

Permanent up-regulation of regulatory T-lymphocytes in patients with head and neck cancer

ANNE K. SCHOTT*, RALPH PRIES* and BARBARA WOLLENBERG

Department of Otorhinolaryngology, University of Schleswig-Holstein Campus Lübeck, D-23538 Lübeck, Germany

Received December 3, 2009; Accepted January 26, 2010

DOI: 10.3892/ijmm_00000436

Abstract. Various immune functions of different types of immune cells are strongly impaired in patients with head and neck squamous cell carcinoma (HNSCC). Regulatory T-lymphocyte cells (Tregs) have been suggested to be involved in the immunomodulation of immune responses and contribute to HNSCC progression and immune escape. 'Naturally' occurring CD4⁺CD25⁺ Tregs represent a small fraction within the different subsets of regulatory T cells, which are known to inhibit numerous immune functions of different types of immune cells. In this study, the cellular ratio of CD4⁺CD25^{high} Tregs to the entire population of CD4⁺ T-lymphocytes was analyzed with respect to different stages of tumor progression and disease. Our data indicate a significantly high increased abundance of CD4⁺CD25^{high}CD127^{low} Tregs in the peripheral blood of patients with HNSCC, which in addition show modulated expression levels of various functional proteins. Surprisingly, increased Treg levels were found even in patients with no active disease several years after tumor resection, with no significant correlation to the individual tumor stage. Additionally, increased levels of chemokine CCL22, which mediates migration of Tregs to the tumor, and upregulation of the corresponding receptor protein CCR4 were observed in HNSCC. Our data strongly suggest that HNSCC leads to a permanent shift of Treg levels with hardly recognizable recovery rates.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common tumors and standard treatment has only slightly improved the 5-year survival rate of patients with HNSCC over the last 40 years (1-6).

Different studies have shown that the HNSCC micro-environment strongly impairs different types of immune cells

such as natural killer (NK) cells (7), monocytes (8) and T-lymphocytes (9). It has been suggested that regulatory T-lymphocytes (Tregs) play an important role in the suppression of anti-tumor immune responses and thus strongly participate in the immune escape process (10-19).

Tregs are divided into several subtypes, the so called 'naturally' occurring CD4⁺CD25⁺ Tregs and 'induced' or 'adaptive' Tregs, all of which are different in development, phenotype, cytokine secretion and immune function (20,21). Naturally occurring CD4⁺ Tregs constitute a small fraction of the overall CD4⁺ T cell population and constitutively co-express CD25, a high affinity IL-2 receptor α -subunit, on a high level (CD25^{high}) (22).

Natural CD4⁺CD25^{high} Tregs arise in the thymus as a sub-population of T-lymphocytes by the escape from apoptotic death during negative selection (23) and high-avidity interactions of their T cell receptors with self-peptides presented by MHC class II molecules expressed on thymic stroma cells (24,25). Tregs appear to inhibit a variety of immune functions, such as the proliferation and cytokine secretion of CD4⁺ T cells as well as the lytic granule release by CD8⁺ effector cells (12,26). Moreover, Tregs modulate the function and maturation of dendritic cells, monocytes and B-lymphocytes and the cytotoxic effects of NK cells (12,27,28). Besides playing an important role in the regulation of immunotolerance and the prevention of autoimmune diseases (29), elevated proportions of regulatory T cells were found in different types of cancers, such as lung, breast (30), prostate cancer (31) and B-cell non Hodgkin lymphoma (32), and also in HNSCC (33). Furthermore, Tregs have been shown to be associated with a reduced survival in patients with ovarian carcinoma (34).

It has been suggested, that tumor-related factors induce and expand the accumulation of regulatory T cells in cancer-bearing hosts (35). According to Curiel *et al* (34), ovarian tumor cells and microenvironmental macrophages produce CCL22/macrophage derived chemokine (MDC), a chemokine that induces the migration of regulatory T cells through corresponding CCL22 chemokine receptor CCR4 to tumor sites and impair anti-tumor immune responses.

Recent studies have suggested that CD4⁺CD25⁺ T cells are subdivided into suppressive CD4⁺CD25^{high} Treg cells as well as CD4⁺CD25^{low} T cells, which are non-suppressive, antigen-experienced activated CD4⁺ T cells (36). In addition, the α -chain of the IL-7 receptor (CD127) is supposed to be a specific marker protein for suppressive Tregs, namely CD4⁺CD25^{high}CD127^{low} (37). It was recently demonstrated that Tregs are

Correspondence to: Dr Barbara Wollenberg, UK-SH, Klinik für HNO, Ratzeburger Allee 160, D-23538 Lübeck, Germany
E-mail: barbara.wollenberg@uk-sh.de

*Contributed equally

Key words: head and neck cancer, T-lymphocyte cells, immunomodulation

Table I. Clinicopathologic features (sex, age, tumor site, TNM-stage, post-surgery since) of patients with HNSCC included in the study.

A, Sixteen presurgery patients with active disease characterized by sex, age, tumor site and TNM-stage

Sample no.	Sex	Age	Tumor site	TNM-stage
TB479	m	43	epipharynx	cT4bcN3cM0
TB527	f	66	oropharynx (relapse)	pT3N0cM0
TB530	m	49	hypopharynx (relapse)	pT3pN2bcM0
TB533	m	55	hypopharynx/larynx	pT4apN1cM1
TB534	m	69	hypopharynx/larynx	cT3-4cN2ccM1
TB535	m	76	larynx (relapse)	pT3pN0cMx
TB536	m	71	oropharynx	cT3cN2acM0
TB537	m	64	larynx/hypopharynx	pT4pN2bcM0
TB538	f	57	hypopharynx	cT4acN2ccM1
TB539	m	81	oral cavity	cT3cN2cM0
TB541	m	56	vocal cord right	pT2cN0cM0
TB543	m	64	larynx	pT3pN0cM0
			oropharynx	pT2pN0cM0
TB544	m	77	larynx (relapse)	cT4cN2bcM1
TB545	m	41	larynx (relapse)	pT4apN2bcM0
TB546	m	44	CUP	TxN2bM0
TB782	m	61	oropharynx	pT4apN2ccM1

