5–12 and 7.5–9.6% of lymphoid cells in TS/A-pc tumors and draining LN, respectively, in six different experiments; Fig. 4, *A* and *B*). In TS/A-pc tumors, variable proportions of CD25⁺ cells coexpressed CD4⁺ and predominantly had a CD25^{low} phenotype. In tumor draining LN, most CD25⁺ cells were CD4⁺ and CD25^{bright}. In addition, the majority (~85%) of CD25⁺ cells from draining LN coexpressed Foxp3 (Fig. 4, *A* and *B*), whereas in TIL, only a minor fraction (4.1–5.2% in four different experiments) coexpressed CD25 and Foxp3. Intriguingly, in TIL, a CD25⁺Foxp3^{low} population (4–6.5%) was also found, whereas only 0.5% of draining LN cells had this phenotype (Fig. 4, *A* and *B*).

We then isolated CD4⁺CD25⁺ cells by immunomagnetic purification (>85% purity) from both tumor draining LN and TIL, although the latter with a lower yield. Consistent with the two-color immunofluorescence data, >80 and ~60% of CD4⁺CD25⁺ cells purified from draining LN or TIL, respectively, were Foxp3⁺ and therefore represented bona fide Treg cells (Fig. 4, *A* and *B*). RT-PCR analyses showed that CD4⁺CD25⁺ cells from TS/A-pc draining LN expressed GITR, Foxp3, and IL-10 mRNA, which appeared less expressed or undetectable in CD4⁺CD25⁺ cell frac-

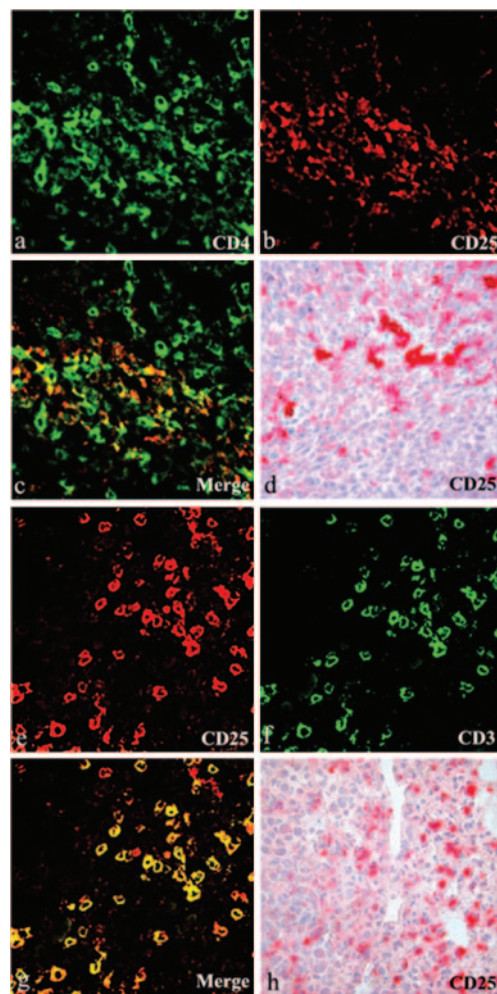


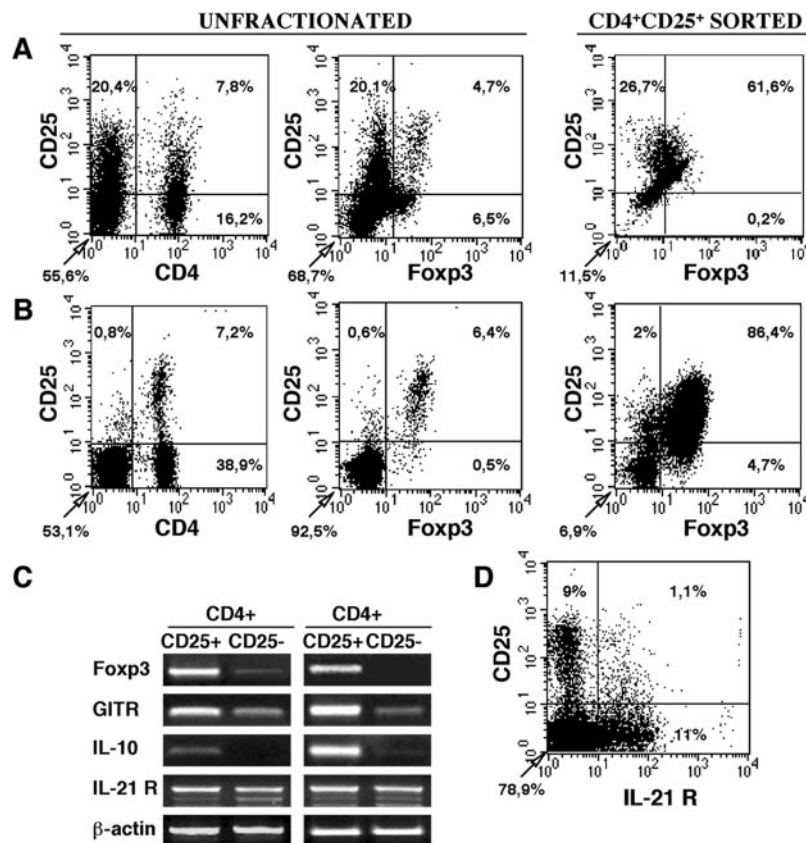
FIGURE 3. Infiltration of TS/A-pc s.c. tumors and lung metastases by CD25⁺ cells. Confocal microscopic analyses revealed that TS/A-pc tumor, developed 8 days after s.c. tumor cell injection, was infiltrated, particularly at the peripheral zones, by CD4⁺ (a; green stained)/CD25⁺ (b; red stained) cells, as evidenced in the merged image (c; yellow-orange stained). CD25⁺ cells (d; red stained) also infiltrated TS/A-IL-21 tumor. Most CD25⁺ cells present in TS/A-pc tumors (e; red stained) coexpressed CD3 (f; green stained) as revealed by the yellow color in the merged image (g). CD25⁺ cells (h; red stained) were also found in the context of experimental lung metastases developing 17 days after i.v. TS/A-pc cell inoculation. (magnification, $\times 400$).

