Irradiated CIITA-positive mammary adenocarcinoma cells act as a potent anti-tumor-preventive vaccine by inducing tumor-specific CD4⁺ T cell priming and CD8⁺ T cell effector functions

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Abstract

In the present study, we investigated the possibility to use irradiated, non-replicating class II transcriptional activator (CIITA)-transfected tumor TS/A cells as a cell-based vaccine. Eighty-three percent of TS/A-CIITA-vaccinated mice were completely protected from tumor growth and the remaining 17% displayed significant reduction of tumor growth. In contrast, only 30% of mice injected with irradiated TS/A parental cells were protected from tumor growth, whereas the remaining 70% of animals remained unprotected. Immunity generated in the TS/A-CIITA-vaccinated mice correlated with an efficient priming of CD4⁺ T cells and consequent triggering and maintenance of CD8⁺ CTL effectors, as assessed by adoptive transfer assays. Important qualitative differences were observed between the two cell-based vaccines, as TS/A-CIITA-vaccinated mice developed a CTL response containing a large proportion of anti-gp70 AH1 epitope-specific cells, completely absent in TS/A-vaccinated mice, and a mixed Tₘ₁/Tₘ₂ type of response as opposed to a Tₘ₂ type of response in TS/A-vaccinated mice. Finally, in TS/A-CIITA-vaccinated mice, a statistically significant reduction in the percentage and absolute number of CD4⁺ CD25⁺ T regulatory cells as compared with those of untreated mice with growing tumors (P < 0.001) or mice vaccinated with TS/A parental cells were observed. These results let to envisage the use of CIITA-transfected non-replicating tumor cells as a vaccination strategy for prevention and, possibly, adjuvant immunotherapy in human settings.

Introduction

It is widely accepted that the immune system can recognize and respond against neoplastic cells (1). However, the clinical history of a cancer patient often demonstrates the failure of the immune system to eliminate the tumor. Several approaches have been investigated to modify such a behavior and in particular the effectiveness of newly induced or already existing, but anergized, anti-tumor immune responses. Among these, attention has been addressed to tumor-specific MHC-I-restricted peptide vaccination, dendritic cell (DC) vaccination, means to increase the availability of soluble mediators, including cytokines and chemokines and adoptive transfer of ex vivo expanded tumor-specific immune cells (2,3,4,5). Most of the above approaches were aimed at triggering and/or amplifying anti-tumor-specific CTL as these cells are believed to be the major effectors against cancer cells. Unfortunately, CTL responses were generally weak and unable to control tumor growth in most patients (3). It is now generally accepted that this is mostly due to poor tumor-specific, MHC class-II-restricted CD4⁺ T cell help generated in tumor-bearing patients (6, 7) since it is known that CD4⁺ Tₘ₁ cells are required both for priming and for clonal expansion of specific CTL following re-encounter with antigen (8,9,10,11).

A crucial initial step of the anti-tumor immune response, such as CD4⁺ Tₘ₁ cell priming may be altered, for example, by the action of the tumor microenvironment, which in many cases either by default (scarce tumor-associated antigen expression and/or presentation) or by active action (pro-tumor...
Irradiated CIITA-positive tumor cells as vaccine

The function of several hematopoietic cell types, including lymphoid and myeloid cells, can be polarized in the tumor microenvironment to suppress specific immune control, particularly CD4+ Tc cell triggering and expansion and by consequence also CTL expansion. Among them, we find regulatory CD4+CD25+ Tc cells (13), tumor-associated macrophages (14), myeloid-derived suppressor cells (15) and type II NK T cells (16). To obtain effective preventive or therapeutic immune-based approaches, direct targeting to these polarized cells may be a suitable approach to trigger both tumor-specific Tc and cytotoxic T effector cells.

We have recently shown that the TS/A mammary adenocarcinoma tumor cells genetically engineered to stably express the MHC class II transcriptional activator (CIITA) (17, 18) result in a stable and strong expression of all repertoire of MHC class II molecules as well as associated structures as Invariant chain and DM. When injected in vivo, these tumor cells are rapidly and efficiently rejected. Most importantly, tumor-rejecting animals are protected by a subsequent challenge with the highly tumorogenic, untransfected TS/A parental cells (TSA-pc) (17). Careful immunological analysis indicated that CD4+ Tc cells were absolutely required to trigger the tumor-specific response and to maintain the main anti-tumor effector cells, the CD8+ CTLs (18). In the TS/A-CIITA animals, the tumor microenvironment changes drastically when compared with the one observed in TS/A-pc-bearing mice, with a rapid infiltration of CD4+ T cells, followed by infiltration of CD8+ T cells and a strong infiltration of neutrophils. The tumor tissue rapidly becomes necrotic because of an extensive tumor cell death. Interestingly, CD4+ T cells in draining lymph nodes and spleen display a polarized Th1 phenotype (18).

Thus, this approach appears as an attractive way to investigate new strategies of anti-tumor immune vaccination both in preventive and, possibly, in therapeutic settings. Toward this direction, it is important to evaluate the applicability of this approach to human cancer. Certainly, in humans, it is ethically impossible to immunize with live tumor cells. On the other hand, a ‘dead cellular vaccine’ could be more ethically acceptable, provided it can be safely proved to be efficacious.

