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ORIGINAL ARTICLE

Therapeutic efficacy of antitumor dendritic cell vaccinations correlates with persistent Th1 responses, high intratumor CD8+ T cell recruitment and low relative regulatory T cell infiltration

Gregory Driessens · Laurence Gordower · Lise Nuttin · Patrick Stordeur · Didier Blocklet · Dominique Egrise · Thierry Velu · Catherine Bruyns

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Abstract Despite the increasing number of immunotherapeutic strategies for the treatment of cancer, most approaches have failed to correlate the induction of an antitumor immune response with therapeutic efficacy. We therefore took advantage of a successful vaccination strategy—combining dendritic cells and irradiated GM-CSF secreting tumor cells—to compare the immune response induced against 9L gliosarcoma tumors in cured rats versus those with progressively growing tumors. At the systemic level, the tumor specific cytotoxic responses were quite heterogeneous in uncured vaccinated rats, and were surprisingly often high in animals with rapidly-growing tumors. IFN-γ secretion by activated splenic T cells was more discriminative as the CD4+ T cell-mediated production was weak in uncured rats whereas high in cured ones. At the tumor level, regressing tumors were strongly infiltrated by CD8+ T cells, which demonstrated lytic capacities as high as their splenic counterparts. In contrast, progressing tumors were weakly infiltrated by T cells showing impaired cytotoxic activities. Proportionately to the T cell infiltrate, the expression of Foxp3 was increased in progressive tumors suggesting inhibition by regulatory T cells. In conclusion, the main difference between cured and uncured vaccinated animals does not depend directly upon the induction of systemic cytotoxic responses. Rather the persistence of higher CD4+ Th1 responses, a high intratumoral recruitment of functional CD8+ T cells, and a low proportion of regulatory T cells correlate with tumor rejection.

Keywords T cells · Tumor immunity · Vaccination

Abbreviations

CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
Treg	Regulatory T cells
TIL	Tumor-infiltrating lymphocytes

Introduction

The relationship between the immune system and tumor development is complex. Cancer immunotherapy has largely focused on eliciting tumor antigen-specific cytotoxic T cells (CTL), historically thought to be critical for tumor rejection to occur [22]. Although CD8+ T cell responses can be observed in patients treated with antigen-specific vaccines, they do not correlate with tumor rejection,

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G. Driessens (✉) · L. Nuttin · T. Velu · C. Bruyns
LCCE-IRIBHM, Faculty of Medicine,
Université Libre de Bruxelles (ULB), route de Lennik 808,
1070 Bruxelles, Belgium
e-mail: g_driessens@hotmail.com

C. Bruyns
e-mail: cbruyns@ulb.ac.be

L. Gordower · P. Stordeur · T. Velu
Department of Medical Oncology, Erasme Hospital,
Université Libre de Bruxelles (ULB), route de Lennik 808,
1070 Brussels, Belgium

D. Blocklet · D. Egrise · T. Velu
Department of Nuclear Medicine, Erasme Hospital,
Université Libre de Bruxelles (ULB), route de Lennik 808,
1070 Brussels, Belgium

likely due to central and peripheral mechanisms of T cell tolerance [21, 26, 30]. These observations highlight the difficulties encountered in elucidating the key components of the immune response that are essential for effective anti-tumor immunity.

Recent studies monitoring patients antitumor response have emphasized the potential advantage of using vaccines that provide naturally processed, MHC class I and II-restricted peptides capable of engaging both CD8+ and CD4+ T cells [28]. The CD4+ Th1 T cell subtype, characterized by the secretion of IFN-γ, is primarily responsible for activating and regulating the development and persistence of CTL [17]. Previous studies in mice have shown that the in vivo induction of CTL responses, especially those mediated through cross-priming of exogenous antigens by host antigen-presenting dendritic cells (DC) is dependant on a CD4 Th response [2, 33]. More recently, it was demonstrated that CD4+ T lymphocytes play a pivotal role in the generation of functional CD8+ memory T cell responses to viral or acute infections [15], and are important for their long-term maintenance [38]. CD4+ T cells are also essential for the activation of memory CTL into tumor killer cells [10]. However, relatively little is known about the generation and maintenance of CD4+ memory T cells. With regard to antitumor immune responses, CD4+ Th cells are required for the induction of CTL and are needed during the effector phase of tumor rejection [14, 25].

Interestingly, only a handful of studies have examined the link between the therapeutic efficacy of a vaccine and its ability to induce the migration of both CD8+ and CD4+ T cells within the tumor microenvironment. For example, Galon et al. [9] showed recently that the density of T cell infiltration predicted the clinical outcome for patients with colorectal cancer. In a murine colon carcinoma model, intratumoral expression of CCL17 chemokine was shown to induce tumor regression by increasing the number of TIL [16].

Recent research also emphasized the important role of regulatory T cells (Treg), for their ability to potently suppress antitumor immune reactions in vivo [1, 41]. Indeed, depletion of Treg was shown in several animal models to enhance tumor immunity elicited by vaccines. Moreover, in humans, Treg appear to be present at an increased frequency in the peripheral blood and tumor microenvironment of patients with a wide array of malignancies, the latter predicting a shortened survival for patients with ovarian cancer [4]. Treg cells constitute 5–10% of peripheral CD4+ T cells in normal mice and humans. Their generation and function depend upon the expression of the transcription factor *forkhead box p3* (Foxp3) [13]. While the exact mechanism of Treg-induced suppression has not been identified, possibilities include direct cell contact through binding of cell surface molecules such as CTLA-4 or local secretion of immunosuppressive cytokines such as TGF-β and IL-10.

Vaccination with irradiated whole tumor cells genetically modified to secrete GM-CSF were shown, in numerous animal and preclinical tumor models, to generate potent antitumor immune responses, both humoral and cellular (CD4+, CD8+ and CD1d-restricted NKT-mediated), through improved tumor antigen presentation by recruited dendritic cells [5, 35]. While GM-CSF gene-engineered vaccines were effective in the prophylactic tumor setting, they were unable to control the growth-rate of established tumors [11, 18]. As we previously reported, we were able to cure approximately 60% of rats with pre-established 9L gliosarcoma tumors through vaccination with irradiated GM-CSF secreting tumor cells co-injected DC [6]. Here, we attempted to identify the crucial parameters responsible for tumor rejection, and to compare and contrast the immune response generated in control non-vaccinated rats with those induced in cured or uncured vaccinated ones. Our results indicate that at the systemic level, the main difference between cured and uncured vaccinated animals did not relate to the induction of cytotoxic response but rather depended on the persistence of higher CD4+ Th1 responses in cured animals. Moreover, at the tumor level, a more robust recruitment of CD8+ T cells and a lower proportion of Foxp3+ Treg/CD3+ T cells observed within regressing tumors appeared as the principal events leading to successful vaccine therapy.

