Advances in Brief

Increase of Regulatory T Cells in the Peripheral Blood of Cancer Patients[1]

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Abstract

Purpose: T cells constitutively expressing both CD4 and CD25 are essential for maintenance of self-tolerance and therefore have been referred to as regulatory T cells (Treg). Experimental tumor models in mice revealed that Tregs are potent inhibitors of an antitumor immune response. The current study was designed to determine whether cancer patients exhibit an expanded Treg pool.

Experimental Design: The frequency of Tregs in the peripheral blood of 42 patients suffering from epithelial malignancies and from 34 healthy controls was determined by flow cytometry. The immunoregulatory properties of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were characterized by proliferation and suppression assays. Cocultures with natural killer (NK) cells were performed to determine the impact of Tregs on NK-mediated cytotoxicity.

Results: Patients with epithelial malignancies show an increase of $CD4^+CD25^+$ T cells in the peripheral blood with characteristics of Tregs, *i.e.,* they are $CD45RA^-$, $CTLA-4^+$, and transforming growth factor β^+. Notably, $CD4^+$ T cells from cancer patients are characterized by an impaired proliferative capacity, which is restored to the extend of CD25-depleted $CD4^+$ T cells from control persons by prior removal of $CD25^+$ T cells. In contrast to $CD4^+CD25^-$ T cells, isolated $CD4^+CD25^+$ T cells from cancer patients were anergic towards T cell receptor stimulation. In addition, $CD4^+CD25^+$ T cells suppressed the proliferation of $CD4^+CD25^-$ T cells. When cultured together with $CD56^+$ NK-cells, $CD4^+CD25^+$ T cells from cancer patients effectively inhibited NK-cell-mediated cytotoxicity.

Conclusions: Thus, we provide evidence of an increased pool of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of cancer patients with potent immunosuppressive features. These findings should be considered for the design of immunomodulatory therapies such as dendritic cell vaccination.

Introduction

The emergence of a tumor results from the disruption of cell growth regulation as well as from failure of the host to provoke a sufficient immunological antitumor response. Indeed, most cancer patients do not develop a satisfactory immunological antitumor response, implicating the existence of tumor-specific immune evasion strategies (1). Until now, several mechanisms have been described. The metastatic growth of tumors has been shown to be associated with a loss of MHC I expression and of other proteins involved in the processing of antigenic peptides (*i.e.,* TAP1), thereby down-regulating the presentation of T cell activating epitopes (2). Other effective strategies are the secretion of tumor-derived immunosuppressive factors, such as IL[3]-10 and TGF-β (3) or the induction of immunotolerance against tumor-specific antigens (4). Recently, the existence of a $CD4^+CD25^+CD45RA^-$ Treg population has been described in rodents and in humans (5–9). In healthy humans, this population accounts for 5–10% of peripheral $CD4^+$ T cells. The observation that Treg-depleted mice develop a broad range of autoimmunopathies suggests that this T cell subset plays a crucial role for the control of T cell-mediated autoimmunity (5). Notably, removal of regulatory T cells can also evoke effective antitumor immunity in mice injected with syngeneic cancer cells (5). Thus, we hypothesized that elevation of Tregs may down-regulate tumor-specific immunity. We therefore performed the current study to determine whether cancer patients exhibit an expanded Treg pool.

Materials and Methods

Patients. After having obtained informed consent, heparinized blood samples were collected from 34 healthy volunteers and 42 patients with malignant tumors. Information regarding patient history and tumor stage was recorded. The following tumor entities were included (Table 1): lung cancer ($n = 16$, 11 in stage IV, 2 in stage IIIa, 2 in stage IIIb, and 1 in stage I); breast cancer ($n = 6$, 1 in stage IV, 1 in stage IIIb, 1 in stage II, and 3 in stage I); colorectal cancer ($n = 9$, 3 in stage IV, 1 in stage III, 2 in stage II, and 3 in stage I); gastric cancer ($n = 3$,

[3] The abbreviations used are: IL, interleukin; TGF, transforming growth factor; Treg, regulatory T cell; PE, phycoerythrin; Ab, antibody; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; FACS, fluorescence-activated cell sorting; DC, dendritic cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor.
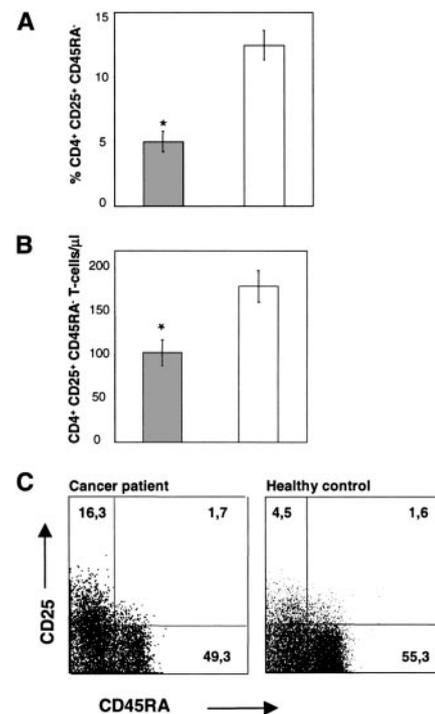
Table 1 Patient characteristics