B, Fourteen postsurgery patients with no evidence of disease having received curative therapies; accessory specification of the post-surgery time frame

Sample no.	Sex	Age	Tumor side	TNM-stage	Post-surgery since
TB525	m	46	oral cavity	pT1cN0pMx	3.0 years
TB526	m	67	hypopharynx	pT3pN3cM0	4.0 years
TB528	m	57	hypopharynx	pT4pN2bcM0	5.0 years
TB529	m	49	oral cavity	pT1pN0cM0	18.0 days
TB531	m	55	nasal cavity	pT1cN0cM0	36.0 days
TB532	f	78	Gl. parotis	pT3pN0cM0	10.0 days
TB768	m	61	larynx	pT1cN0cM0	1.5 years
TB777	m	65	larynx/hypopharynx	cT3cN2bcM0	3.0 years
TB778	m	44	larynx	pT2cN0cM0	7.0 months
TB779	m	53	larynx/hypopharynx	pT4apN1M0	10.0 months
TB780	m	67	larynx	pT2pN1cM0	3.0 years
TB781	m	57	oropharynx	pT1pN2acM0	1.0 year
TB783	m	69	hypopharynx	pT2pN0cM0	2.0 years
			oral cavity	pT1pN0cM0	
TB784	m	74	larynx	pT4pN0cM0	9.0 years

characterized by low levels of CD127, which together with a high expression of CD25 (CD25^{high}) helps distinguish Tregs from activated CD4⁺ T-lymphocytes.

Our investigations demonstrate a strong and permanent increased ratio of circulating CD4⁺CD25^{high}CD127^{low} Tregs within the entire population of CD4⁺ T-lymphocytes through HNSCC, which was found even in patients with no active disease after several years.

Materials and methods

Preparation of peripheral blood samples. After written informed consent, peripheral blood samples were taken from

healthy blood donors and patients with HNSCC. All HNSCC tumor patients had histologically proven HNSCC, either at the time of blood draw or before (summarized in Table I). At the time of blood withdrawal, 16 patients had active disease (AD; presurgery) and 16 patients showed no evidence of disease anymore (NED; post-surgery).

As healthy controls, human Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats provided by the blood bank of the University of Lübeck, Germany. Blood donors were 18-65-year-old healthy men and women who were tested negative for HIV and hepatitis B and C viruses. Serum, used for the CCL22-ELISA, was taken from 21 voluntary donors. Patient characteristics and disease classification are



SPANDIDOS PUBLICATIONS sized in Table I. PBMCs were prepared from buffy Ficoll-Hypaque density gradient centrifugation and subjected to flow cytometric analyses.

Cell culture. Permanent HNSCC cell lines BHY (DSMZ Germany) (38), PCI-1 and PCI-13 (generously provided by T.L. Whiteside, Pittsburgh Cancer Institute) were cultured in DMEM-medium (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10% FCS, 1 mM glutamine and 0.1 mM sodium pyruvate and incubated in cell culture flasks with 37°C and 5% CO₂ in an incubator.

Flow cytometry. Surface antigen staining was performed as described previously (39). Cells were stained with peridinin chlorophyll (CD4-PerCP, BD Biosciences), allophycocyanin (CD25-APC, eBioscience), fluorescein-5-isothiocyanate (CD25-FITC, eBioscience), phycoerythrin-Cy7 (CD45RA-PE-CY7, BD Biosciences), GITR-FITC (eBioscience), CD152, phycoerythrin (CTLA-4-PE, BD Biosciences), phycoerythrin (CD122-Pe, BD Biosciences), phycoerythrin-Cy7 (CCR7-PE-CY7, BD Biosciences), phycoerythrin-Cy7 (CCR4-PE-CY7, BD Biosciences), fluorescein-5-isothiocyanate (CD62L-FITC, BD Biosciences) antibodies as specified in figure legends. For intracellular staining of FOXP3 and CTLA-4 (CD152), PE anti-human FOXP3 Flow Kit from BioLegend was used. For measuring of intracellular CCL22, cells were permeabilized using saponin buffer (PBS, 0.1 saponin, 1% FCS and 1 M HEPES), stained with monoclonal anti-human CCL22/MDC antibody (R&D Systems) and the corresponding secondary antibody PE goat anti-mouse Ig (BD Biosciences) or APC goat anti-mouse Ig (BD Biosciences). Propidium-iodide was used to determine dead cells. Isotype control monoclonal antibodies were used to estimate the non-specific binding of target primary antibodies to antigens. Samples were analyzed on a FACSCanto (BD Biosciences, Heidelberg, Germany) and data acquisition was performed using the FACSDiva software (BD Biosciences).

Cytokine analysis. The Enzyme-linked immunosorbent assay (ELISA) was used for determination of CCL22/MDC levels in supernatants according to the manufacturer's instructions (Quantikine Human MDC, R&D Systems). This cytokine assay allows the multiplexed quantitative measurement of multiple cytokines in a single small volume of cell culture supernatant or serum. The minimum detectable dose of MDC is typically <62.5 pg/ml. The cytokine array was analyzed by a specialized microplate reader (Microplate Manager 5.2, Bio-Rad) set to 450 nm and revealed data were calculated using the Microplate spectrophotometer (Microplate Manager software, Bio-Rad). Data were expressed as the means \pm SD. Serum, used for the CCL22-ELISA, was taken from 21 single voluntary donors and 19 tumor patients.

Protein analysis. CCL22 protein expression was analyzed in the lysate of PCI-1, PCI-13, BHY (see above), adenoid vegetations (H437), human tongue squamous cell carcinoma (Cal27) and HaCaT. HaCaT is a permanent epithelial (immortalized keratinocyte cell line) cell line from adult human skin and serves as a positive control for CCL22 production (40). The adenoid vegetations (H437) were used as a negative

control. Cell extracts were prepared and solubilized and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) with bovine serum albumin (BSA) as a standard. Aliquots of protein extracts (60 μ g) were run on SDS-PAGE and were then transferred onto a nitrocellulose membrane. Blots were decorated with monoclonal anti-human CCL22/MDC antibody (R&D Systems) and mouse anti- β -actin antibody as loading control (mAbcam).