Materials and methods

Animals

Male inbred Fischer 344 rats, purchased from Charles River Laboratories (l'Arbresle, France) were housed at the Animal Facility at University of Brussels Medical Center, in accordance with European Community guidelines, and used at the age of 10–12 weeks.

Cell lines

The 9L gliosarcoma cell line, syngeneic of Fischer 344 rats was provided by D. Deen and D. Dougherty (Brain Tumor Reasearch Center, University of California, San Francisco, CA, USA). The 9LmGM-CSF subline was generated in our laboratory as previously described [19]. We also used the NK cell target K562, and the MATB cell line, a syngeneic mammary adenocarcinoma. Cell lines were grown at 37°C in a humidified incubator with 5% CO_2, in RPMI 1640 medium supplemented with 10% fœtal bovine serum, 1% L-glutamine, 1% sodium-pyruvate, 1% non-essential amino acids, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Dendritic cell generation

Rat immature DC were generated as previously described [3]. Briefly, bone-marrow cell suspensions were first incubated on Petri dishes coated with normal goat and human serum to deplete the FcR positive and plastic adherent cells and then cultured in presence of 0.5 ng/ml mGM-CSF (Biosource, Nivelles, Belgium) for 8 days in RPMI medium (RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 10% FCS, 1% sodium-pyruvate, 5×10^{-5} mol/l 2-mercaptoethanol and 50 μg/ml gentamicin). DC purity was assessed by testing the expression of the rat dendritic cell marker OX-62 (the integrin α E2) with a specific antibody (Diagnostics Products Corporation, Humbeek, Belgium) and purity was routinely 50–85%.

In vivo combined therapeutic vaccinations

Tumor inoculation and therapeutic vaccinations were performed as described previously [6]. Briefly, 10^5 9L cells were inoculated s.c. in one flank at day 0. Therapeutic vaccines combining 3×10^6 bone-marrow derived DC and 5×10^6 irradiated 9LmGM-CSF cells (80 Gy, ^{137}Cs irradiator) were delivered s.c. in the opposite flank, at days 4, 11 and 18. Control rats did not receive vaccines. Tumor size was measured once a week with a caliper, starting at day 20. The vaccinated uncured rats were sacrificied between 35 and 60 days after tumor inoculation when their tumor reached 9 cm^2 (Fig. 1) and were analyzed for the antitumor immune response. Most of the vaccinated cured rats did not have any evidence of tumor and some showed regression of tumor $\leq$1 cm^2. These animals were also sacrificied between 35 and 60 days after tumor inoculation when tumor regression was completely accomplished. When specified, some cured animals were sacrificied at late time points (>100 days) or earlier during the course of tumor regression, such that they had a small residual tumor burden (~0.5 cm^2).

Anti-9L antibody detection

At the time of sacrifice, blood was collected from control non-vaccinated and from cured or uncured vaccinated rats. Sera from these animals were then examined for the presence of anti-9L antibodies directed against membrane or intracellular determinants respectively, by incubation with either entire or previously fixed and permeabilized 9L cells. Antibody-labelled 9L cells were detected by using a secondary goat anti-rat IgG FITC antiserum and flow cytometry. We also used, following manufacturer's instructions, a clonotyping® system/beads kit for rat isotype determination by flow cytometry (SouthernBiotech, Birmingham, USA).

Tumor-specific cytotoxic immune responses

Enriched splenic T cell suspensions were purified by passage through nylon wool fibre columns. Tumor-infiltrating lymphocytes (TIL) were recovered by enzymatic digestion (with collagenase 1 mg/ml and DNAse 0.1 mg/ml, 4-h at 37°C) of minced tumors, followed by several washings and

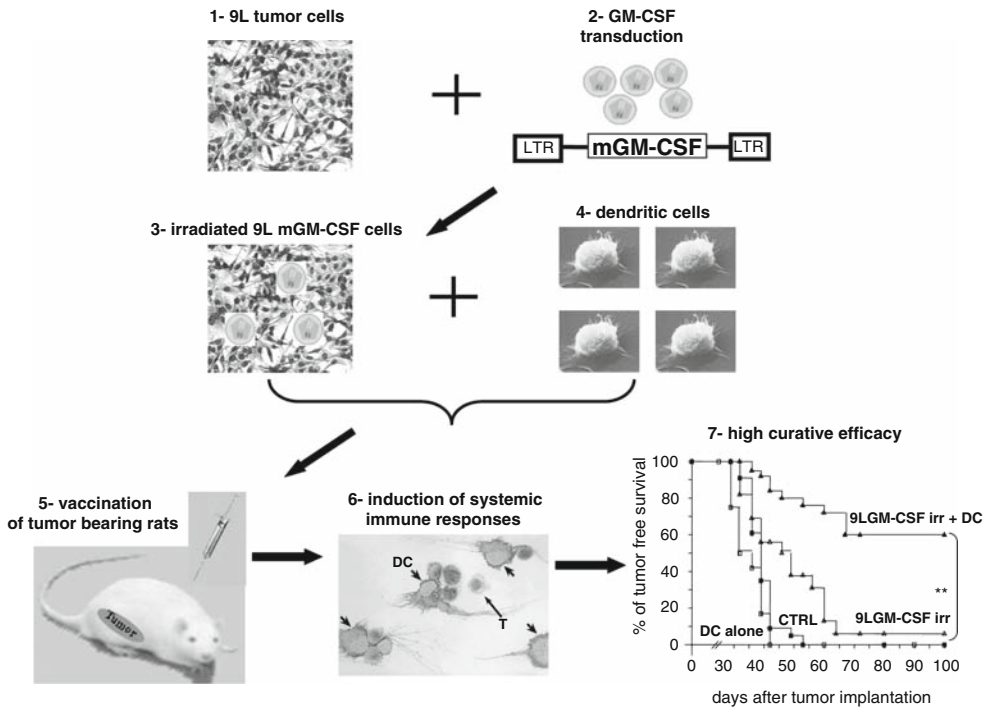

Fig. 1 Schematic representation of the antitumor vaccination strategy. A stable 9L cell line expressing the mouse GM-CSF was first established by retroviral transduction. Tumor-bearing rats were then vaccinated weekly for 3 weeks with irradiated 9LmGM-CSF cells mixed with bone marrow derived DC. Systemic tumor-specific immune responses were induced, able to cure 60% of the rats from their pre-implanted tumor

the elimination of erythrocytes/dead cells by centrifugation on a Ficoll gradient (Histopaque 1083, Sigma). These semi-purified splenic T cells or TIL were then stimulated at 5×10^6 cells/ml in 2 ml complete RPMI medium with 10^5 irradiated (80 Gy) 9L cells. After 5 days of culture, the activated effector cells were tested for lytic activity against the specific 9L target or the MATB third party or K562 NK cell target in a standard 4-h ^{51}Cr-release assay. Results were expressed as percent specific lysis at various effector to target cell ratios (E/T ratios). Meanwhile, after 72 h, aliquots of supernatants were collected from activated spleen cells, frozen, and assayed for IFN-γ content by ELISA (Biosource).