tions (Fig. 4C). In addition, IL-21R mRNA was expressed on both CD25⁺ and CD25⁺CD4⁺ cell subsets. Two-color immunofluorescence analysis revealed that only a minor fraction of CD25⁺ cells coexpressed IL-21R, whereas most IL-21R⁺ cells were CD25⁺ (Fig. 4D).

CD4⁺CD25⁺ cells from draining LN or TS/A-pc s.c. tumors strongly suppressed the proliferation of syngeneic lymphocytes in response to allogeneic stimulation with irradiated H2^b splenocytes (Fig. 5A), whereas MLR proliferation was even increased by the addition of CD4⁺CD25⁺ LN cells under the same experimental conditions. Inhibition of MLR activity correlated with a potent suppression (>90%) of IL-2 secretion in the culture supernatant (data not shown), in keeping with the ability of Tregs to inhibit IL-2 gene expression (4), and addition of exogenous IL-2 (60 ng/ml) restored proliferation (data not shown).

Titration experiments showed that tumor-associated or LN CD4⁺CD25⁺ Tregs were potent suppressors able to inhibit MLR at a 16:1 or 32:1 responder cell/Treg ratio (Fig. 5B). The addition

FIGURE 4. Two-color immunofluorescence analysis performed on cell suspensions from TS/A-pc s.c. tumors (A) or draining LN (B) showed the presence of CD4⁺CD25⁺ and CD25⁺Foxp3⁺ lymphoid cells. A fraction of the CD25⁺ cells from TS/A-pc tumor coexpressed Foxp3 (A), whereas most CD25⁺ cells from draining LN were Foxp3⁺ (B). Cell suspensions were stained with a mixture of anti-CD4-FITC and anti-CD25-PE-conjugated mAbs or with irrelevant isotype-matched FITC or PE-labeled Abs for a negative control (markers for analysis were set to obtain >99% double-negative cells in controls). For analysis of Foxp3 expression, unfractionated or CD4⁺CD25⁺ purified TIL (A) or LN cells (B) were stained with anti-CD25 mAb, then fixed, permeabilized, and stained by an anti-Foxp3 FITC-labeled mAb. Data refer to events gated on lymphoid cell populations. C, Purified CD4⁺CD25⁺ tumor draining LN cells from two different preparations were analyzed for their expression of Foxp3, GITR, IL-10, and IL-21R mRNA expression by RT-PCR analysis. D, Two-color immunofluorescence analysis by IL-21R (green fluorescence) and CD25-PE showed that most IL-21R⁺ cells were contained in the CD25⁺ subset of LN cells.



of rIL-21 at a final concentration of 10 ng/ml failed to reverse the inhibition mediated by CD4⁺CD25⁺ cells in the MLR, whereas at 60 ng/ml, the proliferative response was partially restored. CD4⁺CD25⁺ cells alone failed to proliferate in response to the allogeneic stimulus or anti-CD3 mAb in either the presence or the absence of IL-21 (Fig. 5, B and C), suggesting that IL-21 was unable to support CD4⁺CD25⁺ Treg proliferation, whereas IL-2 induced Treg cell proliferation (Fig. 5C). Because IL-21 failed to restore IL-2 production blocked by CD4⁺CD25⁺ cells (data not shown), it is conceivable that at high concentrations, IL-21 may directly stimulate responder cells, thereby partially overcoming the lack of IL-2. However, because TS/A-IL-21 cells secreted ~2 ng/ml IL-21/10⁶ cells/48 h, it is very unlikely that IL-21 released by vaccine cells might completely overcome CD4⁺CD25⁺ cell-mediated immune suppression. We also found that CD4⁺CD25⁺ or CD25⁺Foxp3⁺ cells were present in transiently growing tumors formed by s.c. injection of viable TS/A-IL-21 cells and in the corresponding draining LN. The CD4⁺CD25⁺ cells could be isolated only from TS/A-IL-21 draining LN and showed significant immune-suppressive activity in allogeneic MLR (data not shown).