Tumor type	Tumor stage	Treg (%)	Age (yr)	Gender
Lung cancer ($n = 16$)	IV	3,5	65	M
	IV	6	59	F
	IV	33,5	58	M
	IV	13,5	68	F
	IV	9	49	F
	IV	9,4	67	M
	IV	7,5	70	M
	IV	10,5	50	M
	IV	12,3	58	M
	IV	13	71	M
	IV	8,8	77	M
	IIIa	2,6	53	M
	IIIa	13,3	65	M
	IIIb	14,3	75	F
	IIIb	16,1	64	M
	I	8,7	59	F
Breast cancer ($n = 6$)	IV	18	71	F
	IIIb	14,2	78	F
	II	9,4	80	F
	I	17,4	44	F
	I	8,3	52	F
	I	9,7	61	F
Colorectal cancer ($n = 9$)	IV	21,6	62	M
	IV	13	67	F
	IV	18,7	82	F
	III	8,6	80	M
	II	18,02	79	M
	II	19,3	74	M
	I	16,5	67	F
	I	12,53	72	F
	I	9,3	75	M
Gastric cancer ($n = 3$)	IV	10,9	75	F
	IIIa	13,2	82	M
	Ib	20,7	52	M
Pancreatic cancer ($n = 2$)	IV	15,8	77	F
	IV	16,7	62	M
Gallbladder carcinoma ($n = 2$)	IV	4,5	77	M
	IV	9,8	75	M
Esophageal cancer	IV	15	55	F
Cervical cancer	IV	5,9	62	F
Ovarian cancer	IV	5,2	47	F
Epipharyngeal cancer	IV	8,7	73	F

Fig. 1 Cancer patients show a significant increase in CD4$^+$CD25$^+$ T cells. *A*, the percentage of CD4$^+$CD25$^+$CD45RA$^-$ T cells in the peripheral blood of 42 cancer patients (□) was determined by flow cytometry and compared with 34 healthy controls (■). The values are given as mean value ± SE. ∗, $P \leq 0.001$ *versus* healthy control (Student's *t* test). *B*, absolute cell counts of CD4$^+$CD25$^+$CD45RA$^-$ T cells of cancer patients (□) and healthy controls (■) as mean value ± SE. ∗, $P \leq 0.01$ (Student's *t* test). *C*, a representative staining for CD25 and CD45RA of CD4$^+$-gated T cells is shown for one cancer patient and one healthy control.

1 in stage IV, 1 in stage IIIa, and 1 in stage Ib); pancreatic cancer ($n = 2$ in stage IV); gallbladder carcinoma ($n = 2$ in stage IV); esophageal cancer ($n = 1$ in stage IV); epipharyngeal cancer ($n = 1$ in stage IV); cervical cancer ($n = 1$ in stage IV); and ovarian cancer ($n = 1$ in stage IV). The mean age of the cancer patients was 66.3 ± 10.4 years, the corresponding healthy controls had a mean age of 52.3 ± 22.4 years. Increasing age has no impact on relative and absolute numbers of Tregs in the peripheral blood (unpublished results).

Patients had received neither immunosuppressive therapy nor chemotherapy for at least 3 months.

Flow Cytometric Analysis. To determine the regulatory cell phenotype, three- and four-color flow cytometry of whole blood or isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells was performed using the following antibodies: anti-CD4; anti-CD11a; anti-CD25; anti-CD28; anti-CD31; anti-CD45RA; anti-CD62L; anti-CD69; anti-CD95; anti-CD154; anti-CTLA-4; and IgG1-isotype control (either FITC-, PE-, peridin chlorophyll

protein- or adenomatous polyposis coli-conjugated, all purchased from BD PharMingen). For whole blood stainings, 50 μl of whole blood were incubated with appropriate amounts of fluorochrome-labeled Abs in the dark at room temperature for 30 min, washed once, and analyzed. Isolated T cells were stained with titrated amounts of Ab and washed once. Anti-TGF-β mAb (R&D, unconjugated) was detected by a PE-labeled rabbit-antimouse mAb (Dako), according to the manufacturer's instructions. Cytoplasmatic bcl-2 was determined by intracellular flow cytometry as follows. After surface staining and lysing of RBCs, cells were permeabilized using Cytofix/Cytoperm solution (BD PharMingen), stained with FITC-anti-bcl-2 mAb (Dako) for 30 min at room temperature and washed once. To compare the phenotype of recently *in vitro* activated T cells with the CD4$^+$CD25$^+$ population, 1 ml of whole blood was stimulated with 10 ng/ml PMA (Sigma) and 0.5 μg/ml Ionomycin (Sigma) for 48 h before FACS analysis. Flow cytometry was performed on a Becton Dickinson FACSCalibur or FACSscan and CellQuest software was used for analysis