Statistical analysis. Statistical analysis was performed using the SPSS 12.0 program (SPSS Inc., Chicago, USA), SigmaPlot 10.0 (Systat Software Inc) and Office 2003 (Microsoft Corporation, Redmont, USA). Data are expressed as mean \pm SD resulting from at least four independent experiments. The achieved results were tested by means of Kolmogorov-Smirnov-test regarding normal distribution. The significance of the results was determined using the Student t-test for independent samples (highly significant, $p < 0.01$; significant, $0.01 \leq p \leq 0.05$; trend, $0.05 < p < 0.1$); otherwise statistical analysis was performed using the Mann-Whitney-U-test.

Results

Increased ratio of Tregs within CD4⁺ T cells in HNSCC patients. The abundance of circulating lymphocytes, CD4⁺-, CD4⁺CD25⁺-, CD4⁺CD25^{high}CD127^{low}- and CD4⁺CD25^{low}CD127^{high}-T cells in the peripheral blood of patients with HNSCC was analyzed using flow cytometry. The presented study included 25 patients with HNSCC and 27 healthy controls, which are summarized in Table I.

Patients with HNSCC showed a highly significant increase of CD4⁺CD25^{high}CD127^{low} Tregs among CD4⁺ T-lymphocytes in comparison with healthy controls ($3.4 \pm 2.5\%$ in HNSCC vs. $1.8 \pm 1.7\%$ in NC; $p < 0.01$) (Fig. 1A and B). Human CD4⁺CD25⁺ cells are subdivided in suppressor CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells, which are non-suppressive, antigen-experienced activated CD4⁺ T cells (36). A low expression of CD127 (CD127^{low}) together with a high expression of CD25 (CD25^{high}) is supposed to be specific for suppressive Treg cells (37). Our data show that up to 98% of CD4⁺CD25⁺ T cells are non-suppressive CD4⁺CD25^{low} T cells and normal controls displayed significantly higher percentages of CD4⁺CD25^{low}CD127^{high} T cells relative to HNSCC patients ($98.2 \pm 1.7\%$ in NC vs. $96.6 \pm 2.5\%$ in HNSCC; $p < 0.01$). In the TNM-stages, no significant correlation between tumor stage and the level of circulating Treg cells was observed in the peripheral blood of HNSCC tumor patients.

Phenotypic analysis of CD4⁺ T cell subsets. Circulating CD4⁺CD25^{high}CD127^{low} lymphocytes from HNSCC patients and healthy controls were analyzed concerning the expression of functional proteins forkhead box P3 (FoxP3), membrane glucocorticoid-induced tumor-necrosis factor receptor family-related gene (GITR), immunoregulatory protein cytolytic T-lymphocyte-associated antigen 4 protein or CD152 (CTLA-4), chemokine receptors CCR7 and CCR4, the 'homing receptor' CD62L, CD45RA and the β -chain of the IL-2-receptor CD122. In patients with HNSCC 48.3% of the analyzed CD4⁺CD25^{high}CD127^{low} Treg cells expressed the transcriptional activator

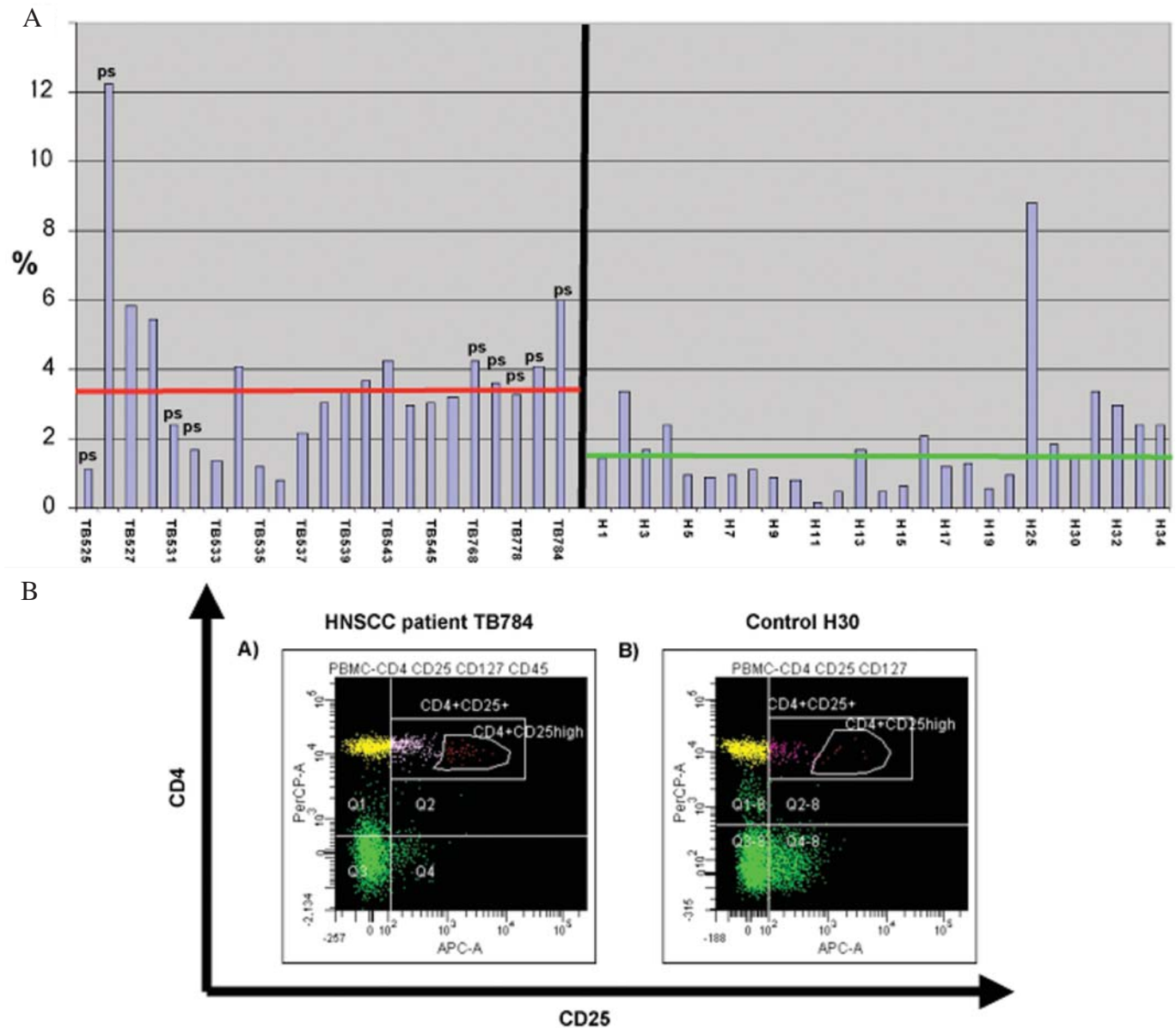
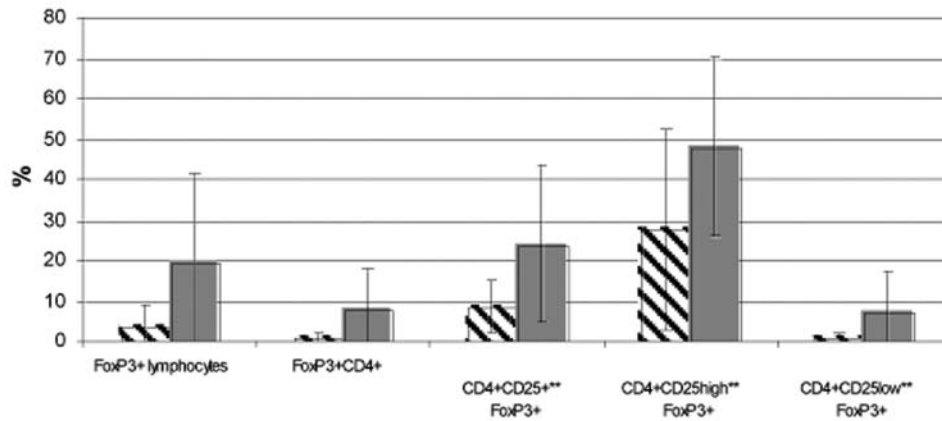


