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CD25\(^+\) Regulatory T Cell Depletion Augments Immunotherapy of Micrometastases by an IL-21-Secreting Cellular Vaccine\(^1\)

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IL-2 is an IL-2-like cytokine, signaling through a specific IL-21R and the IL-2R \(\gamma\)-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-\(\gamma\) 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-\(\gamma\)-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-\(\gamma\)-. 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.

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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). The F1F cells were transfected with 10 μg of pLl-21RESneo 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 TMB, 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 autopathologically confirmed.

Animals (five to seven mice per group) were injected i.v. with 5 × 104 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 × 105 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 μg 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 (LST4; clone YTS.191.1.2; Sera-lab) or anti-CD25 Ab (anti-IL-2Ra; 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-PE 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 (ab3268; Abcam) and a goat FITC-conjugated goat anti-rabbit Ig (Santa Cruz Biotechnology) were used. Flow3 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− (105) responder splenocytes from naive BALB/c mice (H2b) were plated in the presence of various numbers of CD4+ CD25+ or CD4+ CD25− cells and 106 lethally irradiated stimulator splenocytes from C57Bl−6j mice (H2b) 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 μCi/well [3H]thymidine (Amersham Biosciences). Cultures were then harvested onto glass fiber filters and counted in a beta counter.

RT-PCR analysis of Fos-p, GTR, 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 Fos-p (5′-CAG CTG CCT ACA GTG CCC CTA-3′ and 5′-CATT TGG CCA GCA GTG AGG-3′), GTR (5′-TCT CGA TGC TCT GTG TGC TG-3′ and CGT GGC ACA GGC AAC ACA C-3′), IL-21R (5′-CCAC CTCAA AACTT CACCTTC and 5′-TGC TCTCAGGACGAGGAAAG-3′) or IL-10 (5′-GCC ACT CGT CCG CTT AC-3′ and 5′-ACT CTT CAC CTG TCT 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 (105 cells/ml) were restimulated in vitro for 5 days at 37°C in the presence of the gp70-derived L1-restricted AHI peptide (40, 41) (SPSYFYHYQF; Primm) at 1 μg/ml. Cytolytic activity was evaluated by a standard 3H 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 × 104/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 AHI-induced IFN-γ production was assessed by ELISPOT on spleen cells from naive or cured mice restimulated in vitro for 5 days with either AHI 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 × 105 viable TS/A-pc and were randomized into three groups. The TS/A-IL-21 cells were transfected with a second PE-conjugated anti-CD25 Ab (anti-IL-2Rα; clone 3C7;
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 = $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 gp70 env 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

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**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 $5 \times 10^4$ 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 μg/ml of the AH1 epitope of gp70env. Data are expressed as the percent lysis of ^51Cr-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 μg/ml of either peptide. Spots were counted using a stereomicroscope.
and B-CD3 had a CD25low phenotype. In tumor-draining LN, most CD25
portions of CD25 and therefore represented bona fide Treg cells (Fig. 4, cells purified from draining LN or TIL, respectively, were Foxp3 experiments; Fig. 4, in TS/A-pc tumors and draining LN, respectively, in six different revealed that a substantial fraction of these CD25 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\textsuperscript{low} phenotype. In tumor-draining LN, most CD25+ cells were CD4+ and CD25\textsuperscript{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\textsuperscript{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 twocolor 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 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\textsuperscript{6} cells/ml. B. One day after induction of micrometastases by i.v. injection of 5 x 10\textsuperscript{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.

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, ×400).
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-2/10⁶ 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 CD4⁺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-2Ra (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 μg of anti-CD25 depletion 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 μg 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 Ld-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 expressed 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⁻ population was also
evident in TIL. Similar Foxp3-expressing CD25⁻/H11002 cells have recently been shown to constitute a peripheral reservoir of Treg-committed cells, which can be recruited to the CD25⁺/H11001 pool upon activation stimuli (49). Thus, it is plausible that such cells may be recruited at the TS/A-pc tumor site and then differentiate in the tumor microenvironment into CD25⁺/H11001 Foxp3⁺/H11001 cells.

By in vitro assays we showed that IL-21 could partially revert the immune-suppressive activity of CD4⁺/H11001 CD25⁻/H11001 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⁺/H11001 CD4⁺/H11001 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 micrometastases 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⁻/H11001 cell depletion is not selective for Tregs, this treatment strongly reduced the number of Foxp3⁺/H11001 cells in tumor draining LN, suggesting that the cooperative effects achieved with
were not needed in the IL-21-based immunotherapy described in mice predepleted of CD25 mAb and the TS/A-IL-21 vaccine. Other reports showed that mediated antitumor effects (34–36). Indeed, both CTLs and NK cells induce gp70 Ag-specific CTL responses (35), which become

References

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Disclosures

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