Quantification of Absolute Cell Numbers. Absolute cell numbers were determined using TruCOUNT Tubes (BD PharMingen) and a lyse-no-wash method according to the manufacturer's instructions. In brief, 50 μl of whole blood were
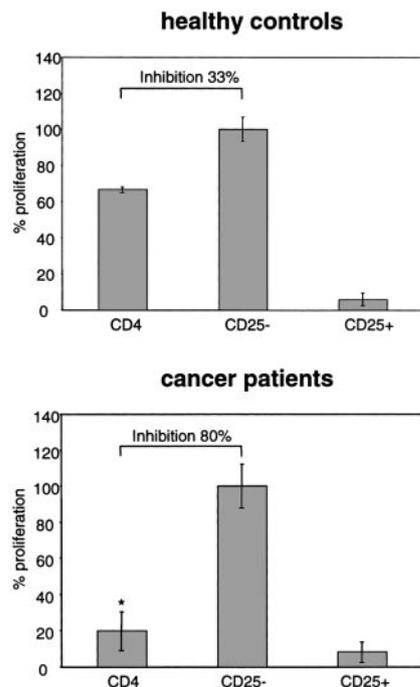
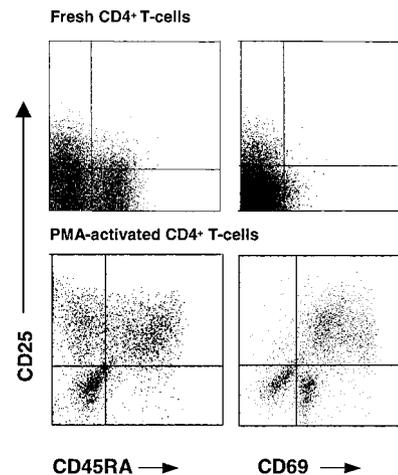*Fig. 2* Increased inhibition of CD4$^+$ T cell proliferation by CD4$^+$CD25$^+$ T cells of cancer patients. The proliferative response of freshly isolated CD4$^+$ T cells of 7 cancer patients and 3 healthy controls activated with plate-bound OKT3. [^{3}H]thymidine incorporation was determined in triplicates before and after depletion of CD25$^+$ T cells as well as of the isolated CD4$^+$CD25$^+$ T cells. *, $P \leq$ 0.01 for CD4$^+$ T cells *versus* CD25-depleted T cells. Proliferation of CD25-depleted CD4 T cells was considered as 100%. Notably, total proliferation rates of CD4$^+$CD25$^-$ T cells from both groups reached comparable levels between 17,000 and 40,000 cpm. The values are given as mean value $\pm$ SEM.

Fig. 3 CD4$^+$CD25$^+$ T cells express distinct surface markers as compared with activated T cells. *A*, fresh (*top panel*) and PMA-activated (*bottom panel*) whole blood of cancer patients was stained for CD4, CD25, CD45RA, and CD69. One representative staining is shown for three independent experiments.

stained for CD3, CD4, CD25, and CD45RA, lysed with 450 μl of FACS lysing solution (BD PharMingen), and directly analyzed on the flow cytometer. Total cell numbers were calculated by gating on reference beads.

Cytokine Assays. For intracellular analysis of cytokine production anti-IL-4-PE, anti-IL-10-PE, and anti-IFN-γ-FITC mAbs as well as the corresponding isotype-controls were used (all purchased from BD PharMingen). Briefly, 10^6 isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were activated with 10 ng/ml PMA, 0.5 μg/ml Ionomycin, and 1 μl/ml GolgiPlug (BD PharMingen) for 4 h. Cells were washed, fixed and permeabilized (Cytofix/Cytoperm solution; BD PharMingen), and stained with titrated amounts of cytokine-specific antibodies.

Cell Isolation and Generation of DCs. Either CD4$^+$ or CD56$^+$ cells were purified from peripheral blood by Ficoll-Paque density gradient centrifugation followed by isolation with immunomagnetic beads (Miltenyi Biotech). In the case of isolated CD4$^+$ T cells, CD4 beads were detached and stained with anti-CD25 beads, followed by positive and negative selection (purity of CD4$^+$CD25$^+$ population: >85%; CD4$^+$CD25$^-$ population: >98%, CD56$^+$ population: >98%).