Intracytoplasmic cytokine secretion

Cells from 3-day co-cultured splenic T cells/irradiated 9L cells were processed for a cell-type-dependant detection of intracytoplasmic secretion of IFN-γ, IL-4 or IL-10. Briefly, the cells were first restimulated for 5 h with PMA at 20 ng/ml (Sigma-Aldrich, Bornem, Belgium) and ionomycin (at 500 ng/ml, Sigma-Aldrich) in presence of Golgi Plug (BD Biosciences, Erembodegem, Belgium), an inhibitor of Golgi transport. Cells were then washed and labelled with either anti-CD3, anti-CD4, anti-CD8 or NKR-P1A (anti-NK cells) antibody (BD Biosciences). Finally, the cells were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) before being incubated with an anti-rat IFN-γ, IL-4 or IL-10 antibody (BD Biosciences). Samples were analyzed by flow cytometry for intracytoplasmic cytokine production by different cell subsets.

Real-time RT-PCR

Tumors from non-vaccinated rats and from vaccinated cured (with regressive tumors) or uncured (with progressive tumors) rats were collected at specified time points for quantitative reverse transcription PCR analysis of CD3, CD4, CD8, IFN-γ and Foxp3 gene expression. β_2-microglobulin was used as a non-modulated reference gene. Frozen tissues were first homogenized to powder with a mortar, in liquid nitrogen and kept at -80°C in lysis buffer (MagNA Pure LC mRNA Isolation Kit II, Roche Applied Science). The mRNA extraction and isolation was done using the automated MagNA Pure LC Instrument system (MagNAPure LC mRNA Isolation Kit II, Roche Applied Science) following manufacturer's instructions. A one step real-time quantitative RT-PCR technique using the RNA Master Hybridization Probes Kit (Roche Applied Science) was used to quantify the different mRNAs as described previously [36]. The primers and fluorescent probes for rat CD3, CD4, CD8, IFN-γ and β2-microglobulin were purchased from Applied Biosystems (Lennik, Belgium). The following primers and probe for the rat Foxp3 were designed with the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and purchased from Eurogentec (Seraing, Belgium): forward, 5′-ACCTTTCCAGAGTTCTTCCACA-3′; reverse, 5′-GTGGTTTCTGAAGTAGGCGAAC-3′; probe, 6Fam-TCACCTATGCCACCCTCATCCGA-Tamra-p. The following primers were used to amplify Standard Foxp3 sequence: forward, 5′-CTTCAGACAGCTTGTTTGCT-3′; reverse, CGAAACTCAAATTCATCTACG-3′. All the primers utilized did not amplify genomic DNA (data not shown). Data were collected using the Light Cycler Data Analysis software (Roche). A standard curve was generated for each tested gene with a dilution serie of a reference cDNA sample. The software determines the relative quantity of each sample by using the threshold cycle (C_T). Data are expressed as normalized gene expression, which represents for each sample the relative number of mRNA copies of a specific gene per 10^3 copies of the β_2-microglobulin or CD3 gene, in order to refer respectively to total cell content or more specifically to T cells.

Statistics

Data from real-time RT-PCR, flow cytometry, migration assays and cytotoxic assays were statistically analyzed with the unpaired *t* test, using a Welch correction when appropriate.

Results

We previously demonstrated within the rat 9L gliosarcoma model, the high therapeutic efficacy of a novel vaccination strategy combining DC and irradiated GM-CSF-secreting tumor cells [6]. Using this approach, we succeeded in curing approximately 60% of the rats with pre-implanted 9L tumors (Fig. 1). Nevertheless, although all animals were syngeneic, from same origin or age and equally vaccinated, some were cured when others were not. We thus took advantage of this rat vaccination model to identify the crucial parameters leading to tumor eradication and analyzed in detail the immune response that was induced in vaccinated cured (bearing regressive tumors or tumor free) or uncured (bearing progressive tumors).

Anti-9L antibody responses are similarly observed in all cured and uncured vaccinated rats

One feature of GM-CSF-secreting tumor cell vaccines is their ability to induce the production of tumor-specific antibodies in treated rats. We thus tested sera from non-vaccinated or vaccinated tumor-bearing rats for the presence of

antibodies directed against either membrane or intracellular determinants of 9L cells. Anti-9L antibodies were highly present in sera from vaccinated rats as compared to tumor-bearing non-vaccinated rats. However, no significant differences in anti-9L antibody titers or isotypes were evident when comparing animals cured or uncured of their 9L tumors (data not shown).

High systemic tumor-specific cytotoxic responses are induced in both cured and uncured rats

We next investigated the relationship between the in vivo therapeutic outcome and the systemic induction of 9L-specific cytotoxic responses. A number of animal models examining various immunotherapeutic approaches have linked the emergence of systemic immune-mediated cytolytic capabilities with tumor eradication. However, the presence and function of CTL in uncured vaccinated animals has rarely been investigated. To further elucidate the mechanisms responsible for failed immune-mediated 9L rejection in our model, spleens from control non-vaccinated and from cured (tumor-free) or uncured vaccinated rats were harvested at a late stage of tumor progression following vaccination when the therapeutic outcome in the animals was clinically obvious (day 35–60 after 9L implantation). Splenic T cells were semi-purified and re-stimulated in a 5-day co-culture with irradiated 9L cells and then tested for cytotoxic activity in a chromium release assay. Results from these experiments confirmed that the 9L-specific lysis by T cells was nearly undetectable in unvaccinated control rats (Fig. 2a, d) and always elevated in cured vaccinated rats (Fig. 2c, d, $p < 0.001$ compared with control group), even when these tumor-free animals were sacrificed one year after vaccination (data not shown). Interestingly,