In this study, we have used lethally irradiated TS/A-CIITA cells (TS/A-CIITA vaccine) to investigate the effectiveness of this approach in inducing protective tumor immunity. We show that immunization with TS/A-CIITA vaccine results in tumor rejection in a high percentage of mice (83%) in comparison to that obtained with non-replicating TS/A parental cells (TS/A-pc vaccine) (30%). Importantly, we show that TS/A-CIITA-vaccinated mice have anti-tumor Tc1/Tc2 mixed Tc responses and strong tumor-specific CTL response. A substantial part of the tumor-specific CTL response is directed against the AH1 epitope of gp70, a glycoprotein of an endogenous defective murine retrovirus (19). This specificity is not found in CTLs arising from vaccination with irradiated TS/A-pc. Finally, we defined for the first time the ability of TS/A-CIITA vaccine to modulate the percentage and the absolute number of Treg cells in draining lymph nodes as compared with tumor-bearing mice or with mice vaccinated with TS/A parental cells.

Materials and methods

Cell lines, CIITA transfection and cell surface phenotyping

TS/A murine breast adenocarcinoma (20), C26 colon carcinoma and F1F fibrosarcoma cell were cultured in DMEM medium supplemented with l-glutamine, HEPES and 10% fetal bovine serum, in a 5% CO2 atmosphere at 37°C. Transfection of TS/A with CIITA, isolation of stable CIITA-expressing clones and MHC cell surface phenotype of transfectants have been previously described (17, 18).

Animal model, experimental conditions and cell purification procedures

Five-week-old female BALB/c (H-2k) mice were purchased from Harlan (Udine, Italy). Animals were injected subcutaneously (s.c.) three times at 2- to 3-week intervals, with 1 x 10^6 irradiated (20 000 rad) TS/A-pc or CIITA-transfected (TS/A-CIITA) tumor cells. Tumor challenge with the standard tumorogenic dose (10^3 tumor cells) of TS/A-pc was carried out s.c. in the opposite flank of vaccinated animals. Tumor size was measured using a caliper at weekly intervals and was expressed as a multiple of the wider and smaller tumor diameters. For Winn assay experiments (see below), an in vitro T cell subset purification was carried out. Spleen cells from mice vaccinated with irradiated TS/A-pc or irradiated TSA-CIITA tumor cells were treated with either CD4+ or CD8+ T cell isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or CD8α+ T cell isolation Kit (Miltenyi Biotec GmbH). Cell purification efficiency was assessed by immunofluorescence and cytometric analysis (Beckman Coulter, Milan, Italy) by direct staining for CD4 (FITC-conjugated, clone RM4-5, CALTAG Laboratories, Carlsbad, CA, USA) or CD8 (PE-conjugated, clone 5H10, CALTAG Laboratories) and was always >95%. Appropriate isotype controls (FITC rat IgG2a FITC and PE rat IgG2b, respectively, CALTAG Laboratories) were used.

Immunohistochemical analyses

Groups of three mice each, consisting of naive mice, or mice 2 weeks after vaccination protocol with either irradiated TS/A-pc or irradiated TS/A-CIITA tumor cells, were challenged s.c. with TS/A-pc. At 5 and 10 days from TS/A-pc tumor challenge, mice were euthanized. Six micrometre-thick cryostat sections were air dried and fixed in cold acetone for 10 min. Immunostaining was performed using a streptavidin–biotin–alkaline–phosphatase complex staining kit (Bio-Spa Division, Milan, Italy) and naphthol-AS-MX-phosphate and Fast-Red TR (Sigma, St Louis, MO, USA) to visualize binding sites. The mAbs used were anti-CD4 (RK1.5), anti-CD8 (2.43), anti-DCs (DEC-205, clone NLD5-145), anti-polymorphonucleate (PMN) Ly-6G (Gr-1, clone RB6-8C5), anti-macrophage (clone MOMA-1) and anti-NK (anti-asialo-GM1), all from Immunokontact, Lugano, Switzerland. The sections were incubated with the primary antibody overnight at 4°C. The red
reaction product was obtained using a mixture of 2 mg of naphtol-AS-MX-phosphate dissolved in 200 µl of N,N-dimethylformamide (Sigma) and diluted in 9.8 ml of 0.1 M Tris–HCl buffer, pH 8.2, and 1 mM levamisole (Sigma). Immediately before use, 10 mg of Fast-Red TR salt (Sigma) was added. Gill’s hematoxylin was used as a counterstain and the sections were mounted in glycerol (Dako, Carpinteria, CA, USA). Quantitative studies of stained sections were performed independently by three researchers in a blind fashion. Cell counting was carried out in 8–12 randomly chosen fields under a Leica Wetzlar light microscope (Germany) at ×400 magnification, 0.180 mm² per field.

**Adoptive immunity transfer experiments (Winn assay)**

Mice vaccinated with either irradiated TS/A or irradiated TS/A-CIITA tumor cells were challenged after 2 weeks from the last vaccination, in the opposite flank with live TS/A-pc cells. After 3 weeks, mice were sacrificed, their spleen was removed and a spleen cell suspension was prepared (17, 18). Total or in vitro-purified splenocytes (see above) were admixed with TS/A-pc tumor cells (100:1 ratio) and co-injected into naive animals. The results were expressed as percent of survival versus time, as well as tumor size over time.