DCs were generated from peripheral blood of healthy volunteers as described previously (10). In brief, PBMCs were

isolated by Ficoll density gradient centrifugation. Monocytes were isolated by plastic adherence and cultured in RPMI 1640 supplemented with 10% human serum, 800 units/ml granulocyte macrophage colony-stimulating factor, and 1000 units/ml IL-4. On day 7, nonadherent cells were transferred to fresh 6-well plates and 10 ng/ml IL-1β, 10 ng/ml tumor necrosis factor α, 1000 units/ml IL-6, and 1 μg/ml prostaglandin E$_2$ were added for maturation. For allogeneic stimulation, mature DCs were harvested at day 9 and irradiated (40 Gy).

Expansion of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ Cells. Isolated cells were cultured in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Isolated T cells (10^6) were stimulated with 100 units/ml IL-2 and 20 ng/ml anti-CD3 mAb (OKT3; Sigma) in the presence of irradiated (40 Gy) autologous PBMC (10^6/ml). IL-2 was supplemented every 3 days, and cells were restimulated with OKT3 and feeder-PBMCs every 7 days. The expansion of CD4$^+$CD25$^+$ T cells and the respective CD4$^+$CD25$^-$ T cells was quantified by counting cell numbers on days 0, 5, 10, 15, and 20 using trypan blue exclusion for the calculation of absolute cell numbers. Unless otherwise indicated, expanded CD4$^+$CD25$^+$ T cells were cultured for 10 days.

Proliferation Assays. CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells (10^5) were cultured in the presence of 10^5-irradiated allogeneic DCs or stimulated with plate-bound anti-CD3 mAb (2 μg/ml). After 4 days, [^{3}H]thymidine was added for an additional 16 h of culture before the incorporation of [^{3}H]thymidine was assessed. To determine whether the increase of CD4$^+$CD25$^+$ T cells is also of functional importance, we performed proliferation assays using 10^5 immunomagnetically selected CD4$^+$ T cells from cancer patients and healthy controls either before or after depletion of CD25$^+$ T cells (CD4$^+$CD25$^-$ T cell fraction).

Suppression Assays. To determine regulatory properties, cocultures of 5 × 10^4 CD4$^+$CD25$^+$ (freshly isolated or expanded) and CD4$^+$CD25$^-$ T cells from cancer patients that were stimulated either by allogeneic DCs in a ratio of 1:1:1 or

Fig. 4 CD4$^+$CD25$^+$ T cells from cancer patients exhibit a Treg-phenotype. *A,* isolated and expanded CD4$^+$CD25$^+$ (*dark line*) and CD4$^+$CD25$^-$ T cells (*light line*) were stained with anti-CD28, anti-CD95, anti-CD154, or the corresponding isotype control (*dashed line*). One representative result is shown for three independent experiments. *B,* surface expression of CTLA-4 and TGF-β and intracellular staining of bcl-2 of isolated CD4$^+$CD25$^+$ T cells (*dark line*), CD4$^+$CD25$^-$ T cells (*light line*), or the corresponding isotype control (*dashed line*). One representative result is shown for at least three independent experiments. *C,* intracellular cytokine production of CD4$^+$CD25$^+$ (*dark line*) and CD4$^+$CD25$^-$ T cells (*light line*) was determined by anti-IL4, anti-IL-10, and anti-IFN-γ mAbs after 4 h of PMA stimulation. The isotype control staining is shown by the *dashed line*.
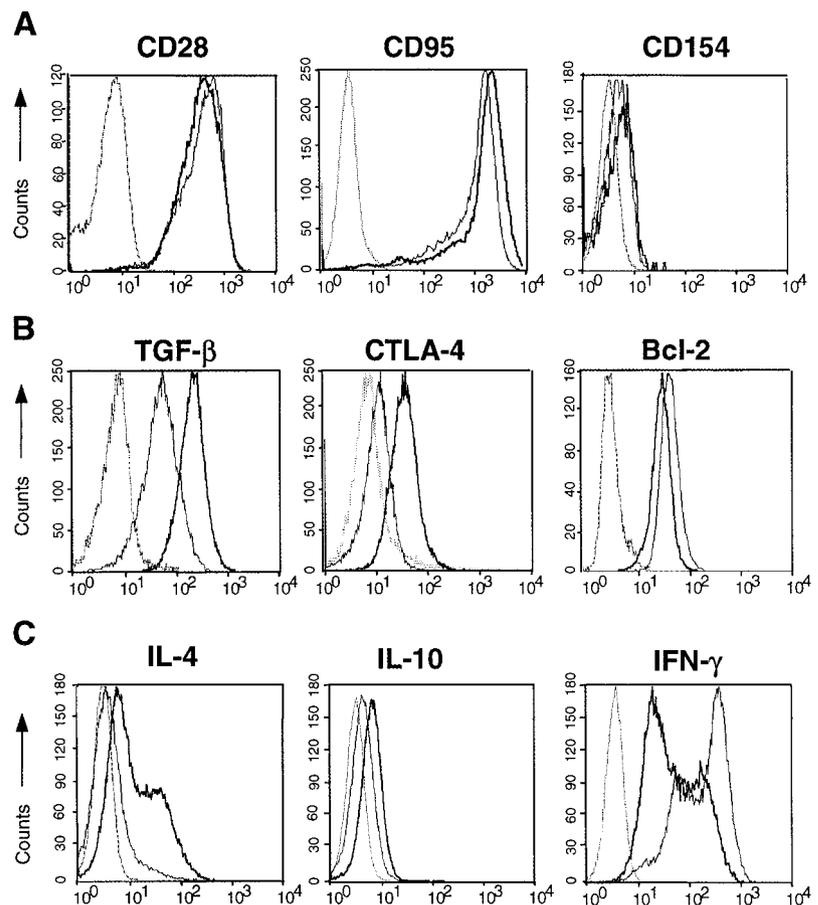
by plate-bound anti-CD3 mAb (2 μg/ml) were performed. Proliferation was measured after 4 days by [^{3}H]thymidine incorporation.

