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$CD4^+/CD25^+$ Regulatory Cells Inhibit Activation of Tumor-Primed $CD4^+$ T Cells with IFN-γ-Dependent Antiangiogenic Activity, as well as Long-Lasting Tumor Immunity Elicited by Peptide Vaccination[1]

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$CD25^+$ regulatory T (T reg) cells suppress the activation/proliferation of other $CD4^+$ or $CD8^+$ T cells in vitro. Also, down-regulation of $CD25^+$ T reg cells enhance antitumor immune responses. In this study, we show that depletion of $CD25^+$ T reg cells allows the host to induce both $CD4^+$ and $CD8^+$ antitumoral responses following tumor challenge. Simultaneous depletion of $CD25^+$ and $CD8^+$ cells, as well as adoptive transfer experiments, revealed that tumor-specific $CD4^+$ T cells, which emerged in the absence of $CD25^+$ T reg cells, were able to reject CT26 colon cancer cells, a MHC class II-negative tumor. The antitumoral effect mediated by $CD4^+$ T cells was dependent on IFN-γ production, which exerted a potent antiangiogenic activity. The capacity of the host to mount this antitumor response is lost once the number of $CD25^+$ T reg cells is restored over time. However, $CD25^+$ T reg cell depletion before immunization with AH1 (a cytotoxic T cell determinant from CT26 tumor cells) permits the induction of a long-lasting antitumoral immune response, not observed if immunization is conducted in the presence of regulatory cells. A study of the effect of different levels of depletion of $CD25^+$ T reg cells before immunization with the peptide AH1 alone, or in combination with a Th determinant, unraveled that Th cells play an important role in overcoming the suppressive effect of $CD25^+$ T reg on the induction of long-lasting cellular immune responses. ***The Journal of Immunology*****, 2003, 171: 5931–5939.**

Regulatory T (T reg)[3] cells have a key role in the maintenance of immune tolerance to both self- and foreign Ags. In particular, a minor population (10%) of $CD4^+$ T cells, which coexpresses the IL-2R α-chain (CD25), has been described to be crucial for the control of autoreactive T cells in vivo (reviewed in Ref. 1). Thus, it has been shown that upon Ag stimulation, this $CD4^+/CD25^+$ cell population potently suppresses the activation/proliferation of other $CD4^+$ or $CD8^+$ cells in vitro (2, 3). The mechanism of suppression seems to be the inhibition of IL-2 transcription in the effector populations. Indeed, suppression can be abrogated by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production by means of anti-CD28 Ab (4). However, the exact mechanism by which $CD25^+$ T reg cells exert their suppressive effects remains unknown. Although some research groups have reported the need of cell-to-cell contact between suppressor and responder cells to exert the inhibitory function (3, 5) and that secreted cytokines are not required (6), other groups have reported that the suppressive effect of $CD25^+$ T reg cells is mediated by soluble factors and do not require cell-to-cell contact (7, 8).

T cell-mediated immunotherapy represents a promising treatment for cancer. The absence of efficient tumor-specific immune responses in cancer patients can be related to a deficient APC function or to T cell tolerance/ignorance toward tumor Ags (9, 10). It has been postulated that $CD4^+/CD25^+$ immunoregulatory cells may be engaged in continuously up-regulating the activation thresholds of other T cells, thereby avoiding effective generation of tumor immunity while inhibiting autoimmunity (2, 3, 11, 12). Thus, breaking immunological tolerance may allow effective induction of tumor immunity. Several reports have documented the potential role of $CD25^+$ T reg removal for the induction of tumor rejection (4, 13–15). Also, depletion of $CD25^+$ T reg cells leads to the activation of otherwise silent tumor-specific $CD8^+$ cells (4, 13, 14) as well as tumor-nonspecific $CD4^-CD8^-$ NK-like effector cells (4). In addition, recent publications have documented that $CD25^+$ T reg cells may contribute to the control of memory $CD8^+$ T cell responses (16, 17). These reports suggest that inhibition of $CD25^+$ T reg cell action might have a beneficial effect on the induction of antitumor immunity when combined with different strategies of vaccination.

In this work, we have investigated the effect of in vivo administration of anti-CD25 mAb on the induction of antitumor $CD4^+$ and $CD8^+$ T cell responses against the BALB/c colon cancer CT26 (18). We have also tested the efficacy of $CD25^+$ cell depletion in combination with peptide vaccination protocols using a cytotoxic T cell determinant (TCd) (18) and Th cell determinants (THd) (19), both of them derived from the sequence of murine leukemia

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[3] Abbreviations used in this paper: T reg, T regulatory cell; TCd, cytotoxic T cell determinant; THd, Th cell determinant; MuLV, murine leukemia virus.

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virus (MuLV) gp70 envelope protein that is a tumor rejection Ag expressed by CT26. We have found that removal of CD25$^+$ cells permits the induction of both CD4$^+$ and CD8$^+$ antitumor T cells in response to CT26 tumor challenge. Interestingly, we have found that the induced CD4$^+$ T cells are able to reject CT26 cells, an MHC class II-negative tumor. The mechanism of action of these CD4$^+$ cells is mediated by IFN-γ production which exerts anti-angiogenic effects. In addition, we have found that CD25$^+$ cell depletion favors the induction of antitumor memory T cell responses after vaccination with TCd and THd peptides. In the present study, we analyze the capacity of activated Th cells to overcome the suppressive effect of CD25$^+$ T reg on the induction of long-lasting cellular immune responses.

Materials and Methods

Peptides

Peptide SPSYVYHQF (from now on AH1) containing a TCd expressed by CT26 cells and presented by H-2L^d MHC class I molecules (18) and LVQFIKDRISVVQA (from now on p320–333) containing a Th epitope (19) for BALB/c MHC class II background, both derived from MuLV gp70 envelope protein, were synthesized by the solid phase method of Merrifield (20) using a manual multiple solid-phase peptide synthesizer as described in Ref. 21. At the end of the synthesis, peptides were cleaved, deprotected, and washed six times with diethyl ether. They were lyophilized and analyzed by HPLC. The purity of peptides was above 80% as judged by HPLC.

Mice

Six-week-old female BALB/c mice were purchased from IFFA Credo (Barcelona, Spain). A breeding pair of RAG2$^{-/-}$ (mice deficient in T and B cells) with BALB/c background was obtained from The Jackson Laboratory (Bar Harbor, ME), and were bred and maintained in pathogen-free conditions. Mouse experimentation followed institutional guidelines and was approved by the intrainstitutional ethical committee.