We then tested the ability of CD4⁺CD25⁺ Tregs isolated from TS/Apc draining LN to suppress IFN- γ production in response to the AH1 peptide by T cells from naive mice or mice cured by TS/A-IL-21 vaccination. AH1 failed to induce IFN- γ production by splenocytes from naive mice, even after secondary in vitro restimulation. In contrast, splenocytes from immune mice showed an increased number of IFN- γ -secreting cells, which were significantly decreased when CD4⁺CD25⁺ cells were present in the system (at a 10:1 responder cell/Treg ratio). IL-21 did not restore the IFN- γ response. These findings indicate that draining LN CD4⁺CD25⁺ cells may inhibit gp70 Ag-specific memory responses.

In vivo depletion of CD25⁺ cells augmented the effect of TS/A-IL-21 cell vaccination

Because of the high activity of Tregs in TS/A-pc tumor-bearing mice, we tested whether CD25⁺ cell depletion combined with TS/A-IL-21 cellular vaccine could cooperate to cure micrometastases that had been induced 24 h before starting any treatment. Because IL-21R is expressed on CD25⁺ lymphocytes (Fig. 4D) and functions independently from IL-2R α (CD25), it is conceivable that IL-21 may stimulate immune responses in CD25-depleted mice. As shown in Fig. 6A, a single dose of 500 μ g of anti-CD25 depleting Ab (42) given 1 day after TS/A-pc cells almost completely depleted CD25⁺ cells in draining LN from TS/A-pc tumor-bearing hosts collected 10 days after Ab administration. In addition, Foxp3⁺ cells were significantly depleted by anti-CD25 mAb treatment (from 6.6 to 0.7%) in draining LN (Fig. 6B). Time-course experiments showed that Tregs were restored in LN only ~50 days after Ab treatment (data not shown). Despite this effect, the sole anti-CD25 Ab, administered 24 h after micrometastasis induction, had no effect on the mean survival time of mice (Fig. 6C). However, if anti-CD25 mAb administration was followed 6 h later (30 h after micrometastasis induction) by TS/A-IL-21 vaccination, most (>80%) mice showed complete cure and long-term survival ($p < 0.0001$ vs untreated; $p < 0.002$ vs TS/A-IL-21-treated), indicating a strong cooperative effect of CD25⁺ Treg depletion and the IL-21-based cellular vaccine. All cured mice (nine of nine) showed resistance to another s.c. rechallenge with TS/A-pc performed 100 days after metastasis induction, suggesting that the effective immunotherapy was followed by a long-lasting protective immunity. The effect of the anti-CD25 mAb was dose related, because a single dose of 100 μ g of anti-CD25 mAb combined with