Cytotoxicity Assays. The assay was performed as previously described (11) with slight modifications. In brief, 2×10^6 CD56-selected NK effector cells were preincubated with equal numbers of CD4$^+$CD25$^-$, CD4$^+$CD25$^+$ T cells or medium alone at 37°C for 2 h. PKH-labeled (Sigma) K562 cells were used as target cells. After incubation, 5×10^4 target cells were added and cultured for additional 4 h. Propidium iodide (10 μg) was added immediately before the FACS analysis. The specific lysis was calculated as total lysis − spontaneous lysis.

Results

Quantification of Relative and Absolute CD4$^+$CD25$^+$ T Cell Numbers. In healthy controls ($n = 34$), the mean percentage of CD4$^+$CD25$^+$CD45RA$^-$ cells in the peripheral blood was $4.7 \pm 0.5\%$ of all CD4$^+$ T cells. In contrast, peripheral blood of cancer patients ($n = 42$) exhibited a 2.5-fold increase in CD4$^+$CD25$^+$CD45RA$^-$ T cells ($12.5 \pm 0.9\%$ of the CD4$^+$ population), which is highly significant ($P \leq 0.001$) as compared with healthy controls (Fig. 1, *A* and *C*). Patient characteristics, tumor entities, and stages as well as the respective percentage of CD4$^+$CD25$^+$CD45RA$^-$ T cells are given in Table 1. The number of CD4$^+$CD25$^+$CD45RA$^-$ T cells did not

correlate with either tumor stage or tumor type (data not shown). Quantification of absolute cell counts of 10 cancer patients and 10 control persons revealed that not only the percentage but also the total cell number of CD4$^+$CD25$^+$CD45RA$^-$ T cells per μl was significantly increased in cancer patients ($P \leq 0.01$) as indicated in Fig. 1*B*.

Functional Importance of CD4$^+$CD25$^+$ T Cell Expansion in Cancer Patients. To determine whether the observed increase of the percentage and the total number of CD4$^+$CD25$^+$ T cells in patients with epithelial malignancies is also of functional importance, we performed proliferation assays with selected CD4$^+$ T cells from cancer patients and the respective healthy control persons. Interestingly, CD4$^+$ T-cell proliferation was markedly impaired in cancer patients as compared with healthy controls (Fig. 2). However, depletion of CD25$^+$ T cells before the proliferation assay restored the proliferative capacity of CD4$^+$CD25$^-$ T cells. This increase of CD4$^+$CD25$^-$ T-cells proliferation was significantly higher in patients suffering from cancer when compared with healthy controls and therefore underlines the functional importance of the observed increase of CD4$^+$CD25$^+$ T cells for suppression of CD4$^+$CD25$^-$ T-cell proliferation in cancer patients.

Phenotypic Analysis of CD4$^+$CD25$^+$ T Cells. To further demonstrate that the observed difference is not attributable to an increase in activated T cells, we compared the surface

marker expression of the CD4$^+$CD25$^+$ population with PMA-activated PBMCs. T cells of cancer patients expressing CD4 together with CD25 were mostly CD45RA$^-$ and CD69$^-$. In contrast, a large proportion of PMA-activated CD4$^+$CD25$^+$ T cells was CD45RA$^+$ and CD69$^+$ (Fig. 3).

Phenotypic analysis of isolated and expanded CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells revealed similar expression levels of CD28, CD95, CD154 (Fig. 4*A*), CD11a, CD31 and CD62L (data not shown) (Fig. 4*A*). However, CD4$^+$CD25$^+$ T cells are readily distinguishable from CD4$^+$CD25$^-$ T cells by their elevated expression of CTLA-4 as well as cell surface TGF-β (Fig. 4*B*). In keeping with a recent report (12), we also detected a slightly decreased expression of bcl-2, which has been attributed to the higher susceptibility of Tregs to cytokine deprivation-induced apoptosis (Fig. 4*B*).