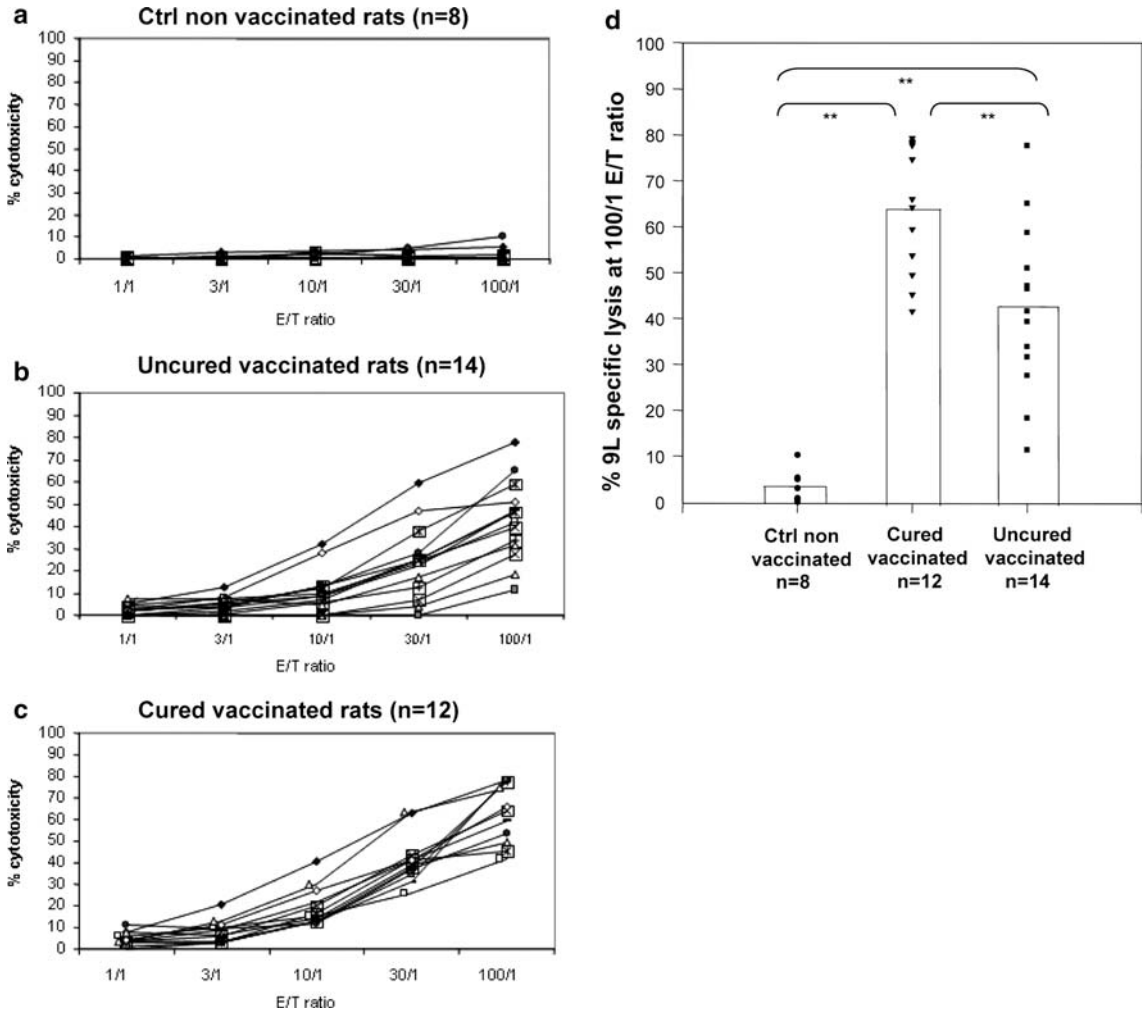

Fig. 2 In vitro detection of 9L-specific cytotoxic responses. Around day 35 after tumor inoculation, T cells were semi-purified from spleens of control non-vaccinated rats (Fig. 2a) or uncured (Fig. 2b) or cured (Fig. 2c) vaccinated rats. They were then stimulated in vitro for 5 days with irradiated 9L cells and tested for cytotoxic activity against the specific target 9L, at different E/T ratio, in a standard 4-h ^{51}Cr-release assay. Each curve represents an individual rat. Figure 2d shows pooled individual and mean values (*histograms*) at the 100:1 E/T ratio from all rats in each group. ** = $p < 0.01$

in uncured vaccinated rats, the 9L-specific cytotoxic responses were quite heterogeneous, as shown in Figure 2b, d. Unexpectedly, half of the uncured vaccinated rats bearing progressively-growing tumors demonstrated high cytotoxic response equivalent to those observed in the cured vaccinated animals. The mean average of cytotoxicity within the uncured vaccinated group (43% at the 100:1 E/T ratio) was however lower than in the cured vaccinated group ($p < 0.002$) and higher than in the control non-vaccinated one ($p < 0.001$). Further chromium release assays done with K562 cells or MATB cells, a third-party syngeneic target, revealed only slight NK activities or non-specific cytotoxic activity, respectively (data not shown).

As another readout of T cell activation, we also measured by ELISA, the IFN-γ production in 72-h culture supernatants of the same 9L-stimulated splenic T cells from non-vaccinated or vaccinated rats. As shown in Fig. 3, we observed clear differences in IFN-γ secretion between cured rats (546 pg/10^6 cells/72 h; $p < 0{,}001$ referred to controls and $p < 0.02$ referred to uncured group) and uncured ones (61 pg/10^6 cells/72 h; $p < 0{,}001$ referred to controls) or control non-vaccinated (4 pg/10^6 cells/72 h).

A lower proportion of activated splenic CD4+ T cells is observed in uncured rats

In order to investigate the cellular source of this IFN-γ production, we analyzed intracellular IFN-γ production by various splenic cell subsets (CD4+ or CD8+ T cells and NK cells). Figure 4a exemplifies a representative experiment examining one rat in each group and demonstrates the near absence of IFN-γ secretion in the non-vaccinated rat, an intermediate production, especially by CD8+ T cells, in the uncured vaccinated rat and a strong CD4+ and CD8+ T cell-mediated production in the cured vaccinated rat. Moreover, this IFN-γ production was quite reduced when the splenic T cells were activated with third-party syngeneic stimulator cells (MATB) instead of specific 9L tumor cells. Figures 4b–d represent for all tested rats in each group and for each different cell subset, the individual and mean percentages of IFN-γ producing cells among all cells belonging to a definite subtype. Together, these data confirm a small proportion of CD4+ T, CD8+ T or NK cells producing IFN-γ in non-vaccinated rats and a quite higher proportion in cured vaccinated rats, i.e. 5% versus 27% for CD4+ IFN-γ+ T cells ($p < 0.01$), 5% versus 34% for CD8+ IFN-γ+ T cells ($p < 0.01$) and 11% versus 40% for IFN-γ+ NK cells ($p < 0.01$). In uncured vaccinated rats, the proportion of CD8+ IFN-γ+ T cells (20%) was intermediate, only slightly decreased as compared to cured rats ($p < 0.01$ referred to control or cured rats) whereas the proportion of IFN-γ+ NK cells (41%, $p < 0.01$ vesus control non-vaccinated rats) was similar to the one of cured vaccinated rats. Surprisingly however, the proportion of CD4+ IFN-γ+ T cells was strongly decreased (9%) in those uncured vaccinated rats ($p < 0.01$ refered to cured vaccinated rats) and reached values close to the ones of control non-vaccinated rats. A similar investigation for IL-4 and IL-10 intracytoplasmic secretion in these three groups of rats revealed weak or no production of these cytokines (data not shown). Collectively, our results indicate that at the systemic level, the main difference between cured and uncured vaccinated rats is a lower proportion of activated splenic CD4+ T cells in uncured ones.