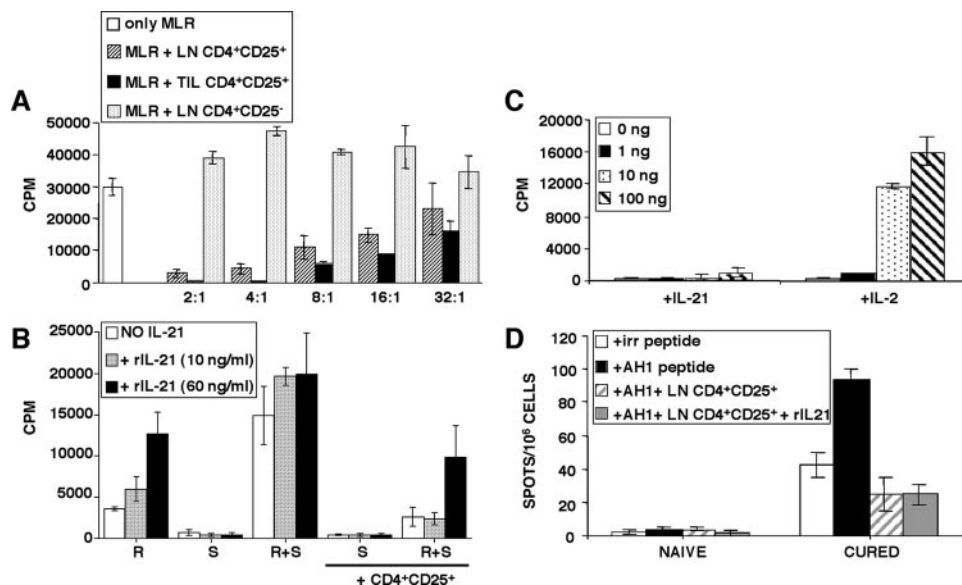


FIGURE 5. CD4⁺CD25⁺ T cells isolated from TS/A-pc s.c. tumor draining LN display potent suppressive effects in allogeneic MLR and also suppress memory responses to in vitro restimulation by the AH1 L^d-restricted peptide. **A**, CD4⁺CD25⁺ cells were purified by stepwise immunomagnetic procedures to a purity >85% and added to MLR cultures (responder cells were CD4⁺CD25⁺ splenocytes from naive BALB/c mice; stimulator cells were lethally irradiated splenocytes from C57BL-6J mice) at responder cell/Treg ratios ranging from 2:1 to 32:1. The CD4⁺CD25⁻ cell fraction from the same LN was used as a control. Proliferation was evaluated in triplicate wells by a [³H]thymidine 24-h pulse at the end of 6 days of culture. **B**, IL-21 partially reversed the suppressive effect of Tregs on allogeneic MLR only at high doses. Ten or 60 ng/ml IL-21 was added to allogeneic MLR in the presence or the absence of Tregs at a 4:1 responder cell/Treg ratio. **C**, Anti-CD3 mAb-activated CD4⁺CD25⁺ Tregs isolated from draining LN proliferate in response to IL-2, but not IL-21. Cells were seeded in anti-CD3 mAb-coated 96-well plates and cultured for 24 h. Then IL-2 or IL-21 was added at concentrations ranging from 1–100 ng/ml. Proliferation was evaluated by a [³H]thymidine 24-h pulse at the end of 6 days of culture. **D**, CD4⁺CD25⁺ LN Tregs inhibited IFN- γ production by AH1 peptide-stimulated spleen cells from long-term cured mice. Spleen cells from naive mice failed to respond to AH1 peptide. IFN- γ was detected by an ELISPOT assay.

TS/A-IL-21 vaccination cured only 28% (two of seven) of the mice (data not shown).

The combined immunotherapy was also effective if the anti-CD25 mAb was administered 48 h after micrometastasis induction, and the first vaccine dose was given 6 h later. In this delayed-onset immunotherapy setting, a significant increase in mean survival time was observed, and 71% of mice had long-lasting cure (Fig. 6D).

The effect of anti-CD25 Ab and TS/A-IL-21 vaccination requires CD8⁺ T cells, NK cells, and IFN- γ

Mice cured by the combined anti-CD25 mAb and TS/A-IL-21 vaccine showed gp70-specific CTL responses and IFN- γ production in response to AH1 peptide stimulation (data not shown). We then studied the roles of CD8 and CD4-positive T cell subsets using anti-CD4 or anti-CD8 depleting Abs. As shown in Fig. 7A, CD4⁺ T cell depletion failed to inhibit the effect of the combined anti-CD25 mAb and TS/A-IL-21 therapy, because 100% of CD4-depleted mice showed long-term survival. By contrast, all mice depleted of CD8⁺ cells developed tumors; however, their mean survival time was longer than that of untreated mice, suggesting the involvement not only of CTLs, but also of other cell types (Fig. 7A). Indeed, NK cell depletion by treatment with anti-asialo-GM1 antiserum reduced the number of cured mice to 14% (Fig. 7A).

The role of IFN- γ was also studied in syngeneic IFN- γ ^{-/-} mice, where TS/A-pc tumor draining LN also showed a CD4⁺CD25⁺ population accounting for ~8% of lymphoid cells (data not shown). As shown in Fig. 7B, no IFN- γ ^{-/-} mice were cured of TS/A-pc micrometastases by TS/A-IL-21 vaccination or the anti-CD25 mAb and TS/A-IL-21 combined immunotherapy protocol (p = NS vs untreated), indicating the critical contribution of IFN- γ to the efficacy of IL-21-based immunotherapy.

Discussion

In this study we show that immunotherapy with irradiated IL-21-transduced TS/A mammary adenocarcinoma cells produces long-term cures in ~20% of syngeneic mice bearing wild-type tumor micrometastases. The therapeutic activity required a vaccine co-expressing the relevant tumor-associated Ags and IL-21, because the use of antigenically unrelated IL-21-secreting cells or non-transduced TS/A-pc failed to cure any mice.

We hypothesized that the poor therapeutic effect of the TS/A-IL-21 cellular vaccine might be related to immune regulatory mechanisms activated by the tumor. In this context, Tregs have been reported to expand in neoplasms and may therefore contribute to tumor-related immune regulation (43–45). Our present data indicate that CD4⁺CD25⁺ T cells were present in TS/A-pc tumors and tumor draining LN and were endowed with potent immune-suppressive properties. Our findings indicate that most CD4⁺CD25⁺ cells isolated from the TS/A-pc tumor draining LN displayed several features of naturally arising Tregs (46): they were CD25^{bright}, they expressed Foxp3, and they also displayed GITR, IL-10, and TGF- β mRNA (Fig. 4 and data not shown).