Cytokine Assays. Next, the cytokine profile of expanded CD4$^+$CD25$^+$ T cells was determined. After 4 h of PMA stimulation, CD4$^+$CD25$^+$ T cells showed an enhanced IL-4 production compared with the CD4$^+$CD25$^-$ population (Fig. 4*C*). In contrast, CD4$^+$CD25$^+$ T cells displayed a marked decrease of IFN-γ production when compared with CD4$^+$CD25$^-$ T cells. Both populations synthesized only little amounts of IL-10 (Fig. 4*C*), which is in line with reports demonstrating that Tregs of healthy humans produce only small amounts of cytokines and that the suppressive effect is unlikely to be because of IL-10 production (6, 8).

Proliferative Capacity in Response to Allogeneic DCs and Polyclonal TCR Stimulation. Functional analysis revealed that Tregs are anergic to TCR stimulation (*i.e.,* by anti-CD3; Ref. 6). However, addition of exogenous IL-2 allowed the propagation of CD4$^+$CD25$^+$ T cells isolated from peripheral blood of cancer patients. Fig. 5 shows typical expansion curves of the two cell populations over a time period of 20 days from a cancer patient and a control person. *In vitro* expanded CD4$^+$CD25$^+$ T cells of cancer patients failed to proliferate in response to allogeneic DCs when exogenous IL-2 was removed (data not shown). The proliferation assays were additionally performed with anti-CD3 mAb stimulation to exclude the potential selection of an alloreactive CD4$^+$CD25$^+$ subpopulation upon stimulation with allogeneic DCs. Again, CD4$^+$CD25$^+$ T cells were anergic, whereas the CD4$^+$CD25$^-$ population showed a marked proliferative response to anti-CD3 stimulation (Fig. 6, *A* and *B*). There is no difference between cancer patients as compared with healthy control persons in terms of the proliferative capacity of Tregs (Fig. 5 and Fig. 6, *A* and *B*). Moreover, Treg-expansion failed to modulate the anergic state of CD4$^+$CD25$^+$ T cells from both groups (Fig. 6, *A* and *B*).

Suppressive Properties of Tregs in Response to Allogeneic DCs or Polyclonal TCR Stimulation. To study the immunoregulatory capacity of CD4$^+$CD25$^+$ T cells from cancer patients before and after expansion, coculture experiments were performed using equal numbers of CD4$^+$CD25$^+$ and autologous CD4$^+$CD25$^-$ T cells, which were either stimulated by allogeneic DCs (data not shown) or by OKT3 (Fig. 6, *A* and *B*). Notably, the isolated CD4$^+$CD25$^+$ T cells decreased the proliferation of CD4$^+$CD25$^-$ T cells, suggesting a role for cancer-associated immunosuppression. Again, there was no difference in the suppressive activity of Tregs from cancer patients and controls, whereas Treg expansion increases the suppressive
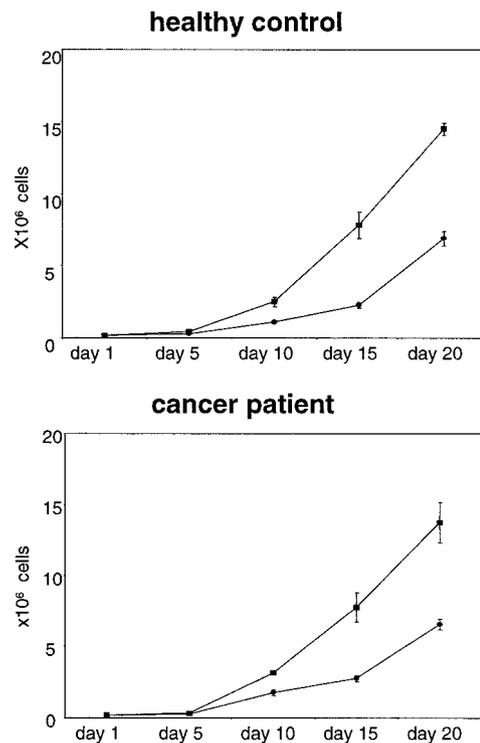

Fig. 5 Expandability of CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells grown in the presence of OKT3 and IL-2. Expansion curves of CD4$^+$CD25$^+$ (●) and CD4$^+$CD25$^-$ (■) T cells over a time span of 20 days from one cancer patient and one control person are shown. Freshly isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were stimulated with OKT3 and IL-2 at day 1. IL-2 was added every 3 days, and cells were restimulated with OKT3 every 7 days. Total cell numbers were determined by trypan blue exclusion in triplicates. The values are given as mean value ± SEM. Similar results were obtained in three independent experiments. One representative example is shown for 3 independent experiments.

properties of CD4$^+$CD25$^+$ T cells. The latter effect was observed for both patients and healthy controls.