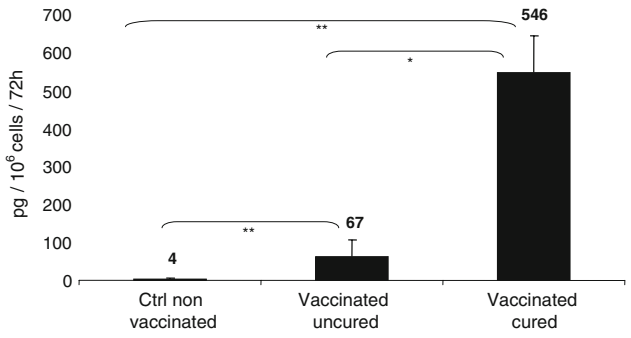

Fig. 3 In vitro IFN-γ secretion by activated splenic T cells. Around day 35 after tumor inoculation, T cells were semi-purified from spleens of control non-vaccinated and uncured or cured vaccinated rats and stimulated in vitro with irradiated 9L cells. After 72 h, aliquots of co-culture supernatants were collected and assayed for IFN-γ content by ELISA. * = $p < 0.02$ and ** = $p < 0.01$

High cytotoxic activity is only detected among TIL of regressive tumors

Since the systemic cytotoxic response induced in vaccinated rats were not necessarily predictive of the therapeutic outcome, we analyzed, at same time point (35 days after 9L tumor implantation), the cytotoxic activity of lymphocytes within the tumor microenvironment of individual rats belonging to the non-vaccinated, vaccinated uncured or cured groups. The difficulty was that the vast majority of the cured vaccinated animals neither developped a palpable tumor or, if they did, their tumor neither exceeded an initial size of 1 cm^2 at first measurement and 0.5 cm^2 when it was definitely identified as being in regression and excisable. Nevertheless, we succeeded to test within two independent experiments, three tumors in regression and six tumors in progression. When processing the tumors, we could observe that the regressing tumors contained mostly necrotic tumor debris, along with many viable TIL. On the contrary, all progressive tumors, whatever their origin, contained a predominance of viable tumor cells and few TIL. Enriched TIL suspensions were thus prepared from all excised tumors and restimulated in culture with irradiated 9L cells for 5 days before being tested for 9L- specific

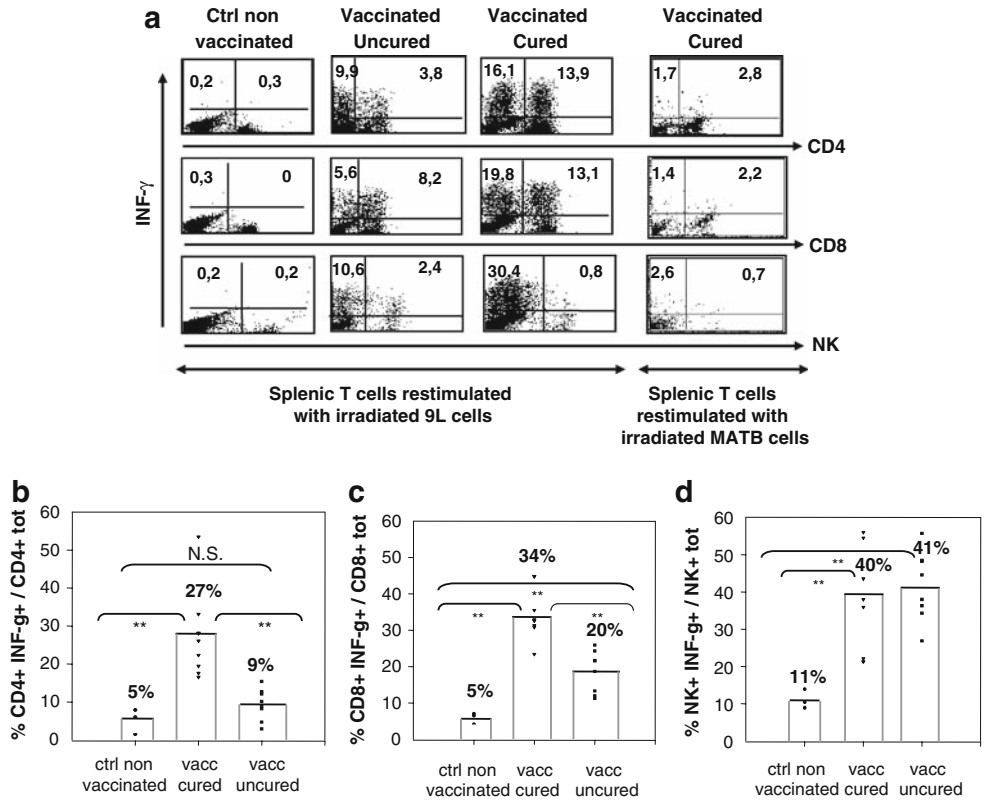

Fig. 4 Intracytoplasmic IFN-γ production by splenic CD4+ T, CD8+ T and NK cell-subsets. Around day 35 after tumor inoculation, T cells were semi-purified from spleens of control non-vaccinated and uncured or cured vaccinated rats and stimulated in vitro for 3 days with either irradiated 9L cells or third-party syngeneic MATB cells before being processed for a cell-type-dependant detection of intracytoplasmic IFN-γ secretion by flow cytometry. Figure 4a shows the results of a representative analysis enrolling one rat in each experimental group. *Numbers* in each quadrant represent the percentages of IFN-γ+ cells. Individual and mean percentages of IFN-γ producing cells among all cells belonging to a definite subtype are represented in Fig. 4b for CD4+ T cells, Fig. 4c for CD8+ T cells and Fig. 4d for NK cells. Each symbol represents the value obtained for an individual rat (control rats $n = 3$, cured vaccinated rats $n = 8$ and uncured vaccinated rats $n = 9$). *Histograms* represent the *mean* values. ** = $p < 0.01$; N.S. = not statistically significant