In vivo depletion experiments

In vivo depletion of CD25$^+$ cells was conducted by i.p. injection of 0.3 mg of anti-CD25 Abs (obtained from rat anti-mouse hybridoma PC61 (American Type Culture Collection, Manassas, VA) and purified as previously described (22)), 4 days before tumor challenge. In some experiments, different doses of anti-CD25 mAb were used to obtain partial depletions. In some experiments, mice were depleted of CD4$^+$ and/or CD8$^+$ cells by i.p. injection of 0.3 mg of anti-CD4 and/or anti-CD8 Abs (obtained from rat anti-mouse hybridomas GK 1.5 and H35.17.2, respectively) on days -1, 0, 1, 6, and 10, as previously described (22), day 0 being the day of peptide immunization. The efficiency of depletions at the day of tumor challenge was assessed by flow cytometry using PBMC isolated from fresh heparinized blood samples by Ficoll-Hypaque centrifugation. In all cases, the levels of depletion were higher than 95%. Depletion of NK cells was conducted by i.p. administration of rabbit anti-Asialo GM1 antiserum (Wako, Neuss, Germany) using 100 μg per dose on days -2, -1, and 3 after challenge with CT26 tumor cells. The efficiency of NK depletions was assessed, at the day of tumor challenge, by flow cytometry using anti CD3 and anti-DX5 Abs. Depletion of CD3$^-$/DX5$^+$ cells was in all cases above 95%. Also, the efficiency of NK depletion was assessed by measuring the remaining lytic activity of spleen cells 3 days after a single injection of rabbit anti-Asialo GM1 antiserum. This was done using Yac-1 cells (American Type Culture Collection) as target cells in a conventional ^{51}Cr release assay (23). A single injection with 100 μg of Ab was able to completely abrogate NK activity (not shown).

Adoptive T cell transfer experiments

CD4$^+$ cells were purified from naive mice or from CD25$^+$ and CD8$^+$ T cell-depleted mice by positive selection using anti-CD4 mAb coated to magnetic beads (Dynal Biotech, Oslo, Norway). Briefly, spleen cells from normal BALB/c mice were incubated with anti-CD4 coated Dynabeads for 1 h at 4°C following manufacturer's instructions. After positive selection using a magnetic concentrator, beads were removed by using DETACHaBEAD (Dynal Biotech). After two washes in PBS, cells were counted and used for adoptive transfer experiments. In some experiments, CD4$^+$/CD25$^+$ cells were purified from a CD4$^+$-enriched cell preparation obtained from naive mice, by incubation of cells with anti-CD25 mAb followed by positive selection using anti-IgG-coated Dynabeads according to manufacturer's instructions (Dynal Biotech). After detachment of beads by overnight incubation at 37°C, cells were washed and counted for adoptive transfer experiments.

Tumor challenge experiments

To measure the antitumoral capacity of the different treatments and/or immunization protocols, mice were challenged by s.c. injection on the right flank with 5×10^5 CT26 tumor cells at different time points. In some experiments animals were immunized with different combinations of peptides in IFA (Difco, Detroit, MI) or IFA alone as described previously (19). Tumor size was expressed as the square value of the small diameter of the tumor, times the value of its larger diameter. Mice were sacrificed when tumor size reached a volume greater than 8 cm^3.

Recognition of CT26 tumor cell lysate by CD4$^+$ T cells from mice exposed to CT26 tumor cells

The presence of antitumoral CD4$^+$ cells was measured in mice 30 days after challenge with CT26 tumor cells. CD4$^+$ T cells were purified from naive mice or from mice previously depleted from CD25$^+$ and CD8$^+$ cells as described above and stimulated in vitro (2×10^5 CD4$^+$ cells/well and 4×10^5 mitomycin-treated spleen cells/well from naive mice) with or without different dilutions of cell extracts from CT26, MC38, P815, A20, NS1, or 18Neo cells. To obtain cell extracts, 10^7 tumor cells or 18Neo cells/ml were lysed by six cycles of freezing and thawing followed by sonication and centrifugation. CT26 colon cancer, MC38 adenocarcinoma, P815mastocytoma, A20 lymphoma, and NS1 myeloma cells were obtained from the ATCC collection (American Type Culture Collection). 18Neo cells are derived from NIH 3T3 cells and were kindly provided by Dr. J. Berzofsky (National Institutes of Health, Bethesda, MD). Culture supernatants were collected after 48 h of culture to measure production of IFN-γ by using a commercial ELISA kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Cell proliferation was assayed after 3 days of culture by measuring [*methyl*-^{3}H]thymidine incorporation. Briefly, the second day of culture 0.5 μCi [*methyl*-^{3}H]thymidine were added to each well and incubated overnight. Cells were harvested (Filtermate 196 harvester; Packard Instrument, Meriden, CT) and incorporated radioactivity was measured using a scintillation counter (Topcount; Packard Instrument).

PCR primers and RT-PCR

Cell extracts from CT26, MC38, P815, A20, NS1, or 18Neo cells were obtained as described above. Total RNA was isolated from cell extracts using Ultraspect RNA isolation kit (Biotex, Houston, TX) following manufacturer's instructions. One microgram of RNA was reverse-transcribed as previously described (24) and the cDNA was amplified with *Taq* polymerase using specific primers for MuLV gp70 env (sense primer: ACCT TGTCCGAAGTGACCG, antisense primer: GTACCAATCCTGTGTG GTCG, to amplify a 594-bp fragment as described (18)) or for β-actin (sense primer: TCTACAATGAGCTGCGTGTG, antisense primer: GGT GAGGATCTTCATGAGGT, to amplify a 314-bp fragment as described (24)). Amplified cDNA was electrophoresed through 1% agarose and visualized under UV after ethidium bromide staining.

Matrigel angiogenesis assay

Angiogenesis assays were conducted by injecting 0.3 ml of ice-cold Matrigel (Collaborative Biomedical Products, Bedford, MA) containing 10 ng/ml vascular endothelial growth factor and 10 ng/ml basic fibroblast growth factor (Peprotech, London, U.K.) mixed with 5×10^5 CT26 cells to BALB/c mice: 1) untreated; 2) depleted of CD25$^+$ and CD8$^+$ T cells, 3) depleted of CD25$^+$ and CD4$^+$ T cells. To study the effect of IFN-γ on angiogenesis, two subgroups of mice, depleted of CD25$^+$ and CD8$^+$ T cells or depleted of CD25$^+$ and CD4$^+$ cells, were injected with anti-IFN-γ Abs at days 0, 2, and 5 after matrigel implantation ($n = 6$ in all groups). Matrigel injections were performed s.c. on the right flank of the mice. Nine days after implantation, plugs were harvested, surrounding tissue was dissected away, and the pellets were liquefied by incubation at 4°C overnight in 300 μl of PBS. The remaining pellets were further disrupted using a mechanic homogenizer. Hemoglobin content was quantified by measuring absorbance of the samples at 410 nm and by comparing with the absorbance of standard hemoglobin solutions.

Statistics

Statistical analysis was conducted using the computer program SPSS for Windows (Chicago, IL). Evaluation of the antiangiogenic effect of CD25$^+$ and CD8$^+$ cell depletion and the differences in the IFN-γ production between groups were done using the Shapiro-Wilk test followed by the Dunnett test.