**Cell-mediated cytotoxicity assay**

Cell-mediated cytotoxicity assay was performed using splenocytes from either TS/A-pc or TS/A-CIITA-vaccinated mice as well as from TS/A tumor-bearing mice. Splenocytes were co-cultured with irradiated TS/A-pc for 5 days and then tested in a standard 4 h ⁵¹Chromium-release assay on TS/A, C26 and F1F target cells as previously reported (17).

**Enzyme-linked immunospot assays**

Frequencies of IFN-γ- or IL-4-producing spleen cells from vaccinated animals were determined by an enzyme-linked immunospot (ELISpot) assay performed with ex vivo total splenocytes after 5-day incubation in vitro with irradiated TS/A-pc, TS/A-CIITA, C26 or F1F tumor cells as antigen, as described (18). A >2-fold increase in number of spots over the control (splenocytes cultured in the absence of tumor cells) was considered as a positive response. In certain experiments, mAbs specific for either MHC class I or class II antigens (see above) were added during the 5-day in vitro co-culture with irradiated tumor cells. Data were expressed as number of spot-forming cells per million of spleen cells.

**Quantification of CD4⁺CD25⁺ Treg cells**

The presence and the proportion of CD4⁺CD25⁺ Treg cells were assessed on tumor-draining lymph nodes. Cell suspensions were first incubated with anti-CD16 mAb to block Fc receptor, then washed and stained with anti-CD4 FITC-conjugated and anti-CD25 PE-conjugated mAbs (CALTAG Laboratories) and analyzed by flow cytometry. Expression of FoxP3, an additional marker of Tregs, was assessed in permeabilized cells by using an FITC-labeled anti-FoxP3 rat mAb (clone FJK-16s, eBioscience, Rome, Italy) (21). A FITC-labeled rat IgG2a mAb (eBioscience) was used as a negative control.

**Statistical analysis**

Statistical analysis was performed by means of one-way and two-way analysis of variance. The Sheffe test was used when post-hoc comparisons were needed.

**Results**

**Irradiated TSA-CIITA tumor cell vaccination induce tumor-specific rejection upon TS/A challenge**

BALB/c mice were injected s.c. three times with lethally irradiated TS/A-pc or clone 32.10.7 TS/A-CIITA tumor cells (18). After 2 weeks from the last injection, mice were challenged in the opposite flank with 10⁶ of TS/A-pc in parallel with naive control mice to assess the ability of rejection and establishment of immunity.

Eighty-three percent of mice that received the TS/A-CIITA-irradiated vaccine were fully protected against tumor challenge (Fig. 1A) and the remaining 17% showed a significant retardation of tumor growth in comparison to non-vaccinated mice (Fig. 1B). On the contrary, only 30% of mice injected with irradiated TS/A-pc resulted protected from challenge (Fig. 1A). In the remaining 70% of animals that were not protected, the kinetics of tumor growth were similar to the one of non-vaccinated mice injected with TS/A-pc (Fig. 1B).

To assess the specificity of the potent anti-tumor immunity induced by irradiated TS/A-CIITA as compared with irradiated TS/A-pc, mice resistant to TS/A-pc challenge were injected with colon carcinoma C26 tumor cells. C26 is a tumor cell line sharing with TS/A the expression of the gp70 envelope protein from a defective murine leukemia virus (MuLV). A gp70 9-amino acid-specific sequence, designated AH1, is a major MHC class I-restricted epitope for CD8⁺ CTLs (19) and we have previously shown that a high proportion of animals rejecting TS/A-pc after being immunized with live TS/A-CIITA cells were also rejecting C26 tumor challenges (17). Twenty percent of TS/A-CIITA-vaccinated mice showed complete protection from C26 challenge, whereas none of the TS/A-pc-vaccinated mice was protected (Fig. 1C). Interestingly, in the 80% unprotected mice showing C26 tumor take, the tumor growth kinetics were substantially retarded as compared with that of mice immunized with irradiated TS/A-pc or with that of non-vaccinated controls (Fig. 1D). Thus, the protective immune response against TS/A-pc tumors in TS/A-CIITA-vaccinated mice was clearly quantitatively and qualitatively different with respect to that obtained in TS/A-pc-vaccinated mice.