Figure 1. Increase of CD4⁺CD25^{high} Treg cells in peripheral blood of HNSCC patients compared with healthy controls. The abundance of circulating CD4⁺CD25^{high} Treg cells in PBMC of 25 HNSCC tumor patients (TB) and 27 healthy controls (H) was analyzed by flow cytometry and compared to the overall level (%) of CD4⁺ T-lymphocytes. (A) Red line, average of CD4⁺CD25^{high} Treg within CD4⁺ T cells in patients with HNSCC; green line, average of CD4⁺CD25^{high} Treg within CD4⁺ T cells in normal controls. Fourteen HNSCC tumor patients are patients with an active disease; 11 patients marked with 'ps' are patients showing no evidence of disease (post-surgery) and oncological treatment of the tumor was carried out between 10 days and 9 years ago. Our data demonstrate strongly increased ratios of CD4⁺CD25^{high} Treg cells to CD4⁺ T lymphocytes in the peripheral blood of patients with HNSCC compared to healthy donors with individually deviating levels. These findings are independent of activity of disease. Patients with HNSCC, TB525-TB539, TB541, TB543-TB546, TB768, TB777-TB779, TB784; normal controls: H1-H20, H25, H28, H30-H34. (B) Example of enrichment in CD4⁺CD25⁺, CD4⁺CD25^{high} and CD4⁺CD25^{low} Treg cells in PBMCs of a single patient with HNSCC (TB784) in comparison with a healthy control (H30). The PBMCs of the HNSCC patient were found to contain 28.6% CD4⁺CD25⁺ Treg cells, that consist of 6% CD4⁺CD25^{high} and 94% CD4⁺CD25^{low} Treg cells relative to 12% CD4⁺CD25⁺ Treg cells, 1.5% CD4⁺CD25^{high} and 98.5% CD4⁺CD25^{low} Treg cells in the normal control.

protein FoxP3, 22.7% expressed GITR, 68.6% CTLA-4, 54.8% expressed CCR7, 86.9% CCR4, 78.5% CD62L, 51.4% CD45RA and 34.96% expressed CD122. Comparing the percentages of the different markers on CD4⁺CD25^{high}CD127^{low} Treg cells in HNSCC patients with healthy controls, there existed no significant difference in the expression of GITR, CTLA-4, CD45RA, CCR4 and CD62L. However, CCR7 showed a tendency ($0.05 < p < 0.1$) for a higher expression of CD4⁺CD25^{high}CD127^{low} Treg cells in HNSCC patients.

On the other hand, the amount of CD122 (interleukin-2, IL-2/IL-15 receptor β -chain), which plays a role in regulating normal lymphocyte development *in vivo*, expressing CD4⁺CD25^{high}CD127^{low} Treg cells were highly significantly increased in healthy controls relative to HNSCC patients (60.8 vs. 35%; $p < 0.01$). Consistent with the significant increase of CD4⁺CD25^{high}CD127^{low} Treg cells in the peripheral blood of patients with HNSCC relative to healthy controls, our data displayed that the amount in percentage of Foxp3 (48.3 vs.



B

Foxp3 on different cells	Average HNSCC in %	Average NC in %	p - value
<i>FoxP3⁺ lymphocytes</i>	20,1	3,9	$p < 0,01$
<i>CD4⁺ FoxP3⁺</i>	8	1,4	$0,01 > p < 0,05$
<i>CD4⁺ CD25⁺</i>	24,3	9	$p < 0,01$
<i>CD4⁺ CD25^{high}**</i>	48,3	27,8	$p < 0,01$
<i>CD4⁺ CD25^{low}** FoxP3⁺</i>	7,2	1,2	$0,01 > p < 0,05$

Figure 2. Expression of Foxp3 expressed as average of percentage with SD in patients with HNSCC (red bar) relative to normal controls (green bar). We analyzed a possible expression of Foxp3 on lymphocytes in general, on CD4⁺, CD4⁺CD25⁺ T cells, CD4⁺CD25^{high}CD127^{low} Treg cells (red box) and on CD4⁺CD25^{low}CD127^{high} T cells. (A) The analyzed cells in PBMCs in HNSCC patients as well as in the normal controls displayed an expression of Foxp3, however the Foxp3 expression is significantly higher in cells in patients with HNSCC. (B) Listing of the frequencies and p-values of cells expressing Foxp3 in patients relative to normal controls.