Results

Effect of in vivo depletion of CD25$^+$, CD4$^+$, or CD8$^+$ cells on the protection against CT26 tumor challenge

Immunization of BALB/c mice with AH1 (a TCd expressed by CT26 cells) is unable to induce a protective CTL response against challenge with CT26 tumor cells (18). This is due to the inability of AH1 to induce a competent Th response (19). However, when mice were depleted of CD4$^+$ cells, immunization with AH1 protected all animals from tumor challenge (Ref. 19 and Table I), paradoxically suggesting that CD4$^+$ T cells were not necessary, or might even be detrimental for the induction of CTL responses. This made us think that elimination of CD4$^+$/CD25$^+$ regulatory cells might be at the origin of this result. To study whether the protective effect of immunization with AH1, in mice depleted of CD4$^+$ cells, was mediated by the elimination of CD4$^+$/CD25$^+$ cells, we selectively depleted this subpopulation by i.p. injection of anti-CD25 Abs previous to challenge with CT26 tumor cells. The efficiency of this depletion was in all cases above 95% (not shown), as assessed by flow cytometry. It was found that CD25$^+$ cell depletion led to a full protection (6 of 6 mice) against tumor challenge (Table II). Interestingly, and in contrast to what was found in CD4$^+$ cell-depleted mice, the protection observed after depletion of CD25$^+$ cells did not require immunization with AH1. Thus, all CD25$^+$ depleted and nonimmunized mice remained protected against CT26 tumor challenge, whereas all CD4$^+$-depleted and nonimmunized mice developed tumors (compare Tables I and II).

To confirm the inhibitory role of CD25$^+$ cells on the induction of a protective cellular immune response against CT26 tumor cell challenge, we conducted adoptive transfer of CD25$^+$ cells. Thus, the same day of challenge with CT26 tumor cells, we infused 5×10^6 CD25$^+$ cells (obtained from naive mice) to AH1-immunized animals, which had been depleted of CD4$^+$ cells. It was found that adoptive transfer of CD25$^+$ cells abrogated the protective effect observed following immunization of CD4$^+$ cell-depleted mice with AH1 (Table III). This result suggests that at least in this model, CD25$^+$ cells are the main inhibitors for the induction of antitumoral immune responses.

CD4$^+$ and CD8$^+$ cells are the main effector cells for the protection against CT26 tumor challenge after depletion of CD25$^+$ cells

To identify the subpopulations of cells responsible for the protection against CT26 tumor grafting in mice depleted of CD25$^+$ cells, we conducted the following experiments of combined cell depletion previous to tumor challenge: 1) CD25$^+$ and CD4$^+$, 2) CD25$^+$ and CD8$^+$, 3) CD25$^+$ and CD4$^+$ and CD8$^+$, and 4) CD25$^+$ and NK cells. It was found that depletion of CD4$^+$ cells in mice depleted of CD25$^+$ had a negative effect on protection against tumor challenge. Thus, only 4 of 12 mice (33%) remained free of tumors after this combined depletion. Similarly, depletion of CD25$^+$ and CD8$^+$ cells had a negative effect on protection, only 11 of 20 mice (55%) remained protected (Fig. 1). By contrast, protection after combined depletion of NK and CD25$^+$ cells was similar to that found in mice depleted of CD25$^+$ T cells only. Combined depletion of CD25$^+$, CD4$^+$, and CD8$^+$ cells, completely abrogated protection (6 of 6 mice developed tumors) indicating that T cells are responsible for protection after depletion of CD25$^+$ cells.

Interestingly, 55% of mice remained protected after combined depletion of CD8$^+$ and CD25$^+$ cells. This result suggests that CD4$^+$ cells by themselves were able to protect mice against challenge with CT26 tumor cells, a tumor which does not expresses MHC class II molecules.

CD4$^+$ cells, induced in the absence of CD25$^+$ and CD8$^+$ cells, produce IFN-γ in response to CT26 tumor Ags

As shown in Fig. 1, 55% of mice depleted of CD25$^+$ and CD8$^+$ cells remained free of tumor after challenge with CT26 tumor cells. However, combined depletion of CD25$^+$, CD8$^+$, and CD4$^+$ cells completely abrogated protection suggesting that in the first case, protection is mediated by CD4$^+$ cells. This fact prompted us to study the capacity of CD4$^+$ cells, induced in the absence of CD25$^+$ and CD8$^+$ cells, to respond to CT26 tumor Ags. For this purpose, we purified CD4$^+$ cells from: 1) nonprotected undepleted mice (control group); 2) from CD25$^+$ and CD8$^+$ cell-depleted mice that remained protected after CT26 tumor challenge, and 3) from CD25$^+$ and CD8$^+$ depleted mice that developed tumors after CT26 tumor challenge. Cells from these three groups were cultured for 48 h in the presence of cell extracts from CT26 or 18Neo cells (see *Materials and Methods*) and the production of IFN-γ was measured by ELISA.

Table I. *Effect of CD4$^+$ and CD8$^+$ cell depletion on the protection after challenge with CT26 tumor cells*

Immunized with	In Vivo Treatment[a]	Mice Protected[b]
AH1	None	0/6
	Anti-CD4	6/6
	Anti-CD8	0/6
None	Anti-CD4	0/6
	Anti-CD8	0/6

[a] Depletion of CD4$^+$ or CD8$^+$ cells was carried out by i.p. injection of anti-CD4 or anti-CD8 mAbs at days −3, −2, and −1, day 0 being the day of immunization.
[b] Mice were challenged with CT26 tumor cells at day 10 after immunization. Percentage of protection was calculated 30 days after challenge with CT26 tumor cells. Similar results were obtained in two independent experiments.

Table II. *Effect of CD25$^+$ cell depletion on the protection after challenge with CT26 tumor cells*

Immunized with	In Vivo Treatment[a]	Mice Protected[b]
AH1	Anti-CD25	6/6
None	Anti-CD25	6/6
AH1	None	0/6
None	None	0/6

[a] Depletion of CD25$^+$ cells was carried out by i.p. injection of anti-CD25 mAbs at day −4, day 0 being the day of immunization.
[b] Mice were challenged with CT26 tumor cells at day 10 after immunization. Percentage of protection was calculated 30 days after challenge with CT26 tumor cells. Similar results were obtained in three independent experiments.

Table III. *Effect of CD4$^+$ cell depletion and adoptive transfer of CD25$^+$ cells on the protection after challenge with CT26 tumor cells*

Immunized with	In Vivo Treatment		Mice Protected[a]
	Depletion	Adoptive transfer	
AH1	Anti-CD4[b]	None	7/7
AH1	Anti-CD4	CD25$^+$ cells[c]	2/7
AH1	None	None	0/7
IFA	None	None	0/7

[a] Mice were challenged at day 11 after immunization. Percentage of protection was determined 30 days after CT26 challenge. Similar results were obtained in two independent experiments.
[b] CD4$^+$ cell depletion was carried out by i.p. injection of anti-CD4 mAbs at days −3, −2, and −1, day 0 being the day of immunization.
[c] Adoptive transfer of CD25$^+$ cells from naive BALB/c mice was carried out at day 9 after immunization.