cytotoxic activities by chromium release assays. Figure 5a illustrates the percentage of specific lysis at the 10:1 E/T ratio, on account of the low number of TIL generally recovered from tumors. Splenic T cells from the same animals were simultaneously tested for 9L-specific lytic capacities (Fig. 5a, b). Our results demonstrate that TIL purified from regressive tumors showed high 9L-specific cytotoxic activity (mean: 32% at E/T ratio 10:1), which was similar or higher than the one seen with splenic T cells from the same animal (mean: 27% at E/T ratio 10:1). On the other hand, TIL from uncured vaccinated rats showed little or no cytotoxicity activity with an average of 5% of 9L cells lysed at E/T ratio 10:1. Interestingly, splenic T cells from all uncured rats demonstrated much higher cytolysis (mean: 16% at E/T ratio 10:1) than their intratumor counterpart and close to the values observed for the cured animals. No intratumoral nor systemic cytotoxic activity was detected in the non-vaccinated rats bearing a progressive tumor. As shown in Fig. 5c, the levels of activated CD3+ TIL recovered were much higher for the regressive tumors (~6%) than for the progressive ones (~1%). TIL were also examined for intracellular IFN-γ production, after a 3-day coculture with irradiated 9L cells. Variable percentages of CD8+IFN-γ+ TIL and CD4+IFN-γ+ TIL were detected in all tumors, independently of their status (data not shown). But, considering the small number of cells infiltrating some tumors, it seems too hazardous to draw firm conclusions from this analysis. The results of this group of experiments clearly demonstrate that the regressing tumor was highly infiltrated by T lymphocytes which showed equal high lytic capacities than their splenic counterparts. In contrast, progressing tumors were weakly infiltrated by poorly functional T cells.

Progressive tumors from vaccinated rats are characterized by a weak recruitment of CD8+ T cells and a high proportion of Foxp3+/CD3+ T cells

The recruitment of Treg in tumors has been shown by others to be linked to a decreased survival [4]. It was possible

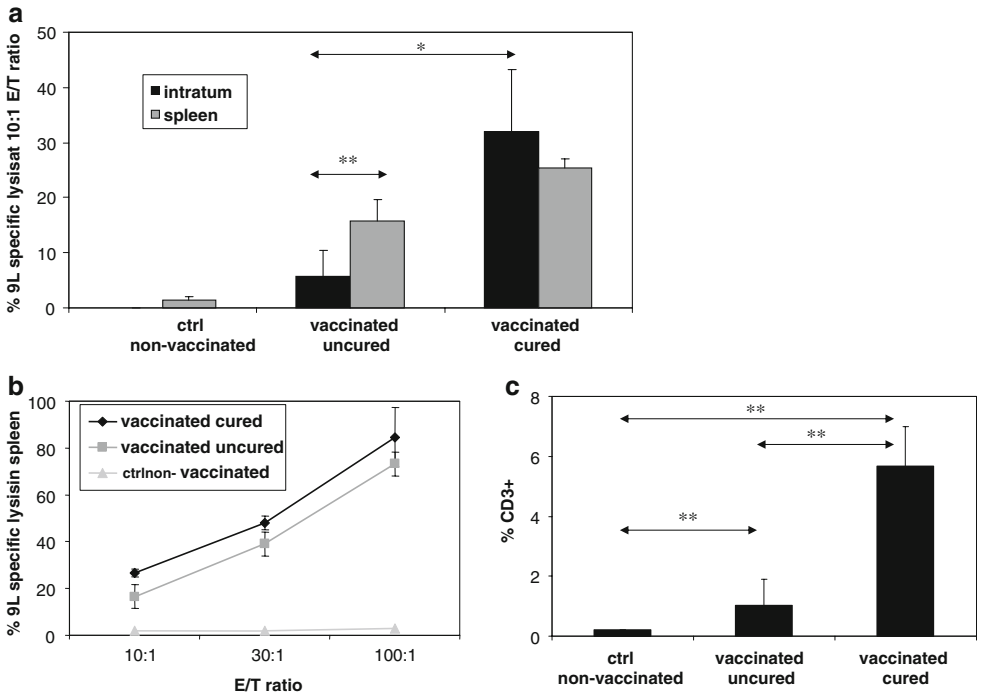

Fig. 5 Comparison between TIL and splenic T cell cytotoxic activity within rats bearing regressive (vaccinated cured, $n = 3$) or progressive tumors (ctrl non-vaccinated, $n = 2$ and vaccinated uncured, $n = 6$). Semi-purified TIL were recovered by enzymatic digestion of minced tumors and centrifugation on a Ficoll gradient. Enriched splenic T cells were purified by passage on nylon wool fibre columns. Both were thereafter stimulated for 5 days with irradiated 9L cells before being tested for 9L specific lytic activity in a standard 4-h ^{51}Cr-release assay. Results were expressed as percent specific lysis at 10:1 effector to target cell ratio (E/T) for TIL and splenic T cells (Fig. 5a) and at various E/T ratios for splenic T cells (Fig. 5b). Figure 5c represents the *percentages* of CD3+ T cells in tumor cell suspensions. * = $p < 0.05$ and ** = $p < 0.01$

that Tregs could be playing a role in inhibiting conventional intra-tumoral T cells in our model. Because our analysis of the tumor microenvironment was limited due to a low number of T cells present, we utilized the real-time PCR to quantify the intratumoral presence of Foxp3+ Treg in relation to other infiltrating T cells.

As a prerequisite, we first examined the 9L cell line or 9L cells purified from ex-vivo resected progressive tumors as well as magnetically purified splenic CD4+CD25+ and CD4+CD25- T cells for the expression of Foxp3 gene. Our RT-PCR data confirmed that Foxp3 was exclusively expressed by CD4+CD25+ T cells and not by CD4+CD25- T cells nor by 9L tumor cells (data not shown). Foxp3 expression by CD4+CD25+ T cells was correlated to functional assays showing their ability to inhibit the proliferation of effector T cells (data not shown). Figure 6a shows pooled data from individual tumors (progressing, regressing or control) that were analyzed by real time PCR for CD3, CD4, CD8, IFN-γ and Foxp3 gene expression. β_2-microglobulin was used as housekeeping gene and was indeed invariant. Results are expressed as relative mRNA copy numbers of one selected gene to thousand copies of β_2-microglobulin or to thousand copies of CD3. In a more illustrative way, Fig. 6b points out the fold variation expression of one selected gene in progressing or regressing tumors from vaccinated rats versus same gene expression in control tumors. Our results confirm an increased intratumoral CD3+ T cell-recruitment in vaccinated rats (progressing or regressing tumors versus control ones: $p < 0.01$). Levels of the CD3 transcript were higher in rats with regressing tumors (regressing versus progressing tumors: $p < 0.01$) and consisted mainly of CD8+ T cells ($p < 0.01$ as compared to progressing or control tumors). This intratumoral migration of CD8+ T cells was a common feature in all regressing tumors. On the contrary, minimal CD8+ T cell-migration was observed in progressing tumors from control non-vaccinated or vaccinated rats. Regarding the intratumoral presence of Treg, the absolute level of Foxp3 expression (Foxp3/Beta-2) was unexpectedly the highest in regressing tumors, probably linked to the increased numbers of T cells within these tumors. There was also a 25-fold higher expression of the CD8 gene and a 3-fold higher expression of CD4 gene correlating with a 3-fold higher IFN-γ expression in regressing tumors as compared to control or progressing ones ($p < 0.05$). Therefore, when comparing the level for Foxp3 transcripts with those for the total T cell population (Foxp3/CD3), we found that this ratio was lower in rats with regressing tumors as