It has been proposed that TGF- β can induce the differentiation of CD4⁺CD25⁻ precursors into functional CD4⁺CD25⁺ Tregs through the induction of Foxp3 (47). Because we found that TS/A-pc expressed TGF- β mRNA and protein (data not shown), in agreement with previous findings (48), it is possible that TS/A-pc tumor-infiltrating CD4⁺CD25⁺ T cells may, at least in part, represent tumor-induced Treg⁻ cells. However, although most LN CD4⁺CD25⁺ cells expressed Foxp3, a population of TIL was CD25⁺ but Foxp3⁻, and may therefore represent activated lymphocytes. Interestingly, a CD25⁻Foxp3^{low} population was also

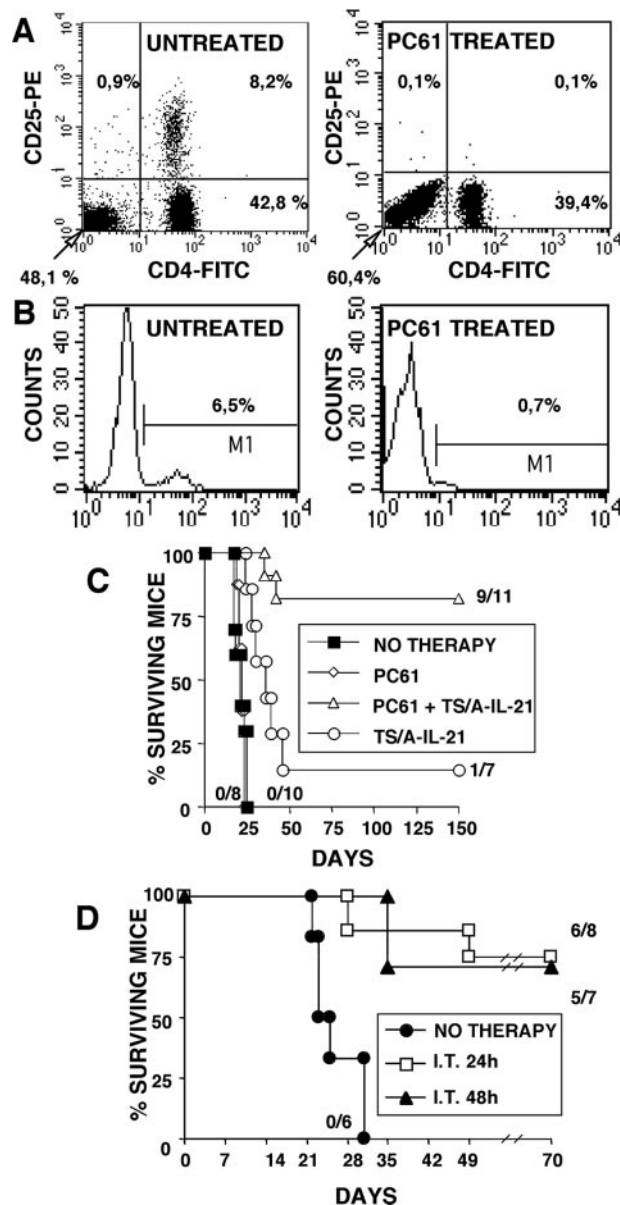


FIGURE 6. Cooperative effect of a single dose of anti-CD25 depleting mAb (PC61) on TS/A-IL-21 vaccine immunotherapy. **A**, Immunofluorescence analysis showed that LN CD25⁺ cells were efficiently depleted by a single dose of 500 μ g of PC61 anti-CD25 mAb i.p. 10 days previously (right panel). Staining of an LN cell suspension from an untreated mouse is shown as a control (left panel). **B**, LN Foxp3⁺ cells were also depleted by anti-CD25 mAb treatment. **C**, One day after induction of micrometastases, mice were randomized into four groups: the first was left untreated, the second received a single i.p. injection of 500 μ g of PC61, the third received four s.c. injections of irradiated TS/A-IL-21, and the fourth received both PC61 and irradiated TS/A-IL-21 vaccine (the first dose of 10^6 cells was injected s.c. 6 h after Ab administration, followed by other doses). **D**, Comparison of the efficacy of immunotherapy combining the anti-CD25 mAb and the TS/A-IL-21 vaccine (I.T.) started 24 or 48 h after micrometastasis induction. The first dose of TS/A-IL-21 vaccine was administered 6 h after anti-CD25 mAb administration.

evident in TIL. Similar Foxp3-expressing CD25⁺ cells have recently been shown to constitute a peripheral reservoir of Treg-committed cells, which can be recruited to the CD25⁺ pool upon activation stimuli (49). Thus, it is also plausible that such cells may be recruited at the TS/A-pc tumor site and then differentiate in the tumor microenvironment into CD25⁺Foxp3⁺ cells.