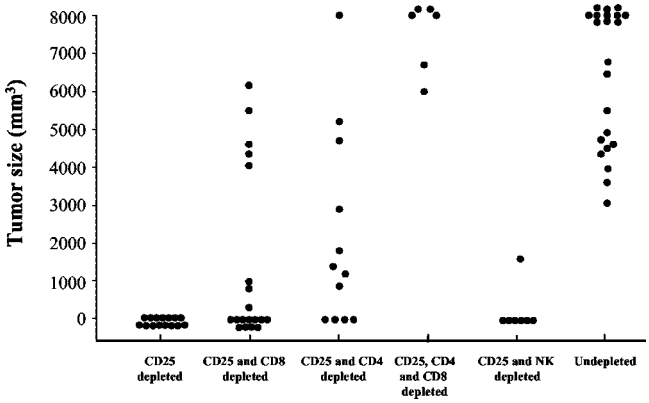

FIGURE 1. Antitumoral effect of CD4$^+$, CD8$^+$, or NK cells, induced in the absence of CD25$^+$ cells. Groups of mice were depleted of: CD25$^+$; CD25$^+$ and CD8$^+$; CD25$^+$ and CD4$^+$; CD25$^+$, CD4$^+$, and CD8$^+$; CD25$^+$ and NK cells; or nondepleted. Mice were challenged with CT26 tumor cells following cell depletion. Tumor size was measured 30 days after tumor challenge. Values correspond to tumor size from each mouse and they were calculated as the square value of the small diameter of the tumor, times the value of the larger diameter.

As shown in Fig. 2*A*, CD4$^+$ cells, from CD25$^+$ and CD8$^+$ depleted mice that remained protected after CT26 tumor challenge, produce high levels of IFN-γ in response to extracts from CT26 tumor cells. These levels were higher than those produced by CD4$^+$ cells from unprotected mice depleted of CD25$^+$ and CD8$^+$ cells (group 3), or from CD4$^+$ cells from naive mice (group 1) ($p < 0.05$). These results suggest that CD4$^+$ T cells, induced after depletion of CD25$^+$ cells, are specific of tumor Ags. To study the specificity of activated CD4$^+$ T cells, a group of CD25$^+$ and CD8$^+$ cell-depleted mice ($n = 5$), which remained protected after CT26 tumor challenge, were rechallenged s.c. with 5×10^5 cells from a different tumor (A20). This new challenge was conducted 45 days after depletion of CD25$^+$ cells, when this subpopulation had reached normal levels. It was found that 4 of 5 mice (as opposed to 0 of 5 from the control group) rejected this second challenge with A20 lymphoma. We also measured proliferation of CD4$^+$ T cells (from the different groups of mice) in response to cell extracts from CT26, MC38, P815, A20, NS1 tumor cells as well as from 18 Neo cells. For this purpose, 30 days after CT26 challenge, CD4$^+$ T cells were isolated from the following groups: 1) mice depleted of CD25$^+$ cells (all protected from tumor challenge); 2) mice depleted of CD25$^+$ and CD8$^+$ T cells, which remained protected after tumor challenge; 3) mice depleted of CD25$^+$ and CD8$^+$T cells, which developed tumors after CT26 challenge, and 4) from undepleted unprotected mice. As shown in Fig. 2*B*, CD4$^+$T cells from mice from groups 1 and 2, were able to proliferate in response to all the cell extracts tested, whereas CD4$^+$ T cells from groups 3 and 4 were not. However, the proliferative response of CD4$^+$T cells to the cell extracts was dependent on the cell extract tested. Thus, it was found that response to CT26, MC38, A20 and NS1 was significantly higher that that observed for P815 or 18Neo ($p < 0.05$). Because the MuLV gp70 env transcript is expressed in a variety of tumor cells and has been described as a tumor Ag (18, 25–27), we tested the presence of gp70 RNA transcripts in the different cell lines by RT-PCR. As shown in Fig. 2*B* (*upper panels*), MuLV gp70 env is expressed by CT26, MC38, A20, and to a lesser extend by P815 cells. We were unable to detect MuLV env expression in NS1 cells, despite the capacity of CD4$^+$T cells to proliferate in response to tumor extracts from NS1. These data suggest that MuLVgp70 is not the only source of the determinants recognized by such antitumor CD4$^+$ T cells.

Protective effect of adoptive transfer of CD4$^+$ cells obtained from mice depleted of CD25$^+$ and CD8$^+$ cells: role of IFN-γ

To study the antitumoral effect of these CD4$^+$ cells, we isolated CD4$^+$ cells from mice depleted of CD25$^+$ and CD8$^+$ cells which remained protected after CT26 tumor challenge as well as from those mice which developed tumors. CD4$^+$ cells from these two

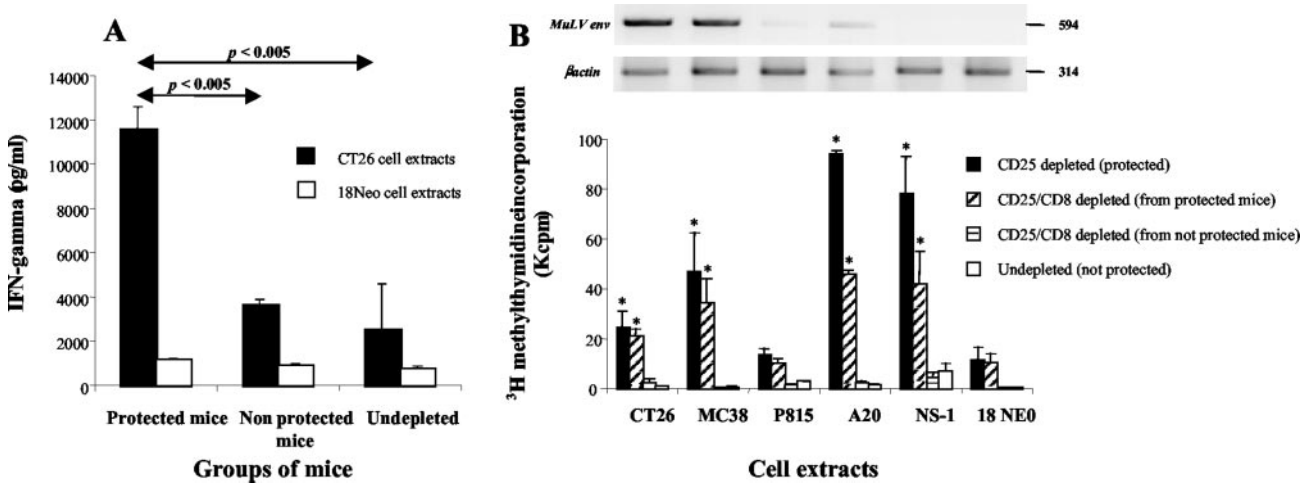

FIGURE 2. IFN-γ production by purified CD4$^+$ cells obtained from different groups of mice after challenge with CT26 tumor cells. *A*, Naive undepleted mice ($n = 5$), or mice depleted of CD8$^+$ and CD25$^+$ cells ($n = 5$), were challenged with CT26 tumor cells. Thirty days after challenge, CD4$^+$ cells from undepleted nonprotected naive mice (control group), as well as from protected or nonprotected mice from the depleted group, were cultured in the presence of extracts from CT26 or 18Neo cells (see *Materials and Methods*). After 48 h of culture, IFN-γ was measured by ELISA in the culture supernatants. *B*, *Upper panels*, Levels of expression of MuLV gp70 by different cell lines as measured by RT-PCR. *Bottom panel*, Proliferation of CD4$^+$ T cells from different groups of mice in response to cell extracts from CT26, MC38, P815, A20, NS1, or 10Neo cells. Mice were depleted of CD25$^+$ cells only; of CD25$^+$ and CD8$^+$ cells; or nondepleted. All groups were challenged with CT26 tumor cells. Thirty days after challenge, proliferation of CD4$^+$ cells was measured in response to the different tumor cell extracts (see *Materials and Methods*). As indicated, mice from the group CD25$^+$ and CD8$^+$ cells are classified as protected or nonprotected depending on the presence or absence of tumors ($n = 5$ in all groups). $*$, A significant difference in proliferation ($p < 0.05$) with respect to the control 18Neo cell extract.