Inhibition of NK Cell-mediated Cytotoxicity. NK cells are an effective part of the innate antitumor response (13). To determine the suppressive effect of CD4$^+$CD25$^+$ T cells on *in vitro* cytotoxicity against tumor cells, we performed cytotoxicity assays with the NK target cell line K562. The latter was cultured together with CD56-selected NK cells in a ratio of 1:40 for 4 h. Preincubation of the effector cells together with CD4$^+$CD25$^+$ T cells for 2 h (ratio 1:1) significantly reduced NK cell-mediated target cell lysis ($P \leq 0.01$). In contrast, after preincubation of NK cells with CD4$^+$CD25$^-$ T cells, only a slight decrease of NK cell-mediated cytolysis could be detected (Fig. 7) compared with NK cells, which were incubated for 2 h in medium alone. These results demonstrate that Tregs from cancer patients are effective inhibitors of NK cell-mediated antitumor responses.

Discussion

The current report provides for the first time functional evidence of a significant increase of Tregs in the peripheral blood of cancer patients. Recent reports underscore that CD4$^+$CD25$^+$ Treg cells do not only play a central role in the

Fig. 6 *In vitro* expanded CD4$^+$CD25$^+$ T cells from cancer patients show an equal suppressive efficacy as compared with control CD4$^+$CD25$^+$ T cells. Freshly isolated and for 10 days expanded, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T-cells from (*A*) healthy control people (*n* = 3) and (*B*) cancer patients (*n* = 14) were activated with plate-bound OKT3 and tested for their ability to proliferate either alone or in cocultures of CD4$^+$CD25$^+$ together with CD4$^+$CD25$^-$ T cells (ratio of 1:1). Proliferation was determined in triplicate cultures by [^{3}H]thymidine incorporation, and proliferation of CD4$^+$CD25$^-$ T cells was considered as 100% (mean ± SEM). *, $P \leq$ 0.05 for CD4$^+$CD25$^-$ *versus* CD4$^+$CD25$^+$/CD4$^+$CD25$^-$ for healthy controls after expansion and cancer patients before and after expansion (Student's *t* test).
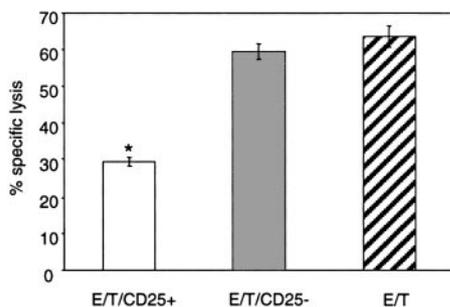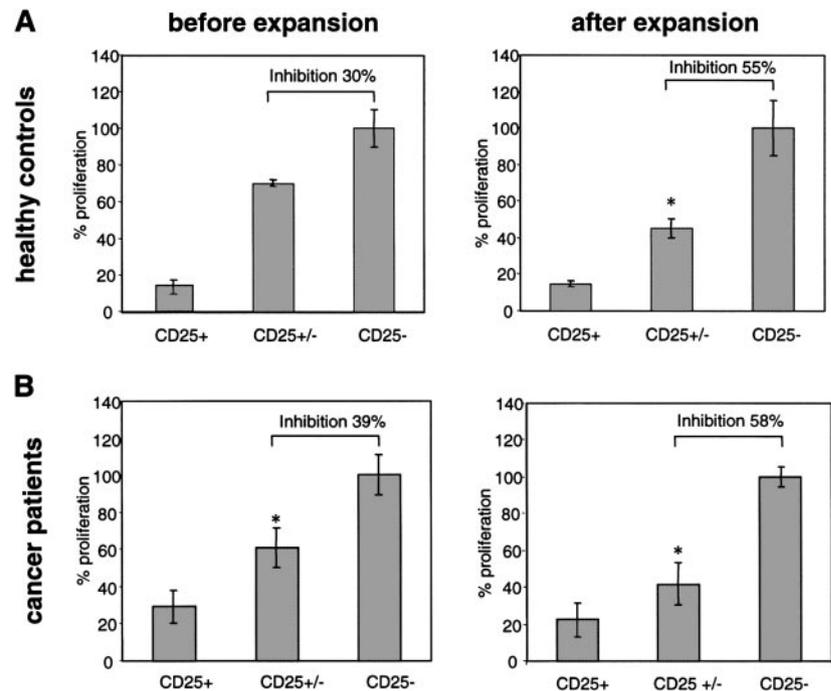
Fig. 7 CD4$^+$CD25$^+$ from cancer patients effectively inhibit NK cell-mediated cytotoxicity. NK effector cells (2×10^6) were preincubated for 2 h with either CD4$^+$CD25$^+$ (□) or CD4$^+$CD25$^-$ (■) T cells in a ratio of 1:1 before 5×10^4 K562 target cells were added for additional 4 h (E:T ratio, 40:1). As control, 2×10^6 NK cells were cultured for 4 h with 5×10^4 target cells after 2 h of preincubation with medium alone (▨). The mean value ± SEM of specific lysis from three independent experiments of 3 patients was calculated as total lysis − spontaneous lysis. *, $P \leq 0.01$ (Student's *t* test).