27.8%; $p < 0.01$) expressing CD4⁺CD25^{high}CD127^{low} Treg cells were also highly significantly increased in these patients (Fig. 2). The transcription factor Foxp3 has been shown to be critical for the development and function of CD4⁺CD25^{high} regulatory T cells. However, recently FOXP3 expression has been shown to be induced upon activation of human CD4⁺ T cells (41) and the expression is not supposed to be restricted to Treg cells. Hence we analyzed a possible expression of Foxp3 in lymphocytes, CD4⁺, CD4⁺CD25⁺ and CD4⁺CD25^{low}CD127^{high} T cells (Fig. 2). We showed that all analyzed cells displayed an expression of Foxp3. However, the percentage of Foxp3 expression was again significantly higher in patients with HNSCC (Fig. 2B).

HNSCC induces a permanent shift of Treg abundance. Analyzed patients with HNSCC were subdivided into patients with an active tumor disease (AD) (n=14) and postsurgery patients (n=11) with no evidence of an active disease (NED) after an oncologic therapy. Oncological treatment (surgery ±radiochemotherapy) of the tumor was carried out between 10 days and 9 years ago.

Surprisingly, the mean ratio of CD4⁺CD25^{high}Foxp3⁺ Treg cells to the total of peripheral blood CD4⁺ T cells was still significantly increased in all analyzed samples, even up to 9 years after active disease and oncologic therapy (Fig. 1A). Our data demonstrate that HNSCC leads to a permanent shift of the Treg abundance with hardly recognizable recovery rates.

Even though we found no difference in the mean ratio of CD4⁺CD25^{high}CD127^{low} Treg cells between patients with AD and NED, we noted that the percentage of GITR and CCR4

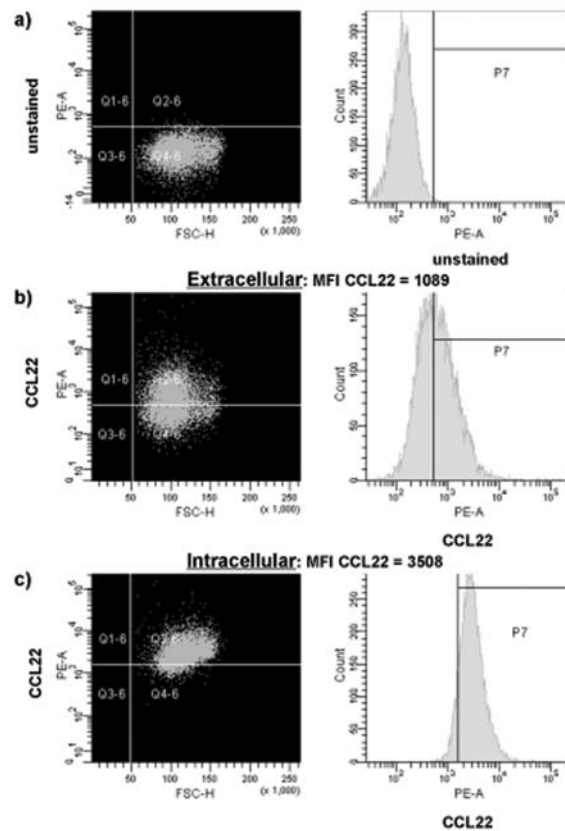
expressing Treg cells were significantly higher among PBMCs from HNSCC patients with an active stage of disease (AD). In addition the expression levels of CCR4 and GITR were significantly higher on the surface of single CD4⁺CD25^{high}CD127^{low} Treg cells in patients with active disease, declared by the mean fluorescence intensity measured by flow cytometry. Moreover, our data displayed, that patients before being treated with an oncological therapy show significantly higher percentages of GITR and Foxp3 expressing lymphocytes, CD4⁺ and CD4⁺CD25^{low} T cells.

CCL22 as a chemoattractant in HNSCC. The ability of HNSCC to autonomously produce CCL22 was investigated using permanent HNSCC cell lines PCI-1 and PCI-13. Flow cytometric analyses demonstrate a significant production of CCL22 (Fig. 3). Furthermore, our data displayed an extracellular expression of CCL22 on the surface of the analyzed HNSCC cell lines, which is highly significantly lower ($p < 0.01$) than the detected intracellular expression (Fig. 3A).

In addition, the CCL22 expression was analyzed in protein lysates of permanent HNSCC cell lines PCI-1, BHY, adenoid vegetations (H437) (Fig. 3B), as well as PCI-13, Cal 27 and HaCaT by Western blotting (Fig. 3B). Fig. 3B demonstrates that CCL22 can be detected in all analyzed permanent cell lines with similar expression levels. The adenoid vegetations (H437) served as a negative control and showed no production of CCL22. The human keratinocyte cell line HaCaT was used as a positive control as described in previous studies (40,42).

To evaluate the relationship between the level of CCL22 and the frequencies of regulatory CD4⁺CD25^{high} T cells in