groups were adoptively transferred (10^7 cells/mice) to BALB/c mice previously depleted from $CD8^+$ cells. As control, a group of mice depleted of $CD8^+$ was injected with saline. The same day of cell infusion, mice were challenged with 5×10^5 CT26 tumor cells. The evolution of tumors was assessed 30 days after. As shown in Table IV, two of the three mice depleted of $CD8^+$ cells that were adoptively transferred with $CD4^+$ cells from protected mice remained free of tumors, whereas all the mice infused with $CD4^+$ cells from unprotected mice or with saline developed tumors. To expand and reinforce these results in a slightly different setting, we adoptively transferred these antitumor $CD4^+$ cells in $RAG2^{-/-}$ mice. Three groups of $RAG2^{-/-}$ mice were adoptively transferred with: 1) antitumor $CD4^+$ cells (seven mice) obtained from mice depleted of $CD25^+$ and $CD8^+$ cells which remained protected after CT26 tumor challenge; 2) $CD4^+$ cells from naive mice (five mice) and 3) with saline only (three mice), the same day of CT26 tumor challenge. As shown in Table V, 5 of 7 mice infused with antitumor $CD4^+$ cells remained free of tumors (71% of protection) whereas only 1 of 5 mice infused with $CD4^+$ cells from naive mice was protected (20% of protection). None of the mice injected with saline were protected (0% of protection). To study the possible role of IFN-γ on this protective effect, a group of $RAG2^{-/-}$ mice adoptively transferred with antitumor $CD4^+$ cells ($n = 4$) was treated at days 0, 2, and 4 after CT26 tumor challenge, with three i.p. injections of 100 μl of ascitic fluid containing neutralizing anti-IFN-γ Abs. This treatment completely abrogated the protective effect of adoptive transfer of antitumor $CD4^+$ cells (4 of 4 mice developed tumors) (Table V).

Secretion of IFN-γ inhibits tumor angiogenesis

As shown in Fig. 3, $CD4^+$ cells induced in the absence of $CD25^+$ and $CD8^+$ cells, produce IFN-γ in response to CT26 tumor extracts. Because it has been recently described that $CD4^+$ T cells can inhibit tumor growth by inhibiting tumor angiogenesis, and that this inhibition is IFN-γ-dependent (28, 29), we conducted in vivo matrigel assays to measure the extent of angiogenesis stimulated by CT26 cells in the following groups of six mice: 1) mice doubly depleted of $CD25^+$ and $CD8^+$, 2) mice doubly depleted of $CD25^+$ and $CD8^+$ and treated with anti-IFN-γ Abs, 3) mice doubly depleted of $CD25^+$ and $CD4^+$, 4) mice doubly depleted of $CD25^+$ and $CD4^+$ and treated with anti-IFN-γ Abs, and 5) undepleted mice. All five groups of mice were injected with matrigel containing CT26 cells as described in *Materials and Methods*.

Table IV. *Effect of adoptive transfer of $CD4^+$ cells (BALB/c)*[a]

Mice	In Vivo Treatment		Mice Protected[b]
	Adoptive transfer	Depletion	
BALB/c	$CD4^+$ from protected mice[c]	$CD8^+$ depleted	2/3
BALB/c	$CD4^+$ from not protected mice[c]	$CD8^+$ depleted	0/3
BALB/c	Saline	$CD8^+$ depleted	0/3

[a] Effect of adoptive transfer of $CD4^+$ cells, from $CD8^+$ and $CD25^+$ depleted BALB/c mice which remained protected after challenge with CT26 tumor cells, to BALB/c mice depleted of $CD8^+$ cells.

[b] Mice were challenged with CT26 tumor cells. Percentage of protection was determined 30 days after challenge. Similar results were obtained in two independent experiments.

[c] $CD4^+$ cells from $CD8^+$ and $CD25^+$ cell-depleted BALB/c mice which remained protected after challenge with CT26 tumor cells were transferred to $RAG2^{-/-}$ mice and treated or nontreated with anti-IFN-γ Abs. Adoptive transfer was carried out the same day of challenge with CT26 tumor cells (day 0), whereas treatment with anti-IFN-γ Abs was carried out at days 0, 2, and 4 after challenge. As controls, $RAG2^{-/-}$ mice were transferred with $CD4^+$ cells from naive BALB/c mice or with saline.

Table V. *Effect of adoptive transfer of $CD4^+$ cells ($RAG^{-/-}$)*[a]

Mice	In Vivo Treatment[b]		Mice Protected[c] (%)
	Adoptive transfer	Anti-IFN-γ	
$RAG^{-/-}$	$CD4^+$ cells from protected mice[b]	No	5/7 (71.4)
$RAG^{-/-}$	$CD4^+$ cells from protected mice[b]	Yes	0/4 (0)
$RAG^{-/-}$	$CD4^+$ cells from naive mice[b]	No	1/5 (20)
$RAG^{-/-}$	Saline	No	0/3 (0)

[a] Effect of adoptive transfer of $CD4^+$ cells, from $CD8^+$ and $CD25^+$ depleted BALB/c mice which remained protected after challenge with CT26 tumor cells, to $RAG2^{-/-}$ mice.

[b] $CD4^+$ cells from $CD8^+$ and $CD25^+$ cell-depleted BALB/c mice which remained protected after challenge with CT26 tumor cells were transferred to $RAG2^{-/-}$ mice and treated or nontreated with anti-IFN-γ Abs. Adoptive transfer was carried out the same day of challenge with CT26 tumor cells (day 0), whereas treatment with anti-IFN-γ Abs was carried out at days 0, 2, and 4 after challenge. As controls, $RAG2^{-/-}$ mice were transferred with $CD4^+$ cells from naive BALB/c mice or with saline.

[c] Mice were challenged with CT26 tumor cells. Percentage of protection was determined 30 days after challenge. Similar results were obtained in two independent experiments.