**Both primed CD4⁺ and CD8⁺ T cells from TS/A-CIITA-vaccinated mice confer highly effective protection in adoptive transfer experiments**

To better evaluate the in vivo relevance of both CD4⁺ and CD8⁺ T cells in triggering the anti-tumor effector functions induced by our vaccination protocols, we carried out adoptive transfer assays (Winn assay) using either whole immune spleen, purified CD4⁺ T cells or CD8⁺ T cells from vaccinated
mice 3 weeks after challenge with live TS/A-pc. Cell-adoptive transfer using whole spleen cells from mice vaccinated with irradiated TS/A-CIITA cells showed 80% of protection from tumor take (Fig. 2A), as compared with 40% protection when total spleen cells from TS/A-pc-vaccinated mice were used (Fig. 2B). Interestingly, when purified T cells were co-injected with TS/A-pc, the rate of protection reached 60 and 40% in animals receiving CD4+ T or CD8+ T cells, respectively (Fig. 2A), as compared with 20% protection in mice receiving CD4+ T cells or CD8+ T cells, respectively, from animals vaccinated with irradiated TS/A-pc (Fig. 2C). Moreover, even by the adoptive cell transfer approach, the kinetics of tumor growth in animals receiving immune total spleen cells, purified CD4+ or CD8+ T cells from TS/A-CIITA-vaccinated mice displayed a significant retardation (Fig. 2B) as compared with the equivalent kinetics in mice receiving cells from TS/A-pc-vaccinated animals (Fig. 2D). These results establish the crucial role of the CIITA-dependent MHC-II expression in TS/A tumor cells in inducing a stronger anti-tumor-protective immune response particularly at the level of CD4+ Th triggering, necessary also for a better induction of CD8+ effector CTLs.

Tumor tissue correlates of rejection in TS/A- and TS/A-CIITA-vaccinated animals

To assess the histologic correlates of immunity, the phenotype and the relative percentage of leukocytes infiltrating the tumor at distinct time periods after TS/A-pc challenge were investigated in mice vaccinated with either TS/A-CIITA or TS/A-pc dead cells. The results of this analysis are summarized in Table 1. Mice vaccinated with TS/A-CIITA (Vac TS/A-CIITA) showed a highly significantly influx of CD4+ cells already at day 5 (P < 0.005) compared with non-vaccinated mice (TS/A-pc) or vaccinated with irradiated TS/A-pc cells (Vac TS/A-pc). At day 10, infiltration of CD4+ cells in TS/A-CIITA-vaccinated mice was more pronounced with respect to TS/A-pc-vaccinated mice and non-vaccinated controls. At this time point, extensive areas of tumor necrosis were seen in the tumor tissue of TS/A-CIITA-vaccinated mice (data not shown). A similar picture was observed also for CD8+ T cells which rapidly infiltrated the TS/A-pc tumor in TS/A-CIITA-vaccinated mice. At this time point, mice vaccinated with irradiated TS/A-pc cells showed a significantly higher CD8+ T cell infiltration when compared with non-vaccinated controls, but definitely much lower than mice vaccinated with irradiated TS/A-CIITA cells. At day 10 after challenge, statistically significant infiltration of CD8+ T cells in the tumor was observed also in mice vaccinated with TS/A-pc cells.

The presence and number of four additional cell types were analyzed in tumor tissue infiltrate. DCs were significantly increased at day 5 in both groups of vaccinated mice with respect to non-vaccinated controls. As observed for CD8+ T cells, however, DC infiltration was stronger in animals vaccinated with TS/A-CIITA and remained significantly higher with respect to the other two groups even at day 10. Macrophage infiltration at day 5 in TS/A-CIITA-vaccinated animals was again significantly higher than the one observed in TS/A-pc-vaccinated or non-vaccinated animals and similar to unvaccinated controls at day 10. Interestingly at this time point, macrophage infiltration was reduced in TS/A-pc-vaccinated mice.

Strong infiltration of PMN was observed in all cases and at both time points analyzed, although stronger infiltration was found at day 5 again in tumors of TS/A-CIITA-vaccinated animals.

Very few NK cells were present in the tumor infiltrates and no significant difference was observed in vaccinated...
animals when compared with each other or with non-vaccinated controls.

**Superior rejection capacity after vaccination with irradiated TS/A-CIITA correlates with anti-tumor CTL activity preferentially directed against the dominant gp70 AH1 epitope**

In order to define the cytolytic T cell function generated after the two distinct vaccination protocols, the ability of the immune spleen cells to kill different tumor cell lines in a standard *in vitro* 4-h $^{51}$Chromium-release assay was evaluated after 1 month from vaccination. Two distinct *in vitro* tumor antigen stimulations were performed: the first consisted in incubating spleen cells with whole TS/A tumor cells to allow the immune cells to react with all possible tumor-associated antigens; the second stimulation was performed, instead, with the gp70 peptide AH1, an immunodominant MHC class I-restricted epitope for CD8$^+$ CTLs, shared between TS/A and C26 tumor cells. When stimulation with whole TS/A-pc tumor cells was used, high CTL responses specific for both TS/A and C26, but not for F1F, were detected in TS/A-CIITA-vaccinated mice (Fig. 3A). These responses were present but at much weaker intensity in spleen cells of TS/A-pc-vaccinated animals (Fig. 3B). After AH1 peptide stimulation, instead, only spleen cells from TS/A-CIITA-vaccinated mice (Fig. 3C) and not TS/A-pc-vaccinated mice (Fig. 3D) showed significant CTL activity against both TS/A and C26 tumors.