A



B

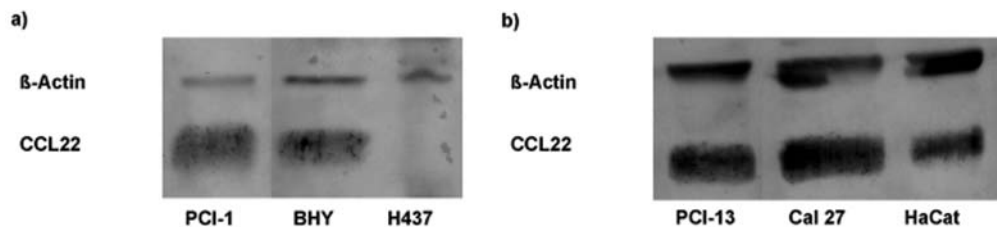


Figure 3. Expression of the chemokine CCL22 by Treg cells and HNSCC cell lines. (A) a-c, Extracellular and intracellular expression of CCL22 on the analyzed HNSCC cell line PCI-1. Permanent HNSCC cell lines PCI-1 and PCI-13 were stained with or without Fix/Perm buffer set for intracellular or extracellular staining respectively and stained with CCL22-antibody inclusive secondary antibody and flow cytometric analysis indicated the production of CCL22 (c). Furthermore our data displayed an extracellular expression of CCL22 on the surface of the analyzed HNSCC cell lines, which is highly significantly lower ($p < 0.01$) (b) than the detected intracellular expression (c). (B) Analysis of the CCL22 protein expression in the lysate of the HNSCC-cell lines PCI-1, BHY, PCI-13 and Cal-27, adenoid vegetations (H437) and the human keratinocyte cell line HaCaT by Western blotting with CCL22-antibody and β -actin and corresponding secondary antibody. β -actin served as a loading control. a, Western blot with the lysate of PCI-1, BHY, adenoid vegetations (H437). CCL22 was detected in PCI-1 and BHY cell lines. The adenoid vegetations (H437) served as a negative control and showed no production of CCL22. b, Cal-27 showed a similar amount of CCL22 relative to PCI-13. The production of CCL22 by the human keratinocyte cell line HaCaT is well known in previous studies, hence we used HaCaT as a positive control for CCL22 production in HNSCC cell lines.

HNSCC patients, we examined the expression of chemokine CCL22/MDC in the serum of 19 tumor patients and 21 healthy blood donors, which are summarized in Table I.

Our data demonstrate, that there are no significant differences of the CCL22 levels in the peripheral blood of tumor patients compared to healthy donors, but only individual deviations. Furthermore, our data show no correlation between HNSCC tumor stage (TNM) and the level of CCL22 in the peripheral blood (Fig. 4).

Since IFN- γ is known to suppress constitutive expression of CCL22 in mature macrophages and dendritic cells (43), we analyzed its influence on the CCL22 production in HNSCC. Therefore, permanent HNSCC cell lines were cultivated in 5 ml DMEM-medium with 2 μ l (4,000 pg/ml) recombinant human IFN- γ for 12, 48 or 72 h, respectively. Cells were analyzed by

flow cytometry and the level of CCL22 before and after incubation with IFN- γ was declared in Mean Fluorescence Intensity (MFI), which is an admeasurement for adhesion of antibodies and for the expression and production of CCL22. The decrease in the CCL22 level after incubation with IFN- γ was specified in percent (Fig. 5). Our data indicate that IFN- γ leads to a reduced expression of CCL22 in HNSCC.

Discussion

In recent years, a variety of immunoregulatory functions and different subsets of Tregs in cancer patients have been described and the concept has emerged that peripheral tolerance to tumors is maintained and enhanced by Tregs (14,19,22,44). In this work we demonstrate that malignant head and neck

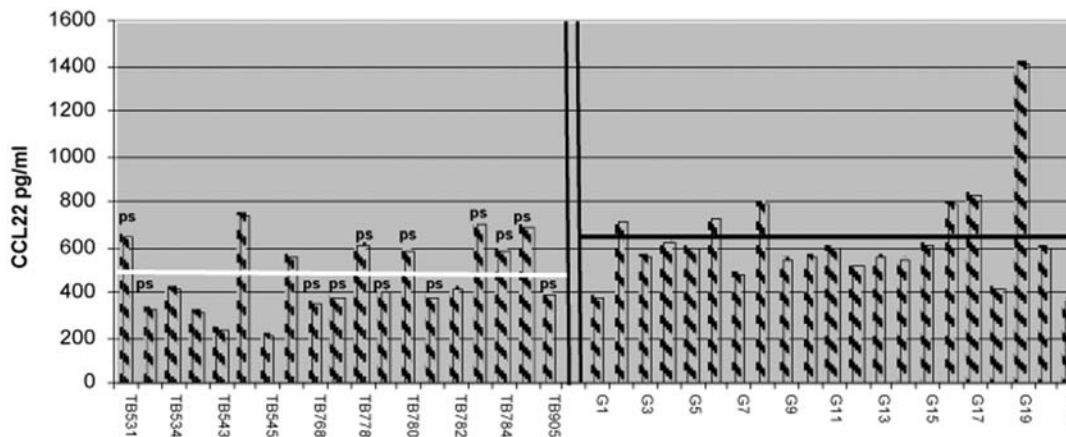


Figure 4. Level (pg/ml) of the chemokine CCL22/MDC in the serum of 19 tumor patients (TB) relative to 21 healthy controls (H). 7 HNSCC tumor patients are patients with an active disease; 12 patients marked with 'ps' are patients showing no evidence of disease (post-surgery) and oncological treatment of the tumor was carried out between 10 days and 9 years ago. The level of CCL22 was measured by Enzyme-linked Immunosorbent Assay (ELISA). The average levels of CCL22 in tumor patients and healthy controls are illustrated by a white and black horizontal line, respectively. The level of CCL22 in the blood samples of tumor patients, active disease or no evidence of disease, does not differ from the level measured in the serum of healthy controls. The CCL22 level did not show any correlation with the TNM-Stage or the number of Treg cells.

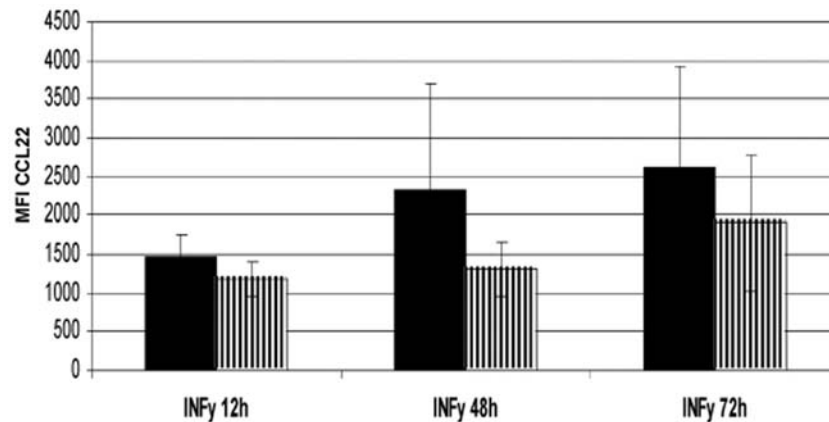


Figure 5. Downregulation of the CCL22 production and CCR4 expression through IFN- γ . The level of CCL22 expression is declared in mean fluorescence intensity (MFI) together with the respective standard deviations. Our data indicate that INF- γ leads to a reduced expression of CCL22 in HNSCC (Blue bar, mean fluorescence intensity of CCL22 without any incubation with INF- γ ; Purple bar, mean fluorescence intensity of CCL22 after incubation with INF- γ).

cancer strongly modulates the abundance of CD4⁺CD25^{high} regulatory T cells in the peripheral blood of HNSCC tumor patients for a long time period.