Hemoglobin content of the matrigel plugs was quantified 9 days after implantation and this value was used as a measure of tumor angiogenesis. As shown in Fig. 3, depletion of $CD25^+$ and $CD8^+$ cells significantly reduces angiogenesis induced by CT26 embedded in matrigel ($p < 0.001$). This inhibition is mediated by IFN-γ production because injection of Abs against IFN-γ is able to restore the levels of angiogenesis observed in nondepleted mice. However, double depletion of $CD25^+$ and $CD4^+$ cells did not have any effect on angiogenesis, suggesting that the main group of cells responsible for the observed antiangiogenic effect were $CD4^+$ T cells induced in the absence of $CD25^+$ cells.

Depletion of $CD25^+$ cells facilitates the induction of antitumor memory T cell responses after peptide vaccination

As described above, depletion of $CD25^+$ cells allows mice to induce a protective T cell immune response against challenge with CT26 tumor cells. However, this protective effect was observed when the challenge was done 10 days after depletion of $CD25^+$

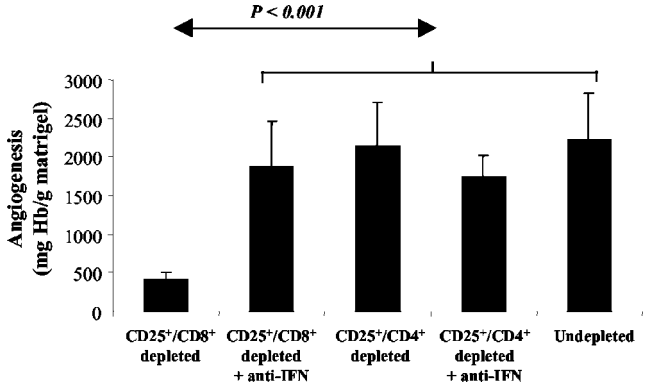

FIGURE 3. IFN-γ-dependent inhibition of tumor angiogenesis in mice depleted of $CD25^+$ and $CD8^+$ cells. Different groups of depleted or nondepleted mice were injected s.c. with CT26 tumor cells embedded in matrigel as described in *Materials and Methods*. As indicated, subgroups of mice depleted of $CD25^+$ and $CD8^+$ cells or of $CD25^+$ and $CD4^+$ cells were injected with anti-IFN-γ Abs at days 0, 2, and 5 after matrigel implantation. Nine days after implantation, plugs were harvested and hemoglobin content was used as a measure of angiogenesis ($n = 6$ in all groups). A similar result was obtained in another independent experiment.

Table VI. *Induction of long-lasting protection against challenge with CT26 tumor cells in mice depleted of CD25$^+$ cells and vaccinated with AH1*

Immunized with	In Vivo Treatment	Day of Challenge of CT26[a]	Percent of Protection (%)
None	Saline	10	0/6 (0)
		20	0/6 (0)
		40	0/6 (0)
		50	0/6 (0)
	Anti-CD25	10	6/6 (100)
		20	6/6 (100)
		40	3/6 (50)
		50	0/6 (0)
AH1	Saline	10	0/6 (0)
		20	0/6 (0)
		40	0/6 (0)
		50	0/6 (0)
	Anti-CD25	10	6/6 (100)
		20	6/6 (100)
		40	5/6 (83)
		50	5/6 (83)

[a] Mice were challenged at days 10, 20, 40, or 50 after treatment with saline or with anti-CD25 mAbs. Similar results were obtained in two separate experiments.

cells. As described previously, mice depleted of CD25$^+$ cells following administration of anti-CD25 mAb Abs recover the normal level of CD25$^+$ cells 35 days after this administration (15, 30). We then studied whether the protective effect following depletion of CD25$^+$ cells was dependent on the period of time elapsed between depletion and challenge with CT26 tumor cells. Thus, we injected CT26 tumor cells at days 10, 20, 40, or 50 after CD25$^+$ cell depletion. Protective immunity in response to CT26 tumor cells was 100% when the challenge was conducted at day 10 after depletion. However, the antitumor efficacy decreases to 50% or to 0% when the challenge with CT26 cells was performed at days 40 and 50 after depletion, respectively (Table VI).

In a previous work from our group, it was observed that immunization of mice with peptide AH1 (a TCd derived from MuLV gp70 envelope protein and expressed by CT26 cells (18)), did not induce a protective CD8$^+$ T cell response despite its partial capacity to delay tumor growth (19). This delay was only observed if CT26 challenge was conducted 10 days after immunization, being undetectable if the challenge was done >20 days after immunization (not shown). However, if CD25$^+$ T cell-depleted mice were immunized with AH1 peptide, 83% of mice remained free of tumors after challenge with CT26 cells, even when the challenge was conducted 50 days after immunization (Table VI). These results suggest that the absence of CD25$^+$ regulatory cells may improve the efficacy of peptide immunization and the establishment of a memory T cell response.

In contrast to immunization with AH1 alone, joint immunization with AH1 and peptide p320–333, a THd peptide derived from MuLV gp70 envelope protein, is able to induce a protective antitumoral CD8$^+$ T cytotoxic cell response against challenge with CT26 tumor cells (19). However, the protective effect induced by this joint immunization only lasts a short period of time. Indeed, we have found a protection of 90% if the challenge with CT26 tumor cells is conducted 10–15 days after immunization (19). By contrast, the protection decreased to only 0–10% if mice were challenged >20 days after immunization (not shown). Because CD25$^+$ regulatory cells seemed to play a negative role in the induction of long-term antitumoral responses after immunization with AH1, we studied the protective effect of immunization of mice with AH1 alone, or in combination with peptide p320–333, under different degrees of depletions of CD25$^+$ regulatory cells. Thus, five groups of mice were treated by i.p. injection of 0, 1, 10, 50, and 300 μg of anti-CD25 mAb Ab. The levels of depletion attained were 0 ± 5%, 33.8 ± 7.5%, 66.2 ± 8.4%, 95.5 ± 1.7%, and 99.3 ± 0.5%, respectively. These groups of mice were immunized with AH1 alone, AH1 plus p320–333 or with IFA only (see *Materials and Methods*). Fifty days after immunization, mice were