These results indicate that vaccination with CIITA-dependent MHC class II-positive-irradiated tumor cells induces a stronger protective immunity which correlate mostly, although not

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**Table 1.** Immunohistochemical analysis of tumor infiltrating cells in TS/A-CIITA- and TS/A-pc-vaccinated mice at two different time points from s.c. live TS/A-pc tumor challenge

| Infiltrating cells | Day 5 | | | Day 10 | | |
|-------------------|-------|-------|-------|-------|-------|
|                   | TS/A-pc$^b$ | Vac TS/A-pc$^c$ | Vac TS/A-CIITA$^d$ | TS/A-pc | Vac TS/A-pc | Vac TS/A-CIITA |
| CD4               | 1.5 ± 1.2 | 3.8 ± 2.3$^a$ | 15.8 ± 6.8$^f$ | 5.6 ± 1.4 | 2.2 ± 1.3 | 22.5 ± 8.2$^f$ |
| CD8               | 1.3 ± 1.0 | 6.4 ± 3.2$^b$ | 19.3 ± 6.9$^f$ | 2.4 ± 0.5 | 1.3 ± 0.7 | 11.4 ± 4.4$^f$ |
| Dendritic cells   | 0.9 ± 0.6 | 8.3 ± 3.1$^b$ | 19.6 ± 7.1$^f$ | 1.4 ± 0.5 | 3.2 ± 1.7 | 8.6 ± 3.4$^f$ |
| Macrophages       | 8.6 ± 2.7 | 7.4 ± 2.6 | 33.6 ± 10.6$^f$ | 9.8 ± 4.5 | 7.5 ± 1.5 | 10.5 ± 2.7 |
| Polymorphonucleates | 18.6 ± 10.2 | 24.4 ± 5.8 | 42.6 ± 13.1$^f$ | 15.5 ± 6.5 | 11.0 ± 2.9 | 17.6 ± 4.7 |
| NK cells          | 3.2 ± 0.7 | 1.3 ± 0.5 | 2.4 ± 0.9 | 4.4 ± 2.0 | 1.8 ± 0.7 | 1.3 ± 0.9 |

$^a$Results are mean ± SD of positive cells per field evaluated on cryostat sections. Cell counts at ×400 magnification in a 0.180-mm$^2$ field. At least 8–10 randomly chosen fields per sample were evaluated.

$^b$Non-vaccinated mice injected with live TS/A tumor cells.

$^c$Mice vaccinated with TS/A-pc and challenged with live TS/A tumor cells.

$^d$Mice vaccinated with TS/A-CIITA and challenged with live TS/A tumor cells.

$^e$$P < 0.005$, significantly different from corresponding values of TS/A-pc tumor growth in BALB/c mice at the same time point.

$^f$$P < 0.005$, significantly different from corresponding values of Vac TS/A-pc tumor growth in BALB/c mice at the same time point.
exclusively, with the generation of CTL effector cells specific for the dominant gp70 AH1 epitope. Interestingly, the immunity generated after vaccination with the class II-negative TS/A-pc, instead, is specific for antigenic determinants distinct from the gp70 AH1 peptide.

TS/A-CIITA-irradiated tumor cell vaccination triggers a mixed anti-tumor Th1/Th2 immune response

We then assessed whether the dramatic change in the tumor microenvironment and the potent immune rejection response generated after TS/A-CIITA dead cell vaccination correlated also with a functional polarization of the Th cells. The frequency, phenotype and functional features of the CD4+ Th cell sub-populations were assessed by ELISPOT assay that measured the frequency of ex vivo spleen cells producing IFN-γ or IL-4 at two distinct time points: after 2 weeks from vaccination (Fig. 4A, C, E and G, white columns) and after 2 weeks from TS/A-pc tumor challenge (Fig. 4A, C, E and G, hatched columns).

Two weeks after vaccination with TS/A-CIITA dead cells, spleen cells stimulated in vitro with TS/A-pc displayed an increased frequency of lymphocytes-producing IFN-γ (Fig. 4A) and, to lower extent, IL-4 (Fig. 4C). Stimulation in vitro with TS/A-CIITA tumor cells dramatically increased the frequency of both IFN-γ- and IL-4-producing cells. A similar pattern was also observed when spleen cells were stimulated in vitro with C26 tumor cells. Increased frequency of IFN-γ, but not of IL-4, -producing cells was observed after in vitro stimulation with the AH1 peptide (Fig. 4A). Interestingly, spleen cells from mice vaccinated with TS/A-CIITA and subsequently challenged with TS/A-pc displayed a significantly increased number of both IFN-γ- and IL-4-producing lymphocytes when stimulated in vitro with TSA-pc, TS/A-CIITA and C26 tumor cells. On the contrary, in vitro stimulation with the AH1 peptide resulted in a strong increase in frequency of IFN-γ-producing lymphocytes but not of IL-4-producing lymphocytes (Fig. 4A and C, compare AH1-specific columns). Of note, incubation in vitro with antibodies specific for either MHC class I or class II of spleen cells from TS/A-CIITA-vaccinated and further TS/A-pc live tumor-challenged mice resulted in a decreased frequency of IFN-γ-secreting cells (Fig. 4B), indicating that both CD4+ and CD8+ T cells were involved in the production of this Th1-like cytokine. On the other hand, the incubation with anti-MHC class II, but not class I, antibodies dramatically reduced the frequency of IL-4-producing cells (Fig. 4D), suggesting that this cytokine was preferentially, if not exclusively, produced by Th2-like CD4+ lymphocytes.