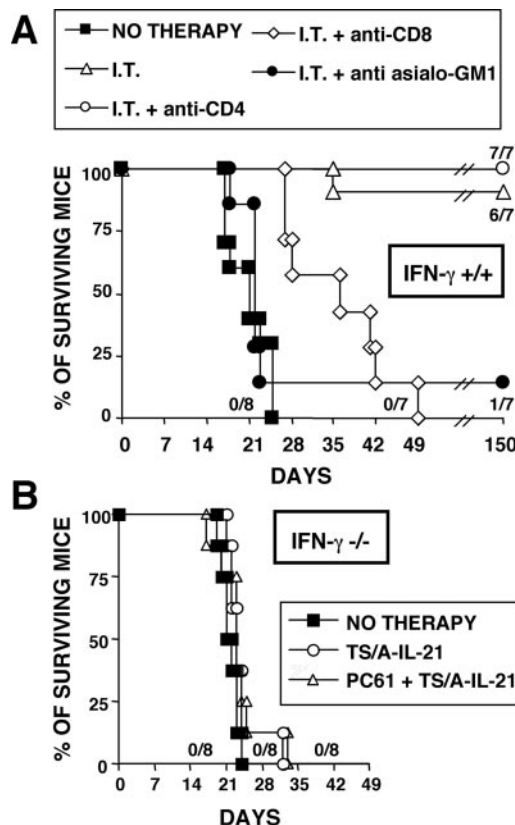


FIGURE 7. The combination immunotherapy by anti-CD25 mAb (PC61) and TS/A-IL-21 vaccine (I.T.) requires CTL, NK cells, and IFN- γ . **A**, Effects of anti-CD8, anti-CD4 anti-asialo-GM-1, or irrelevant Ab on the survival of micrometastases-bearing mice that received the combined anti-CD25 mAb and TS/A-IL-21 treatment. **B**, The combined anti-CD25 mAb and TS/A-IL-21 immunotherapy had no effect on TS/A-pc micrometastases development in IFN- γ ^{-/-} mice.

By in vitro assays we showed that IL-21 could partially revert the immune-suppressive activity of CD4⁺CD25⁺ Tregs from tumor draining LN only at a high concentration, which is unlikely to be reached using IL-21-engineered cells. This high dose effect could be related to the known direct effect of IL-21 on CTL activation and proliferation (24, 31, 32), rather than to Treg inhibition. Moreover, small s.c. tumor nodules induced by TS/A-IL-21 cells and the related tumor draining LN also showed the presence of CD25⁺CD4⁺ cells, which were endowed with immune-suppressive properties (data not shown), suggesting that low dose IL-21 is unable to suppress Treg function in vitro or in vivo. Because we were unable to select TS/A transfectants secreting greater amounts of IL-21, the possibility that high doses of IL-21 might override Treg inhibition in the TS/A-pc micrometastasis model remains to be determined. However, the administration of a single dose of anti-CD25 depleting mAb a few hours before TS/A-IL-21 immunotherapy strongly potentiated the therapeutic effect, leading to a >70% cure rate and long-term immunity to TS/A-pc Ags. This finding appears to constitute an authentic synergistic rather than a merely additive effect, because the administration of anti-CD25 mAb alone had no effect on mouse survival. This finding is consistent with previous reports showing that the administration of anti-CD25 Ab after tumor challenge is usually poorly effective (19, 20). Although CD25⁺ cell depletion is not selective for Tregs, this treatment strongly reduced the number of Foxp3⁺ cells in tumor draining LN, suggesting that the cooperative effects achieved with

the IL-21-secreting cellular vaccine may be related to Treg depletion. Because the biological activity of IL-2 requires cell surface expression of CD25 for high affinity binding, this cytokine cannot conveniently be combined with anti-CD25 mAbs; the CD25-independent IL-2-like immune-stimulating activities of IL-21, in contrast, offer a rationale for combining IL-21-based cellular vaccines with anti-CD25 mAbs. The finding that IL-21R is expressed on CD25⁺ lymphoid cells underscores the feasibility of this approach. In addition, although IL-2 has a role in tolerance (11, 12), IL-21 has been implicated in the development of autoimmune disorders (26, 27) and may thus overcome tolerance constraints, suggesting that IL-21 may represent a more suitable adjuvant for cancer vaccines than IL-2.

The finding that CD4⁺CD25⁺ Tregs from tumor draining LN fail to proliferate in response to IL-21 and alloantigen or anti-CD3 stimulation could imply that IL-21 is not directly involved in the control of Treg proliferation, although some of these cells may express the IL-21R gene. This offers a possible advantage compared with the use of IL-2, which is capable of supporting Treg proliferation. Moreover, our present data confirm that TS/A-IL-21 cells induce gp70 Ag-specific CTL responses (35), which become more effective in mice depleted of Tregs. Several previous reports indicate that IL-21 is a suitable adjuvant for the induction of CTL responses, and that NK cells, too, may participate in the IL-21-mediated antitumor effects (34–36). Indeed, both CTLs and NK cells were involved in immunotherapy combining the anti-CD25mAb and the TS/A-IL-21 vaccine. Other reports showed that CD4⁺ lymphocytes play a role in antitumor immune responses in mice predepleted of CD25⁺ cells (50, 51), whereas CD4⁺ T cells were not needed in the IL-21-based immunotherapy described in this study. It is possible that IL-21, which is a helper-derived factor (24), by-passes the requirement for other factors released by CD4⁺ Th cells. Finally, IFN- γ was also necessary for the immunotherapy combining TS/A-IL-21 and anti-CD25 mAb, because syngeneic IFN- γ ^{-/-} mice bearing TS/A-pc micrometastases could not be cured by this treatment.