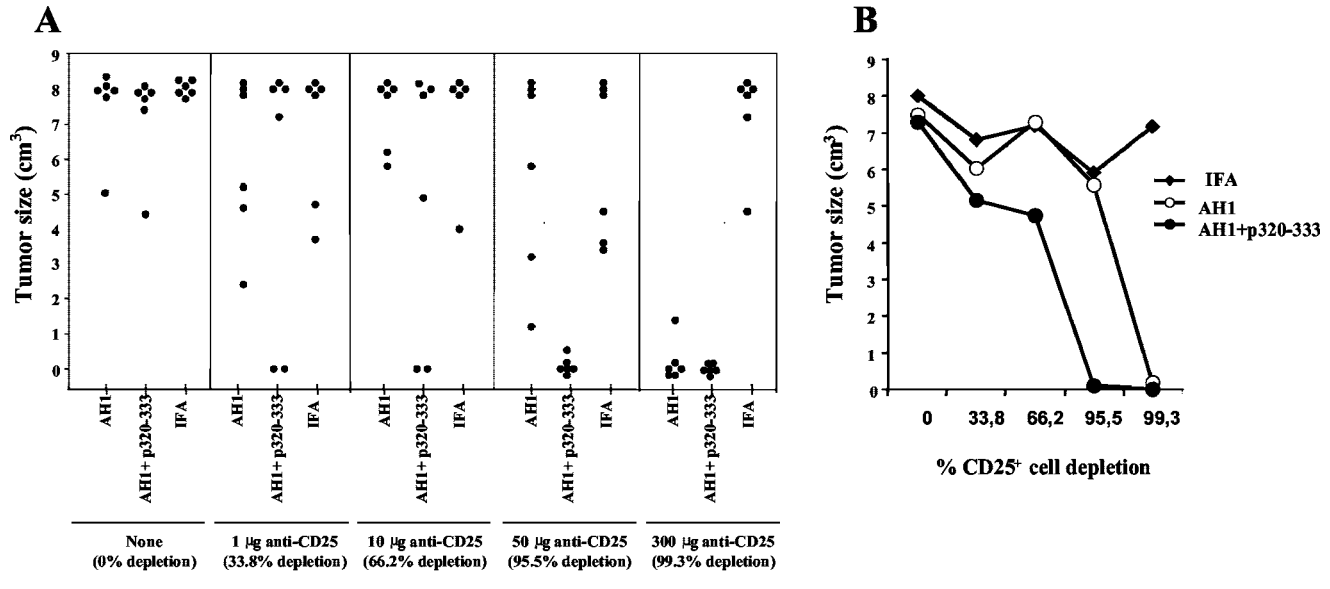

FIGURE 4. Effect of partial depletion of CD25$^+$ cells before peptide vaccination, on the induction of a protective long-lasting antitumoral immune response. Groups of mice were treated by i.p. injection of 0, 1, 10, 50, and 300 μg of anti-CD25 mAb Ab. This afforded depletions of 0 ± 5%, 33.8 ± 7.5%, 66.2 ± 8.4%, 95.5 ± 1.7%, and 99.3 ± 0.5% CD25$^+$ cells, respectively. Mice from these groups were immunized with AH1; AH1 plus p320–333; or with IFA alone, as described in *Materials and Methods*. Fifty days after immunization, mice were challenged with CT26 tumor cells and tumor size was measured 20 days after. *A*, The tumor size of each individual mouse, immunized under the condition shown, is represented with a dot, whereas in (*B*) each point represents the average tumor size from the six mice of each group, plotted against the degree of CD25$^+$ cell depletion.

challenged with CT26 tumor cells and tumor size was measured 20 days after challenge. In Fig. 4*A*, we represent with a dot the tumor size in each individual mouse, whereas in Fig. 4*B* we represent the average tumor size from each group of mice against the degree of $CD25^+$ cell depletion. As shown, immunization with AH1 alone, or with AH1 plus p320–333, after total depletion of $CD25^+$ cells resulted in long-term protection levels of 83 and 100%, respectively.

It is interesting to note that protection induced by AH1 alone, requires the complete absence of $CD25^+$ cells. Indeed, when mice were treated with 50 μg of anti-CD25 Ab, which depletes 95.5% of $CD25^+$ cells, the remaining cells (4.5%) are still able to inhibit the induction of a protective cellular immune response. However, under the same level of depletion of $CD25^+$ cells (95.5%), if AH1 was coimmunized with p320–333, a 100% protection was attained. Moreover, when mice treated with 1–10 μg of anti-CD25 Abs (depletion levels of 33.8 and 66.2%, respectively) were immunized with AH1 plus p320–333 the levels of protection were 33% in both cases. These results suggest that T cell help provided by p320–333 immunization overcomes, at least partially, the suppressive effect of $CD25^+$ regulatory cells.

Discussion

$CD4^+$ Th cells play an important role on the induction of CTL (23, 31–34). However, it has also been described that depletion of $CD4^+$ T cells may favor the induction of CTL responses (14, 22, 35), and enhance the therapeutic efficacy in tumor rejection (14, 35) suggesting that $CD4^+$ T cells play a regulatory role. These results are in agreement with our finding that vaccination with AH1 alone is unable to protect mice from challenge with CT26 tumor cells (18, 19), whereas if this vaccination is conducted after depletion of $CD4^+$ cells, mice are protected. Recent reports have shown that these $CD4^+$ T reg cells express the CD25 marker (3, 11). Also, depletion of $CD25^+$ T reg cells with Abs facilitates the induction of tumor immunity (4, 13–15), a result in agreement with our finding that mice depleted of $CD25^+$ cells are able to reject CT26 tumor cells and that adoptive transfer of $CD25^+$ T cells abrogates this protection. It is interesting to note that, in contrast to what is found after depletion of $CD4^+$ cells, depletion of $CD25^+$ cells permits tumor rejection without the need of a previous vaccination with AH1. It could be argued that absence of $CD25^+$ regulatory cells enhances the immunogenicity of CT26 tumor cells by stimulating tumor-specific $CD4^+$ Th cells, which are now capable of providing efficient T cell help for antitumor CTL induction. However, in the case of complete depletion of $CD4^+$ cells, both T reg and Th cells are depleted. Under this condition, neither a Th response, nor a concomitant CTL response, can be elicited by CT26 tumor cells. However, vaccination with peptide AH1 can bypass the need for T cell help inducing a protective response by direct activation of CTL when their TCR recognize the complex AH1-MHC class I complexes at the surface of APCs.

It has been described that the Ab-mediated depletion of $CD25^+$ T reg cells facilitates the induction of tumor immunity by favoring activation of tumor-specific $CD8^+$ cells (4, 13, 14) as well as tumor nonspecific $CD4^-CD8^-$ NK-like effector cells (4). In the present publication, we show that depletion of $CD25^+$ cells allows the induction of both antitumor $CD4^+$ and $CD8^+$ cells, a result in agreement with the recent publication by Golgher et al. (30). Indeed, simultaneous depletion of $CD25^+$ and $CD8^+$ T cells, or $CD25^+$ and $CD4^+$ T cells, but not triple depletion of $CD25^+$, $CD4^+$, and $CD8^+$ T cells, permits rejection of CT26 tumor cells in an important number of animals. Combined depletion of NK cells and $CD25^+$ cells did not abrogate protection, suggesting that NK cells do not play a determinant role in protection.