The frequency of IFN-γ-producing spleen cells from mice vaccinated with irradiated TS/A-pc tumor cells did not significantly increase in any condition of antigen stimulation in vitro with respect to unstimulated cells, both before and after challenge with live TS/A-pc in vivo (see Fig. 4E). The frequency of IL-4-producing spleen cells, instead, increased, although not dramatically, in TS/A-pc-vaccinated animals when the cells were stimulated in vitro with TS/A-pc, TS/A-CIITA and C26 tumor cells (Fig. 4G), but not with the AH1 peptide, and further increased in spleen cells of vaccinated and TS/A-challenged mice, indicating that this vaccination protocol preferentially induced Th2 type of response. Incubation with anti-MHC-I and anti-MHC-II showed that the major anti-tumor immune response in this case was of the CD4+ phenotype (Fig. 4H).

A quantitative down-modulation of CD4+CD25+ Treg cells is induced by TS/A-CIITA vaccination upon TS/A challenge

In order to assess whether protective vaccination was associated with a variation in the number of CD4+CD25+ regulatory T cells (Tregs), lymphocytes from tumor-draining lymph nodes of mice vaccinated with either irradiated TS/A-CIITA...
or irradiated TS/A-pc cells and further challenged with live TS/A-pc were analyzed 3 weeks after challenge and compared with similarly derived lymphocytes from naive animals or from non-vaccinated and live tumor cell-injected mice. The results of this experiment are summarized in Table 2. In naive BALB/c mice, CD4^+CD25^+ Treg cells represented ~5.5% of the lymph node cells. This value rose to 9.9% in non-vaccinated mice bearing growing TS/A-pc tumors (Table 2, untreated). Importantly, the percentage of CD4^+CD25^+ Tregs was 6.2%, in TS/A-CIITA-vaccinated mice protected from TSA-pc challenge, thus significantly lower than that of untreated mice with growing tumors (P < 0.005) and similar to the percentage observed in naive mice. Interestingly in mice vaccinated with TS/A-pc, the percentage of Tregs was
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Table 2. CD4+CD25+ Treg cell assessment in tumor-draining lymph nodes of vaccinated mice after challenge with live TS/A-pc tumor cells

<table>
<thead>
<tr>
<th>Vaccinationa</th>
<th>No. of mice</th>
<th>CD4+CD25+ (% ± SD)</th>
<th>CD4+CD25+FoxP3+ (% ± SD)</th>
<th>CD4+ (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS/A-CIITA irradiated</td>
<td>3</td>
<td>6.2 ± 0.9</td>
<td>5.7 ± 0.3</td>
<td>58.0 ± 4.7</td>
</tr>
<tr>
<td>TS/A-pc irradiated/prb</td>
<td>3</td>
<td>8.6 ± 0.9</td>
<td>7.5 ± 0.2</td>
<td>53.2 ± 5.9</td>
</tr>
<tr>
<td>TS/A-pc irradiated/tuc</td>
<td>4</td>
<td>9.3 ± 0.8</td>
<td>8.5 ± 0.6</td>
<td>49.3 ± 4.8</td>
</tr>
<tr>
<td>Untreated</td>
<td>4</td>
<td>9.9 ± 1.0</td>
<td>9.1 ± 0.4</td>
<td>52.3 ± 5.3</td>
</tr>
<tr>
<td>Naïve micec</td>
<td>4</td>
<td>5.5 ± 0.3</td>
<td>5.1 ± 0.8</td>
<td>55.5 ± 3.4</td>
</tr>
</tbody>
</table>

aAge-matched mice, either vaccinated or not vaccinated (untreated), were challenged with live tumor cells (for vaccinated mice, 2 weeks after vaccination). Three weeks after challenge, their tumor-draining lymph nodes were phenotypically analyzed for the reported markers.
bMice vaccinated with TS/A-pc and with no sign of tumor (pr, protected) after challenge.
cThese values are statistically significantly higher (P < 0.005) than the value reported for either naïve mice or mice vaccinated with TS/A-CIITA tumor cells.
dMice vaccinated with TS/A-pc and with growing tumors (tu) after challenge.
eNaïve mice neither vaccinated nor injected with live tumor cells.

Discussion

The rationale of using tumor cells expressing CIITA-mediated MHC-II molecules as an anti-tumor vaccine stems from our previous findings indicating that these cells can efficiently induce, in live form, an anti-tumor immune response in syngeneic recipients. Moreover, the amplitude of protective immune response directly correlated with the amount of CIITA-mediated MHC-II expression, and CIITA-transfected cells efficiently processed and presented nominal antigens to antigen-specific CD4+ T cells (18). In order to envisage a possible application of the above approach in human clinical settings, an absolute requirement is to show that similarly modified tumor cells can also induce efficient, specific and long-lasting anti-tumor immunity in non-proliferating form. The present study clearly indicates that irradiated TS/A-CIITA mammary adenocarcinoma cells can be used as a potent and superior cell-based vaccine to induce stable and specific anti-tumor-protective immunity.