In conclusion, our data indicate that the therapeutic activity of a low dose secreting IL-21-based cellular vaccine can be greatly potentiated by a single dose of a depleting anti-CD25 mAb, thereby allowing a strong antitumor effect and the induction of long-term protective immunity.

Disclosures

The authors have no financial conflict of interest.

References

- Atkins, M. B. 2002. Interleukin-2: clinical applications. *Semin. Oncol.* 29: 12–17.
- Rosenberg, S. A. 2001. Progress in human tumour immunology and immunotherapy. *Nature* 411: 380–384.
- Sakaguchi, S. 2004. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.
- Piccirillo, C. A., and E. M. Shevach. 2004. Naturally-occurring CD4⁺ CD25⁺ immuno-regulatory T cells: central players in the arena of peripheral tolerance. *Semin. Immunol.* 16: 81–88.
- Wang, H. Y., D. A. Lee, G. Peng, Z. Guo, Y. Li, K. Niwa, Y., E. M. Shevach, and R. F. Wang. 2004. Tumor-specific human CD4⁺ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20: 107–118.
- Refaeli, Y., L. Van Parijs, C. A. London, J. Tschopp, and A. K. Abbas. 1998. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity* 8: 615–623.
- Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A. C. Feller, and I. Horak. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75: 253–261.
- Sadlack, B., J. Lohler, H. Schorle, G. Klebb, H. Haber, E. Sickel, R. J. Noelle, I. Horak. 1995. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4⁺ T cells. *Eur. J. Immunol.* 25: 3053–3059.
- Willerford, D. M., J. Chen, J. A. Ferry, L. Davidson, A. Ma, and F. W. Alt. 1995. Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3: 521–530.
- Suzuki, H., T. M. Kundig, C. Furlonger, A. Wakeham, E. Timms, T. Matsuyama, R. Schmits, J. J. Simard, P. S. Ohashi, H. Griesser, et al. 1995. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor β . *Science* 268: 1472–1476.
- Furtado, G. C., M. A. Curotto de Lafaille, N. Kutchukhidze, and J. J. Lafaille. 2002. Interleukin 2 signaling is required for CD4⁺ regulatory T cell function. *J. Exp. Med.* 196: 851–857.
- Malek, T. R., and A. L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 4: 665–674.
- Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25⁺CD4⁺ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3: 135–142.
- McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
- Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
- Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4: 330–336.
- Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4: 337–342.
- Li, J., P. Hu, L. A. Khawli, and A. L. Epstein. 2003. Complete regression of experimental solid tumors by combination LEC/chTNT-3 immunotherapy and CD25⁺ T-cell depletion. *Cancer Res.* 63: 8384–8392.
- Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* 59: 3128–3133.
- Sutmoller, R. P., L. M. van Duivenvoorde, A. van Elsland, T. N. Schumacher, M. E. Wildenberg, J. P. Allison, R. E. Toes, R. Offringa, and C. J. Melief. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25⁺ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.* 19: 823–832.
- Stephens, G. L., R. S. McHugh, M. J. Whitters, D. A. Young, D. Luxenberg, B. M. Carreno, M. Collins, and E. M. Shevach. 2004. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4⁺CD25⁺ T cells. *J. Immunol.* 173: 5008–5020.
- Emens, L. A., R. T. Reilly, and E. M. Jaffee. 2005. Breast cancer vaccines: maximizing cancer treatment by tapping into host immunity. *Endocr. Relat. Cancer* 12: 1–17.
- Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin-21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408: 57–63.
- Sivakumar, P. V., D. C. Foster, and C. H. Clegg. 2004. Interleukin-21 is a T-helper cytokine that regulates humoral immunity and cell-mediated anti-tumour responses. *Immunology* 112: 177–182.
- Leonard, W. J., and R. Spolski. 2005. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat. Rev. Immunol.* 5: 688–698.
- Ozaki, K., R. Spolski, R. Ettinger, H. P. Kim, G. Wang, C. F. Qi, P. Hwu, D. J. Shaffer, S. Akilesh, D. C. Roopenian, et al. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J. Immunol.* 173: 5361–5371.
- King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117: 265–277.
- Ozaki, K., K. Kikly, D. Michalovich, P. R. Young, and W. J. Leonard. 2000. Cloning of a type I cytokine receptor most related to the IL-2 receptor β chain. *Proc. Natl. Acad. Sci. USA* 97: 11439–11444.
- Asao, H., C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, and K. Sugamura. 2001. Cutting edge: the common γ -chain is an indispensable subunit of the IL-21 receptor complex. *J. Immunol.* 167: 1–5.
- Kasaian, M. T., M. J. Whitters, L. L. Carter, L. D. Lowe, J. M. Jussif, B. Deng, K. A. Johnson, J. S. Witek, M. Senices, R. F. Konz, et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity* 16: 559–569.
- van Leeuwen, E. M., L. E. Gamadia, P. A. Baars, E. B. Remmerswaal, I. J. ten Berge, and R. A. van Lier. 2002. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8⁺ T cells. *J. Immunol.* 169: 5838–5843.
- Wang, G., M. Tschöi, R. Spolski, Y. Lou, K. Ozaki, C. Feng, G. Kim, W. J. Leonard, and P. Hwu. 2003. In vivo antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer Res.* 63: 9016–9022.
- Ma, H. L., M. J. Whitters, R. F. Konz, M. Senices, D. A. Young, M. J. Grusby, M. Collins, and K. Dunussi-Joannopoulos. 2003. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN- γ . *J. Immunol.* 171: 608–615.
- Di Carlo, E., A. Comes, A. M. Orenco, O. Rosso, R. Meazza, P. Musiani, M. P. Colombo, and S. Ferrini. 2004. IL-21 induces tumor rejection by specific CTL and IFN- γ -dependent CXC chemokines in syngeneic mice. *J. Immunol.* 172: 1540–1547.