We show that circulating CD4⁺CD25^{high} Tregs in HNSCC patients possess a characteristic phenotype determining their regulatory function such as CD25⁺, transcriptional activator Foxp3⁺, CTLA-4⁺ (cytotoxic T-lymphocyte-associated antigen 4; CD152), glucocorticoid-induced tumor necrosis factor receptor (GITR⁺), CD45RA⁺, the chemokine receptors CCR7⁺ and CCR4⁺ and the 'homing' receptor CD62L⁺. Significantly increased levels not only of CD4⁺CD25^{high}Foxp3⁺ Treg cells, but also of Foxp3 expressing lymphocytes, CD4⁺ T lymphocytes, CD4⁺CD25⁺ and CD4⁺CD25^{low}CD127^{high} T cells were found in patients with HNSCC compared to healthy controls. In different solid tumors, the accumulation of Foxp3⁺ Tregs has been associated with reduced survival rates of tumor patients (34,45-47). Hence the greater number of Foxp3 expressing Treg cells may reflect the increased suppressive activity seen in patients with HNSCC (33). However, it is

important to mention that according to Hori *et al* (48) the expression of Foxp3 on T-lymphocytes does not necessarily implicate suppressive abilities.

Our data demonstrate the long-term character of tumor modulated Treg abundance and the CCL22-level in patients with HNSCC. These data are in accordance with other recent studies, which implicate that malignant tumors, independently from the disease stage, site or nodal involvement, have a strong impact on the homeostasis of lymphocytes long after the tumor is removed, even in the absence of any chemo- or radiotherapeutic treatments (9,49). Even though we found no difference in the mean ratio of CD4⁺CD25^{high}CD127^{low} Treg cells between patients with AD and NED, we noted however that levels of GITR and CCR4 expressing Treg cells were significantly higher among PBMCs from HNSCC patients with an active stage of disease (AD). GITR as well as CCR4 can be upregulated on T cells upon activation and TCR and CD28 engagement (50). The increased expression of the receptor on single Treg cells in patients with an active disease

might be consistent with a higher response of Treg cells to the chemoattractant CCL22. Furthermore, the fact that patients with active disease show a significant increase of GTR expressing Treg cells is of special interest, because GTR is a typical Treg surface marker, that is supposed to abrogate Treg cell mediated suppression upon stimulation of GTR (51). But according to a previous (52) while GTR triggering weakens suppressor activity, it can induce Treg proliferation and expansion (53,54). Furthermore, after GTR co-stimulation, Treg cells are supposed to regain their suppressive activity. This fact can imply, that the final outcome of a transient inhibition by GTR of suppressive activity and an increase of Treg proliferation could result in an overall increase of suppressor activity (52).

CCL22 binds to and activates the G-protein-coupled CCR4, a CC chemokine receptor, that is primarily expressed by CD4⁺ and CD8⁺ lymphocytes with a Th2 phenotype and CD25⁺ regulatory T cells (55-59), but also by natural killer cells, dendritic cells and macrophages (60). CCR4 is supposed to be important for regulating immune balance (61). Curiel *et al* (34) have shown, that tumor cells and microenvironmental macrophages in ovarian carcinoma produce CCL22, which mediates migration of regulatory T cells to the tumor. We showed in our study, that HNSCC is also able to autonomously produce CCL22.

Our data demonstrate that IFN- γ is able to downregulate CCL22 production in HNSCC. Interferon- γ is a representative of Th1 cytokines (62) and it is secreted by Th1 cells, dendritic cells and natural killer cells. INF- γ has antiviral, immunoregulatory and anti-tumor properties. Immune responses in HNSCC tumor patients have been shown to be biased toward the secretion of Th2 cytokines, which prevent effective anti-tumor Th1 immune responses and so facilitate the production of CCL22 and in succession the recruitment of Tregs to tumor sites (63,64).

In conclusion, the increase of regulatory T cells in HNSCC tumor patients might stand for a reduced anti-tumor immunity and therefore contribute to an optimized tumor immune evasion and progression or recurrence of head and neck cancer.

Acknowledgements

We are grateful to Ewelina Szymanski and Brigitte Wollmann for skillful support in several parts of this work and all members of the Department of Otorhinolaryngology for helpful discussions and a comfortable atmosphere. Furthermore, we thank T.L. Whiteside (Pittsburgh Cancer Institute) for providing the permanent HNSCC cell lines used in this work. This work was supported by grants of the following funding bodies, Mildred-Scheel-Stiftung (Deutsche Krebshilfe), the Monika-Kutzner-Stiftung, the Werner-and-Klara-Kreitz-Stiftung and the Rudolf-Bartling-Stiftung.