Several authors have investigated the potential of non-replicating or dead tumor cells, with or without adjuvants, to act as a vaccine to prevent tumor growth both in the experimental animal and in human [reviewed in refs (5,22,23,24)]. In most cases, the very low clinical response of vaccinated individuals switched the interest of investigators toward alternative approaches of cell-based vaccination, whereby the vaccine was not the tumor cell but rather an antigen-presenting cell, such as autologous DCs, loaded with tumor cell extracts or putative tumor-associated antigens, as these cells are believed to efficiently process and present antigens for optimal triggering of the immune response [reviewed in ref. (25)].

However, in none of the previous approaches, the comparison between the efficacy of non-replicating MHC-II-positive versus MHC-II-negative isogenic cells in inducing a protective anti-tumor immune response was assessed. By using our protocol of immunization, 83% of mice injected with TS/A-CIITA vaccine resulted fully protected from live TS/A-pc tumor challenge as compared with 30% protected mice when TS/A-pc vaccine was used as immunizing agent. Moreover, tumors growing in the 17% non-rejecting animals vaccinated with TS/A-CIITA displayed a significant reduced growth kinetics compared with the 70% unprotected mice vaccinated with TS/A-pc. In addition, in TS/A-CIITA, but not in TS/A-pc,-vaccinated mice, anti-tumor immunity was extended to the C26 colon adenocarcinoma, which shares with TS/A-pc the expression of important tumor antigens. The cross-immunity was manifested at the level of both protection (20% full rejection) and retarded tumor growth (the remaining 80% of mice with C26 tumor take). These results unambiguously establish that the presence of CIITA-induced MHC-II molecules in tumor cells is instrumental even in non-replicating cells to trigger optimal antigen-specific anti-tumor response capable to protect in vivo the vaccinated animals from tumor take upon challenge. Within this frame, CIITA-induced MHC-II-positive tumor cells may act themselves as surrogate antigen-presenting cells since they have this capability (18), or they may be engulfed as necrotic and/or apoptotic bodies by DCs and ‘fuel’ DCs with preformed MHC-II–antigenic peptide complexes with additional and superior stimulating capacity for CD4+ tumor-specific T cells, as it has been recently suggested (26). Whatever the mechanism, it appears, therefore, that CIITA, via its action on MHC-II expression, may act as a potent and specific ‘adjuvant’ (27), inducing specific immunity against tumor-associated antigens thus bypassing the need of identifying the antigens relevant for the tumor rejection, at least in our experimental model.
The crucial role played by the efficient CD4+ T priming was apparent both by functional experiments and by immunophenotypic analysis of the tumor microenvironment. Adoptive cell transfer experiments demonstrated that CD4+ T cells from TS/A-CIITA-vaccinated mice rejecting TS/A-pc challenge could confer protection from tumor growth to 60% of naïve animals co-injected with live TS/A-pc tumor cells. This protection was even higher than that generated by adoptive transfer of CD8+ T cells from the same vaccinated animals (40%) and quantitatively and qualitatively higher from the one generated by adoptive transfer of either CD4+ or CD8+ T cells from TS/A-pc-vaccinated mice. As we recently found in a different anti-tumor model of therapy-induced tumor vaccination, it is possible that the stronger protective ability of primed CD4+ as compared with primed CD8+ T cells observed in adoptive transfer experiments is predominantly due to the superior capacity of these cells in triggering naive tumor-specific CTL precursors and in maintaining their cytolytic function (21). In support of this hypothesis, we found that the protective response generated in TS/A-CIITA-vaccinated mice strongly correlated with the presence of a major CTL population specific for an immunodominant epitope of the gp70 glycoprotein from MuLV. This specificity was virtually absent in the CTL effectors generated after TS/A-pc vaccination. Thus, the presence of an efficient CD4+ Tn response could rescue and orient the repertoire of CTL effectors toward a similarly more efficient protective response against the tumor. However, it may also be possible that immunization with TS/A-CIITA-irradiated tumor cells may promote as well the generation of a subset of cytotoxic CD4+ T cells. Future studies will further clarify this interesting aspect related to the protective anti-tumor immunity of primed CD4+ Tn cells and will help to distinguish between the hypotheses described above.

Taken together, these results clearly identify in the expression of CIITA-mediated MHC class II molecules in tumor cells the major element associated to the priming of anti-tumor CD4+ Tn cells and thus reiterate the fundamental importance of these cells in the optimal induction of a protective anti-tumor immunity (28, 29).

Immunohistochemistry of tissue section from TS/A-CIITA-vaccinated mice challenged with live TS/A-pc showed a large infiltration particularly of CD4+ and CD8+ T cells, in comparison to TS/A-pc vaccinated. Moreover, infiltration of DCs, macrophages and PMN, consistently present in mice vaccinated with either TS/A-CIITA or TS/A-pc, was more abundant in TS/A-CIITA-vaccinated animals. The early massive infiltration of tumor tissue with CD4+ T cells observed in TS/A-CIITA-irradiated mice is reminiscent of a similar aspect of the infiltrate found in animals rejecting TS/A-CIITA live tumor cells (18). Thus, the expression of MHC-II molecules in tumor cells correlates with both the efficient priming of tumor-specific CD4+ T cells and their faster and massive migration in the tumor tissue upon challenge with live TSA-pc.