36. Moroz, A., C. Eppolito, Q. Li, J. Tao, C. H. Clegg, and P. A. Shrikant. 2004. IL-21 enhances and sustains CD8⁺ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *J. Immunol.* 173: 900–909.
37. Nanni, P., C. De Giovanni, P. L. Lollini, G. Nicoletti, and G. Prodi. 1983. TS/A: a new metastasizing cell line from a BALB/c spontaneous mammary adenocarcinoma. *Clin. Exp. Metastasis* 1: 373–378.
38. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 259: 1739–1742.
39. Meazza, R., P. L. Lollini, P. Nanni, C. De Giovanni, A. Gaggero, A. Comes, M. Cilli, E. Di Carlo, S. Ferrini, and P. Musiani. 2000. Gene transfer of a secretable form of IL-15 in murine adenocarcinoma cells: effects on tumorigenicity, metastatic potential, and immune response. *Int. J. Cancer* 87: 574–581.
40. Gri, G., C. Chiodoni, E. Gallo, A. Stoppacciaro, F. Y. Liew, and M. P. Colombo. 2002. Antitumor effect of interleukin (IL)-12 in the absence of endogenous IFN- γ : a role for intrinsic tumor immunogenicity and IL-15. *Cancer Res.* 62: 4390–4397.
41. Huang, A. Y., P. H. Gulden, A. S. Woods, M. C. Thomas, C. D. Tong, W. Wang, V. H. Engelhard, G. Pasternack, R. Cotter, D. Hunt, et al. 1996. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon derives from an endogenous retroviral gene product. *Proc. Natl. Acad. Sci. USA* 93: 9730–9735.
42. Lowenthal, J. W., P. Corthesy, C. Tougne, R. Lees, H. R. MacDonald, and M. Nabholz. 1985. High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol.* 135: 3988–3994.
43. Viguier, M., F. Lemaitre, O. Verola, M. S. Cho, G. Gorochoy, L. Dubertret, H. Bachelez, P. Kourilsky, and L. Ferradini. 2004. Foxp3 expressing CD4⁺CD25^{high} regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J. Immunol.* 173: 1444–1453.
44. Liyanage, U. K., T. T. Moore, H. G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein, P. S. Goedegebuure, et al. 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol.* 169: 2756–2761.
45. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
46. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
47. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4⁺CD25[−] naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
48. Cavallo, F., E. Di Carlo, M. Butera, R. Verrua, M. P. Colombo, P. Musiani, and G. Forni. 1999. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin-12. *Cancer Res.* 59: 414–421.
49. Zelenay, S., T. Lopes-Carvalho, I. Caramalho, M. F. Moraes-Fontes, M. Rebelo, and J. Demengeot. 2005. Foxp3⁺ CD25[−] CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc. Natl. Acad. Sci. USA* 102: 4091–4096.
50. Casares, N., L. Arribillaga, P. Sarobe, A. Dotor, J. Lopez-Diaz de Cerio, I. Melero, J. Prieto, F. Borrás-Cuesta, and J. J. Lasarte. 2003. CD4⁺/CD25⁺ regulatory cells inhibit activation of tumor-primed CD4⁺ T cells with IFN- γ -dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J. Immunol.* 2003. 171: 5931–5939.
51. Golgher, D., E. Jones, F. Powrie, T. Elliott, and A. Gallimore. 2002. Depletion of CD25⁺ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur. J. Immunol.* 32: 3267–3275.