Many studies, including our own, have described the role of $CD4^+$ T cells in providing help for the induction of CTLs (23, 31–34, 36). However, $CD4^+$ T cells can coordinate other antitumor effector pathways independent of CTLs (37). We have found that $CD4^+$ T cells, obtained from mice depleted from $CD25^+$ and $CD8^+$ cells that remained protected after CT26 tumor challenge, produced high amounts of IFN-γ in response to CT26 tumor Ags in vitro. However, it was also found that activated $CD4^+$ T cells proliferated, not only in response to extracts from CT26, but also, in response to extracts from MC38, A20, P815, NS1, and 18Neo cells. Several interpretations might explain this finding: 1) first, depletion of $CD25^+$ T reg cells might down-regulate the activation threshold of $CD4^+$ T cells specific for Ags from the cell extracts. In other words, proliferations in response to cell extracts (Fig. 2*B*) might be the result of an in vitro induction of T cells by these extracts, and not of an in vivo induction after challenge with CT26 tumor cells. However, we do not favor this interpretation because when mice depleted of $CD25^+$ and $CD8^+$ T cells that rejected CT26 tumor cells were challenged with a second tumor (A20), once the level of CD25+ T reg cells was restored, these mice rejected the tumor, a result in agreement with that recently reported by Golgher et al. (30). 2) A second interpretation might be related to traces of other Ags from the FBS where CT26 tumor cells are grown. These Ags would induce a T cell response in vivo that would be expanded in vitro during the proliferation assays. If this were the case, a similar level of proliferation should be expected for all cell extracts. However, because proliferation of $CD4^+$ T cells in response to CT26, MC38, A20, and NS1 cell extracts was significantly higher than that in response to extracts from P815 or 18Neo cells ($p < 0.05$), we do not favor this interpretation either. 3) A third possibility might be related to a cross-reaction between shared Ags. Thus, using RT-PCR, we studied if the gp70 *env* gene was an Ag shared by the cell lines used in our study. It was found that the gp70 gene was expressed in CT26, MC39, A20, and to a lesser extent in P815 cells, but not in NS1 or in 18Neo cells. Thus, gp70 alone cannot explain all the differences in proliferation, suggesting that besides gp70, other tumor Ags might be involved in tumor rejection mediated by $CD4^+$ T cells. In summary, although we favor that the observed proliferation from Fig. 2*B* is most likely related to shared Ags between cells, additional experiments are needed to identify these shared Ags and to establish whether they are autoantigens shared by normal tissues.

Adoptive transfer of these antitumor $CD4^+$ cells, conducted in the present study using BALB/c mice as well as in $RAG2^{-/-}$ mice, demonstrate that these T cells are able to protect mice against tumor challenge, and that this protective effect is mediated by IFN-γ. In agreement with results reported by other research groups (28, 37, 38). In this study, we show that in the absence of $CD25^+$ T reg cells, an IFN-γ-producing antitumor $CD4^+$ cell subpopulation emerges in response to tumor cells, exerting potent IFN-γ-dependent antiangiogenic effects.

Because IFN-γ has multiple biological activities, and its receptor (IFN-γR) is expressed in almost all cell types (39, 40), this cytokine might have direct effects on tumor cells such as: 1) inhibiting cell proliferation or sensitizing cells to apoptosis (41, 42); 2) up-regulating MHC class I expression and thereby increasing tumor cell lysis (43); 3) up-regulating MHC class II expression on tumor cells (44, 45), 4) stimulating NK activity (46), or 5) inducing the expression of angiogenesis inhibitors, like IFN-γ-inducible protein-10, by tumor and stromal cells (47). We have not found any inhibitory effect of IFN-γ on proliferation of CT26 tumor cells in vitro even at doses of 7500 U/ml (around 3×10^6 pg/ml) (not shown). Up-regulation of MHC class I molecules and the susceptibility to CTL-dependent lysis may not be the mechanisms of

action of IFN-γ produced by CD4$^+$ cells because mice were depleted of CD25$^+$ and also of CD8$^+$ T cells. Moreover, adoptive transfer of these CD4$^+$ T cells to RAG2$^{-/-}$ (mice deficient in T and B cells) protected against tumor challenge. When we tested the expression of MHC class II molecules in CT26 cells after incubation with different concentrations of IFN-γ, it was found that even at 2000 IU/ml cytokine, CT26 cells did not acquire detectable MHC class II expression (not shown). In addition, when we measured the cytotoxic activity of purified CD4$^+$ T cells from protected mice, against IFN-γ-treated CT26 cells, no activity was found even at an E:T ratio of 100:1 (not shown). Because alternatives 1–4 do not seem to be relevant to explain the protective effect of IFN-γ produced by CD4$^+$ cells, and as shown in Fig. 3, IFN-γ inhibits angiogenesis, we favor the hypothesis that IFN-γ-dependent antiangiogenic activity may constitute an important effector mechanism explaining the antitumor activity of these CD4$^+$ T cells.

Depletion of CD25$^+$ T reg cells per se allows the immune system to mount an efficient antitumoral immune response in mice against tumors that are poorly immunogenic (4, 14, 15, 30). However, when we conducted tumor challenge 50 days after depletion of CD25$^+$ T cells, once the population of CD25$^+$ T reg cells was restored, all challenged mice developed lethal tumors. Thus, the beneficial effect of depleting CD25$^+$ T reg cells only takes place shortly after depletion of these cells, when their levels are still very low, suggesting that vaccination immediately after depletion of CD25$^+$ T reg cells might greatly enhance the ability of the host to mount a protective antitumoral immune response. This strategy might even permit to induce antitumoral responses using the class I epitope only, because as shown in our work, a single administration of anti-CD25 mAb before immunization with AH1 permits the induction of a long-lasting protective antitumor immune response (83% of protection). However, in the absence of depletion of CD25$^+$ cells, AH1 is unable to induce a protective response against challenge with CT26 tumor cells (18) due to the incapacity of this peptide to induce a competent Th response (19). Our results are in accordance with two recent publications suggesting that the presence of CD4$^+$/CD25$^+$ cells may restrict or control memory T cell responses (16, 17). In addition to the results reported by Kursar et al. (16), we show in this study that depletion of CD25$^+$ T cells, previous to a single immunization (priming immunization), permits peptide vaccination to induce a protective long-lasting antitumoral effect which is not observed in the presence of CD25$^+$ reg cells. We are aware that maintaining a CD25$^+$ T reg-depleted status for a long period of time, may have the risk of developing autoimmunity (48–50). Also, depletion of T cells expressing the CD25$^+$ marker would not only eliminate T reg cells, but also would deplete activated lymphocytes, including antitumor-specific CD4$^+$ and CD8$^+$ T cells, a situation that might facilitate tumor growth (14). For these reasons, depletion of CD25$^+$ cells should probably be reserved to de novo vaccination under conditions of great caution.

When the antitumor efficacy after immunization with AH1 alone is compared with that attained after coimmunization with AH1 and Th peptide p320–333, under different levels of depletion of CD25$^+$ T reg cells, it is clear that Th cells play an important role to overcome the suppressive effect of CD25$^+$ T reg cells. Thus, we have found that the protective effect of vaccinating with AH1 alone requires total depletion of CD25$^+$ cells previous to immunization with AH1, and is very inefficient (0% protection) in the presence of as low as a 4.5% of total CD25$^+$ cells. However, coimmunization of AH1 and p320–333 allows total protection under both conditions, and reaches a considerable long-lasting protective response (33%) even in the presence of a 66.2% of total CD25$^+$ T reg cells. To our knowledge, our results describe for the first time that Th cells may play an important role in overcoming the suppressive effect of CD25$^+$ T reg on the induction of cellular immune responses. These results might be of great relevance when developing strategies of vaccination against cancer.

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