Further functional and phenotypic characterization of the lymphocyte sub-populations, present in mice undergoing cell vaccination, showed that spleen cells from TS/A-CIITA-vaccinated mice displayed a mixed Tn,1/Tn,2 phenotype with increased frequency of both IFN-γ- and IL-4-producing cells. This increase was even more dramatic in spleen cells from TS/A-CIITA-vaccinated mice challenged with live tumor cells. A major component of the IFN-γ (Tn,1-like) increased frequency was attributable to both CD4+ and CD8+ T cells, as demonstrated by inhibition with anti-MHC class II and class I antibodies. Interestingly, a large component of the CD8 CTL repertoire was specific for the AHT1 epitope of gp70 glycoprotein. On the contrary, the frequency of IFN-γ-producing spleen cells from mice vaccinated with non-replicating TS/A-pc tumor cells did not significantly increase either before or after challenge with live tumor cells. Instead, an increased frequency of Tn,2-like, IL-4-secreting spleen cells was observed, most likely CD4+ T cells since they were inhibited by anti-MHC class II antibodies. These results demonstrate additional important distinction between the immune responses generated by the two cell-based vaccines and emphasize the role of both Tn,1 and Tn,2 cells in the process leading to an efficient in vivo anti-tumor response in the present experimental setting. These results are partially different from those obtained in mice protected from tumor growth when vaccinated with TS/A-CIITA live tumor cells, in which a quite restricted Tn,1 type of response was observed (18). The reasons of this distinction are under investigation. One possibility is that live, replicating TS/A-CIITA cells either better present or preferentially focus toward a restricted number of antigens the anti-tumor immune response and that these antigens stimulate better Tn,1-type lymphocytes. Although in some tumor systems a polarized Tn,2 response promotes, rather than inhibiting, tumor growth and spread (13), in other tumor models a mixed Tn,1/Tn,2 immune response correlates with the tumor rejection (7, 30, 31). Interestingly, we recently found that a protective Tn,1/Tn,2 type of immune response was also observed in mice protected from tumor growth after a therapeutic procedure that targets tumor vessels and result in intense necrosis of tumor tissue (21, 32). Thus, it is likely that the presence of ‘dead tumor cells’ is a better stimulus for a mixed Tn,1/Tn,2-like response with respect to live tumor cells in those cases in which a protective response is generated. Taken together, the above results should induce to carefully reconsider the rather abused notion that, in vivo, a polarized Tn,1 response is synonymous with protective anti-tumor immune response, whereas a polarized Tn,2 response may even favor tumor growth.

It is widely accepted that CD4+CD25+ Tregs are involved in the control of activation and effectiveness of T cell immune response and in the immune tolerance to tumor (13). Here, we show that tumor-rejecting mice after vaccination with TS/A-CIITA, but not with TS/A-pc, cells display a significant reduction of Tregs in the tumor-draining lymph nodes with respect to tumor-bearing mice. It would appear, therefore, that immunization with TS/A-CIITA results in another important difference with respect to immunization with TS/A-pc. Although this difference is not associated per se to the absolute capacity to mount a protective immune response against TS/A-pc, nevertheless it may be important in favoring the generation and/or the stability over time of a repertoire of anti-tumor T cells which better protect, in quantitative terms, the animals from tumor take and tumor growth upon challenge. These results are reminiscent of our recent findings in a different experimental setting, whereby the
therapeutic treatment with a single-chain fragment variable antibody against an isoform of fibronectin (ED-B) expressed in neoangiogenesis, covalently conjugated with TNF-α, generates a therapy-induced vaccination which protects the mice from subsequent tumor challenge (32) and is associated with consistently reduced number of Tregs. Importantly, the quantitative reduction, but not the function, of Tregs was important in generating the protective phenotype (21). Preliminary experiments seem to confirm this finding even in the present experimental setting (data not shown).

In conclusion, the results presented in this investigation clearly demonstrate the crucial role of the CIITA-dependent MHC-II expression in TS/A tumor cells in inducing a strong protective anti-tumor immunity particularly at the level of CD4+ Treg cell triggering, necessary also for a better induction of CD8+ effector CTLs. This immunity is accompanied by a corresponding modification of the tumor microenvironment in the direction of an anti-tumor milieu capable of supporting recognition and killing of parental tumor cells upon challenge. Both CD4+ and CD8+ T cells are necessary to support tumor rejection. Moreover, acquisition of anti-tumor immunity is accompanied by reduction of CD4+CD25+ Treg.

We believe that our approach may be useful in envisaging new and alternative strategies of immune intervention exportable to human settings at the level of both prevention and, possibly, adjuvant immunotherapy.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CIITA</td>
<td>class II transcriptional activator</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<td>ELISPOT</td>
<td>enzyme-linked immunospot</td>
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<td>MuLV</td>
<td>murine leukemia virus</td>
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<tr>
<td>PMN</td>
<td>polymorphonucleate</td>
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<tr>
<td>s.c.</td>
<td>subcutaneously</td>
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<td>Treg</td>
<td>regulatory T cell</td>
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<td>TSA-pc</td>
<td>TS/A parental cell</td>
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**References**