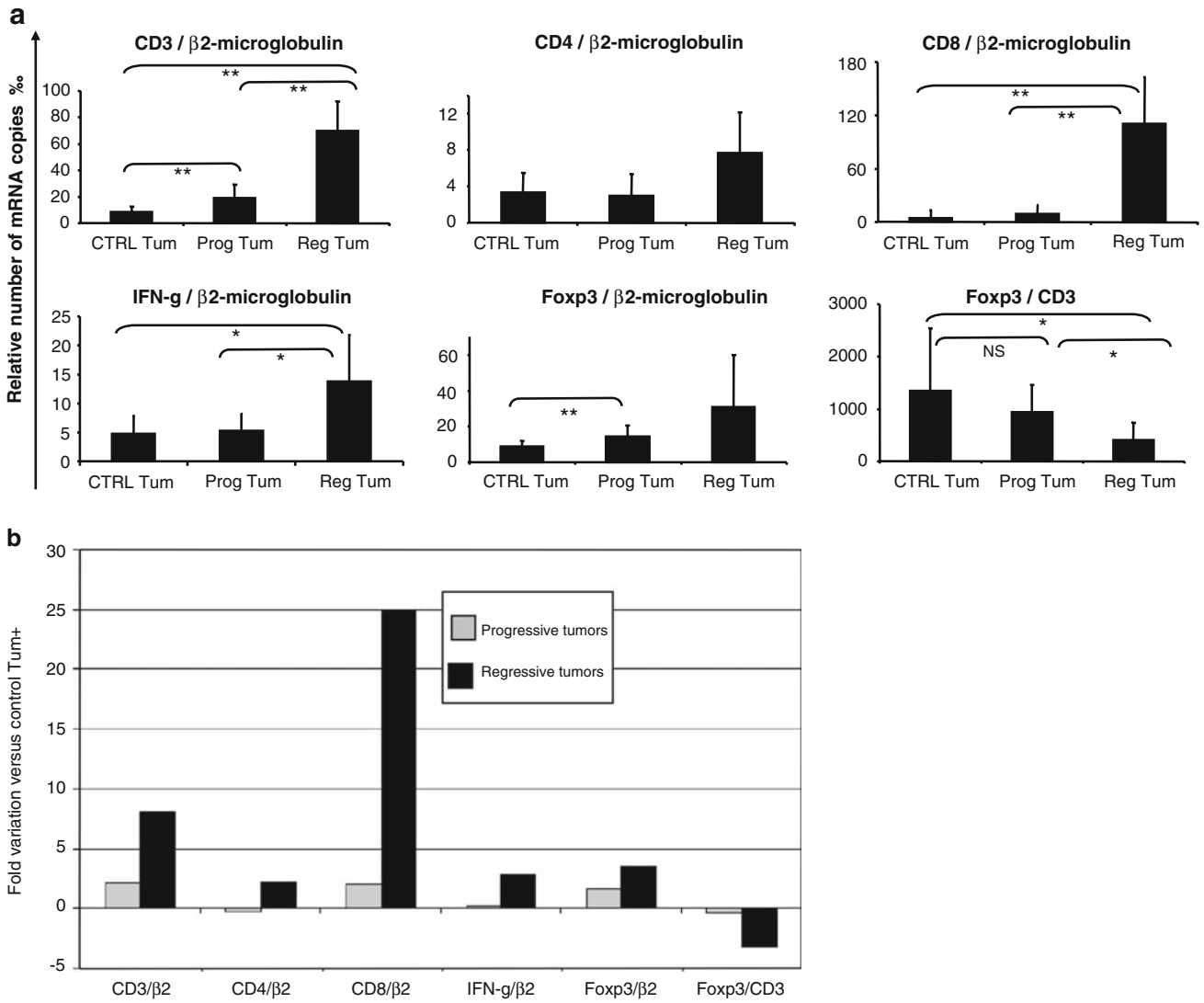

Fig. 6 Real time PCR analysis of the presence of Foxp3+ regulatory T cells in relation to other infiltrating T cells. Individual progressive tumors from control rats or uncured vaccinated rats and regressive tumors from cured vaccinated rats were tested for CD3, CD4, CD8, IFN-γ and Foxp3 gene expression. β_2-microglobulin was used as an indeed invariant housekeeping gene. *Data* in Fig. 6a are expressed as relative mRNA copy numbers of one selected gene to thousand copies of β_2-microglobulin or to thousand copies of CD3. Control tumors (*CTRL Tum*) $n = 30$, progressing tumors (*Prog Tum*) $n = 30$, regressing tumors (*Reg Tum*) $n = 10$. * = $p < 0.05$; ** = $p < 0.01$. Figure 6b illustrates the fold variation expression of one selected gene in progressing or regressing tumors versus same gene expression in control tumors

compared with the other groups. As a whole, real time PCR assays on tumoral tissue proved itself to be a powerfull predictive tool to assess the therapeutic outcome of a tumor.

Discussion

Cancer immunotherapy to date has largely focused on eliciting tumor antigen-specific cytotoxic (CTL) responses [22]. However, the presence of tumor-specific T cells has not always correlated with objective tumor response, suggesting a role for tumor escape mechanisms. Furthermore, recent data have underscored the importance of regulatory T cells in inhibiting anti-tumor immune responses in vivo [41]. However, very few animal or human studies have focused on identifying the crucial parameters leading to tumor eradication following vaccination. In this study, we took advantage of an original therapeutic vaccination strategy associating DC and GM-CSF secreting tumor cell-vaccines we had previously investigated with success in the rat 9L gliosarcoma model [6], to analyze comparatively the immune response that was induced in cured or uncured vaccinated rats and in control non-vaccinated rats.