maintenance of immunotolerance but that Tregs are also potent inhibitors of antitumor immune responses in mice (14–16). Shimizu *et al.* (14) and Onizuka *et al.* (16) both demonstrated the negative impact of Tregs on the generation of tumor-specific CTLs as well as on effector mechanisms mediated by the innate immune response. They showed that Ab-mediated depletion of CD25$^+$ T cells abrogated immunological unresponsiveness against various syngeneic tumors in mice (14, 16). For humans, only few data are available to date (17). In addition to Woo *et al.* (17, 18), who first provided evidence for an increased percentage of CD4$^+$CD25$^+$ Tregs among tumor-infiltrating lymphocytes as well as in the peripheral blood, we report here on the

increase of relative and absolute cell counts as well as on the functional importance of CD4$^+$CD25$^+$ T-cell expansion in cancer patients. It is noteworthy that CD4$^+$ T cells from cancer patients exhibit an impaired proliferative capacity as compared with healthy control persons, an effect that is reversible by prior depletion of CD25$^+$ T cells. This observation additionally supports our hypothesis that the development of a neoplasma promotes the expansion of the CD4$^+$CD25$^+$ Treg pool and/or *vice versa*. In support of our functional data, the cell surface marker profile of the CD4$^+$CD25$^+$ T-cell population in peripheral blood did not indicate the expansion of merely activated T cells because this T-cell subset was mostly CD45RA and CD69 negative. This is in contrast to activated, partly CD45RA and CD69-positive CD4$^+$CD25$^+$ T cells. After *in vitro* expansion of isolated CD4$^+$CD25$^+$ T cells from cancer patients, the cells retained their Treg phenotype, being positive for CD11a, CD28, CD31, CD45R0, CD62L, and CD95 and negative for CD154. Moreover, in contrast to CD4$^+$CD25$^-$ T cells, propagated CD4$^+$CD25$^+$ T cells expressed cell surface TGF-β and CTLA-4, which is in line with recent reports demonstrating the expression of these two molecules on Tregs (8, 19). However, the precise role of CTLA-4 and TGF-β for the functional properties of Tregs still remains controversial (6, 19, 20). The expandability of CD4$^+$CD25$^+$ T cells from cancer patients is comparable with healthy controls, demonstrating that under the selected culture conditions Tregs from cancer patients are not characterized by an impaired proliferative capacity. Additional analysis of the functional properties by proliferation assays before and after Treg expansion revealed that CD4$^+$CD25$^+$ T cells are anergic toward TCR stimulation and that they exert potent immunoregulatory functions, as the proliferative response of CD4$^+$CD25$^-$ T cells was significantly inhibited. There was no difference in either the proliferative or the sup-

pressive properties of CD4$^+$CD25$^+$ T cells from patients and controls. Both groups showed an enhanced antiproliferative effect on CD4$^+$CD25$^-$ T cells, which is in line with recent reports showing an increase of the suppressive activity of Tregs after *in vitro* expansion (21). Moreover, we focused on the effect of Tregs on the cytotoxic potential of CD56$^+$ NK cells, which play a pivotal role for the innate antitumor response (13). NK-mediated cytotoxicity was significantly impaired after preincubation with CD4$^+$CD25$^+$, but not with CD4$^+$CD25$^-$ T cells, which corroborates recent data from an experimental mouse tumor model (14).

In summary, our observations provide additional insight into the regulatory mechanisms responsible for immunosuppression in human cancer, which may facilitate local tumor growth and metastasis. Hematogenic metastasis often represents the fatal step during the course of malignancy, which may be significantly enhanced by the suppression of blood-borne immunosurveillance mechanisms. Moreover, Tregs may also negatively impact the effectiveness of immunotherapies such as tumor-targeted monoclonal antibodies (*e.g.,* trastuzumab, rituximab). Finally, depletion of Tregs may become a successful anticancer strategy. In fact, in a mouse model, the efficacy of a therapeutic vaccine against melanoma was substantially improved by depletion of Tregs before challenging the animals with granulocyte colony-stimulating factor- or IFN-α-producing tumor cells (15, 20). In conclusion, manipulation of Tregs in terms of their frequency and functional activity should be added to the therapeutic armentarium for enhancing tumor immunity in humans.

Acknowledgments

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