The major advantage of GM-CSF tumor cell vaccines is their ability to enhance tumor antigen presentation through local recruitment of DC and macrophages. The resulting

protective immunity is mediated by B cells, CD4+ T cells, CD8+ T cells and CD1d-restricted NKT cells [5, 35]. Accordingly, we have detected anti-9L antibody responses in all vaccinated rats, but without difference between those cured or uncured of their tumor. As expected, we also have always observed a strong tumor-specific T cell response in spleens of cured vaccinated rats, even one year after the complete regression of their tumor mass. No significant CTL response was detected in spleens from tumor-bearing control non vaccinated rats. Unexpectedly, in uncured vaccinated rats, the cytotoxic response induced by vaccination was quite heterogeneous and half of the animals, developing fast growing tumors, had as high CTL activity as the ones observed in the cured group. In general thus, the systemic cytotoxic response detected in vaccinated rats did not allow to predict the therapeutic outcome. It is interesting to note that Rosenberg and colleagues have recently reported that in melanoma patients, tumor progression can occur despite the induction of very high levels of tumor antigen-specific CD8+ T cells [31]. Moreover, although CD8+ T cell responses are frequently observed in patients treated with an antigen-specific vaccines, they are however often ineffective in establishing complete and durable clinical response [26, 30]. Here, we used for the first time, an animal model mimicking what happens during clinical trials, to identify the crucial parameters leading to tumor eradication following vaccination.

When examining the IFN-γ secretion by 9L-activated splenic T cells, we were surprised to note strong disparities between cured and uncured vaccinated rats. Intracytoplasmic IFN-γ detection assays demonstrated a strong CD8+ and CD4+ T cell-mediated INF-γ secretion in cured vaccinated rats and surprisingly, in uncured ones, a quite lower involvement of the CD4+ T cell subset in the IFN-γ production. Our results thus suggest that, even when a potent CTL activity is present, a CD4+ T cell-mediated helper activity must persist to effectively achieve the destruction of the tumor. Numerous studies have underlined that CD4+ T cells were essential for the initiation of the response (reviewed in [17]) but the role of CD4+ T cells during the effector phase of the antitumor response has long been overshadowed by emphasis on CD8+ cytotoxic T cells. According to very recent results from Plautz's group [39] showing that adoptive transfer of effector CD4+ T cells in combination with effector CD8+ T cells provides synergistic anti-tumor response, we show evidence here that the need for their presence/persistence at late stage of the effector phase of the anti-tumor immune response is required to achieve tumor eradication.

Perhaps more importantly, we analyzed individual tumors from rats belonging to the control non-vaccinated and uncured or cured vaccinated groups for immune effectors capability. Pooled data from flow cytometry, cytolytic assays and real time PCR experiments demonstrated that the regressing tumors were highly infiltrated by T lymphocytes, mainly CD8+, which showed equal high lytic capacities than their splenic counterparts. On the other hand, no or few functional CD8+ T cells were observed in progressing tumors. Two mechanisms have been proposed for the inability of CD8+ TIL to effectively lyse tumor cells: a defect in the cytolytic pathways or an overexpression of inhibitory molecules [20, 34].

As concerns the intratumoral presence of CD4+ T cells, we also observed an increased expression of CD4 and IFN-γ genes in the regressing tumors as compared to the control or progressing ones but lighter than for CD8+ T cells.

The intratumoral migration of CD8+ T lymphocytes is a common feature between all regressing tumors. The same is not true for progressively-growing tumors, suggesting that impaired T cell trafficking in some rats may explain their failure to reject 9L tumors. This may be especially true, as many of the uncured vaccinated mice demonstrated potent splenic cytotoxic activities. TIL have been recognized in various human cancers to be capable of inhibiting tumor growth, and their presence has occasionally been associated with an improved prognosis [27, 32]. Moreover, in accordance with our data showing that IFN-γ secreting CD4+ T cells infiltrated regressing tumors, two recent studies have suggested that concurrent infiltration by Th1 cells have a beneficial effect on clinical outcome [9, 12]. However, very little is known about the factors/mechanisms that drive these T cells to migrate inside a tumor. The higher IFN-γ secretion we detected at the systemic and intra-tumoral level in vaccinated rats bearing regressing tumors could play a determinant role in this process. Indeed, Nakajima et al. have reported that in IFN-γ-deficient mice functional T cells were generated but failed to migrate to tumor sites [23].

The higher intra-tumoral expression of IFN-γ we observed in regressing tumors could also have direct effects on the tumor eradication. It has been previously shown that IFN-γ is crucial for tumor rejection especially by increasing tumor immunogenicity via the up-regulation of the MHC class I pathway of antigen processing and presentation (reviewed in [7]). Moreover, since tumor cells exposed to an environment rich in IFN-γ switch their proteasome from the standard type to the immunoproteasome type (constitutively expressed by DC), having an intra-tumoral expression of IFN-γ could play a role in the generation of relevant antigenic peptides for DC vaccine strategies (reviewed in [37]). Finally, another mechanism by which the effects of IFN-γ on host cells might contribute to the anti-tumor immune response was recently reported by a study showing that IFN-γ is able to abrogate the generation/activation of Treg cells [24]. These observations endorse the hypothesis that the higher number of activated CD4+ T cells secreting

IFN-γ that were recruited in regressing tumors could inhibit the activity of Treg cells present in these tumors.

Indeed, when quantifying by real-time RT-PCR the intra-tumoral presence of Treg, we unexpectedly observed that the absolute level of Foxp3 gene expression was the most increased in regressing tumors. But, since those regressing tumors were also highly infiltrated by CD8+ T cells and moderately by CD4+ T cells as compared to control or progressing tumors, the proportion of Treg within the total intra-tumoral T cell population was significantly decreased. Few studies only have characterized changes in intra-tumoral Treg cell population, especially in response to therapeutic vaccinations. In accordance with the work of Zhou et al. [40], we have observed here an in vivo expansion of Treg cells in response to vaccination, even more pronounced in regressing tumors. However, our data are in favor of recent findings demonstrating that the absolute numbers of Treg cells within the tumor appears to be less important than their relative proportion to conventional T cells with respect to tumor growth [8, 34]. In agreement with a recent paper from Allison's group, the success of our vaccination strategy seems to partially depend on the change in the intratumor ratio of effector (CD4+ but mostly CD8+ TIL) to regulator T cells [29]. Since the in vivo depletion of Treg cells strongly increased the efficacy of GM-CSF-transduced tumor cell vaccines in their model, it could be interesting to investigate in our tumor model if the Treg depletion could first of all, increases the vaccine curative efficacy and secondly, modifies the nature/balance of the immune parameters linked to tumor regression or progression.

In conclusion, our results indicate that the main difference observed between cured and uncured vaccinated rats consists in a lower proportion of activated splenic CD4+ T cells in uncured ones. Moreover, the weak intratumoral migration of CD8+ T cells and the greater proportion of Foxp3+ Treg/CD3+ T cells observed within progressing tumors of vaccinated rats appear as the principal reasons of the failure of the therapy. Since immunological monitoring of many clinical trials has failed to identify a surrogate marker for clinical outcomes, integrating all these parameters should be of importance to evaluate and increase the efficacy of immunotherapy in tumor-bearing patients.

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