References

- Luce D, Guenel P, Leclerc A, Brugere J, Point D and Rodriguez J: Alcohol and tobacco consumption in cancer of the mouth, pharynx, and larynx: a study of 316 female patients. *Laryngoscope* 98: 313-316, 1988.
- Snow GB: Follow-up in patients treated for head and neck cancer: how frequent, how thorough and for how long? *Eur J Cancer* 28: 315-316, 1992.
- Berrino F, Esteve J and Coleman MP: Basic issues in estimating and comparing the survival of cancer patients. *IARC Sci Publ*, pp1-14, 1995.
- St John MA, Abemayor E and Wong DT: Recent new approaches to the treatment of head and neck cancer. *Anticancer Drugs* 17: 365-375, 2006.
- Kraunz KS, McClean MD, Nelson HH, Peters E, Calderon H and Kelsey KT: Duration but not intensity of alcohol and tobacco exposure predicts p16INK4A homozygous deletion in head and neck squamous cell carcinoma. *Cancer Res* 66: 4512-4515, 2006.
- Chin D, Boyle GM, Porceddu S, Theile DR, Parsons PG and Coman WB: Head and neck cancer: past, present and future. *Expert Rev Anticancer Ther* 6: 1111-1118, 2006.
- Bauernhofer T, Kuss I, Henderson B, Baum AS and Whiteside TL: Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer. *Eur J Immunol* 33: 119-124, 2003.
- Kross KW, Heimdal JH, Olsnes C, Olofsson J and Aarstad HJ: Head and neck squamous cell carcinoma spheroid- and monocyte spheroid-stimulated IL-6 and monocyte chemotactic protein-1 secretion are related to TNM stage, inflammatory state and tumor macrophage density. *Acta Otolaryngol* 125: 1097-1104, 2005.
- Kuss I, Hathaway B, Ferris RL, Gooding W and Whiteside TL: Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. *Clin Cancer Res* 10: 3755-3762, 2004.
- Whiteside TL: Immunobiology of head and neck cancer. *Cancer Metastasis Rev* 24: 95-105, 2005.
- McHugh RS and Shevach EM: The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol* 110: 693-702, 2002.
- Miyara M and Sakaguchi S: Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 13: 108-116, 2007.
- Shimizu J, Yamazaki S and Sakaguchi S: Induction of tumor immunity by removing CD25⁺CD4⁺ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163: 5211-5218, 1999.
- Szczepanski MJ, Szajnik M, Czystowska M, Mandapathil M, Strauss L, Welsh A, Foon KA, Whiteside TL and Boyiadzis M: Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. *Clin Cancer Res* 15: 3325-3332, 2009.
- Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT and Whiteside TL: A unique subset of CD4⁺CD25^{high}Foxp3⁺ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* 13: 4345-4354, 2007.
- Whiteside TL: Immunobiology and immunotherapy of head and neck cancer. *Curr Oncol Rep* 3: 46-55, 2001.
- Whiteside TL: Immune responses to malignancies. *J Allergy Clin Immunol* 111: S677-S686, 2003.
- Whiteside TL: Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Semin Cancer Biol* 16: 3-15, 2006.
- Ferrone S and Whiteside TL: Tumor microenvironment and immune escape. *Surg Oncol Clin N Am* 16: 755-774, viii, 2007.
- Zwar TD, van Driel IR and Gleeson PA: Guarding the immune system: suppression of autoimmunity by CD4⁺CD25⁺ immunoregulatory T cells. *Immunol Cell Biol* 84: 487-501, 2006.
- Danese S and Rutella S: The Janus face of CD4⁺CD25⁺ regulatory T cells in cancer and autoimmunity. *Curr Med Chem* 14: 649-666, 2007.
- Wang HY and Wang RF: Regulatory T cells and cancer. *Curr Opin Immunol* 19: 217-223, 2007.
- Von Boehmer H: Mechanisms of suppression by suppressor T cells. *Nat Immunol* 6: 338-344, 2005.
- Nielsen J, Holm TL and Claesson MH: CD4⁺CD25⁺ regulatory T cells: II. Origin, disease models and clinical aspects. *APMIS* 112: 642-650, 2004.
- Valzasina B, Piconese S, Guiducci C and Colombo MP: Tumor-induced expansion of regulatory T cells by conversion of CD4⁺CD25⁺ lymphocytes is thymus and proliferation independent. *Cancer Res* 66: 4488-4495, 2006.
- Thornton AM and Shevach EM: CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188: 287-296, 1998.
- Ghiringhelli F, Menard C, Martin F and Zitvogel L: The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunol Rev* 214: 229-238, 2006.



irina N, Poli A, Michel T, Poos L, Andres E, Hentges F
PUBLICATIONS mmer J: Control of natural killer (NK) cell functions by

- CD4⁺CD25⁺ regulatory T cells. *J Leukoc Biol* 81: 144-153, 2006.
29. Bluestone JA and Tang Q: How do CD4⁺CD25⁺ regulatory T cells control autoimmunity? *Curr Opin Immunol* 17: 638-642, 2005.
30. Pakravan N, Hassan AT and Hassan ZM: Naturally Occurring self-reactive CD4(+)CD25(+) regulatory T cells: universal immune code. *Cell Mol Immunol* 4: 197-201, 2007.
31. Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M, Egevad L and Pisa P: CD4⁺CD25^{high} T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *J Immunol* 177: 7398-7405, 2006.
32. Yang ZZ, Novak AJ, Stenson MJ, Witzig TE and Ansell SM: Intratumoral CD4⁺CD25⁺ regulatory T-cell-mediated suppression of infiltrating CD4⁺ T cells in B-cell non-Hodgkin lymphoma. *Blood* 107: 3639-3646, 2006.
33. Lau KM, Cheng SH, Lo KW, Lee SA, Woo JK, van Hasselt CA, Lee SP, Rickinson AB and Ng MH: Increase in circulating Foxp3⁺CD4⁺CD25(high) regulatory T cells in nasopharyngeal carcinoma patients. *Br J Cancer* 96: 617-622, 2007.
34. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L and Zou W: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10: 942-949, 2004.
35. Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H and Fujii H: CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3(+) regulatory T cells in gastric cancer. *Int J Cancer* 122: 2286-2293, 2008.
36. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A and Zippelius A: Selective survival of naturally occurring human CD4⁺CD25⁺Foxp3⁺ regulatory T cells cultured with rapamycin. *J Immunol* 178: 320-329, 2007.
37. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF and Bluestone JA: CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 203: 1701-1711, 2006.
38. Kawamata H, Nakashiro K, Uchida D, Harada K, Yoshida H and Sato M: Possible contribution of active MMP2 to lymph-node metastasis and secreted cathepsin L to bone invasion of newly established human oral-squamous-cancer cell lines. *Int J Cancer* 70: 120-127, 1997.
39. Sozzani S, Luini W, Borsatti A, Polentarutti N, Zhou D, Piemonti L, D'Amico G, Power CA, Wells TN, Gobbi M, Allavena P and Mantovani A: Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J Immunol* 159: 1993-2000, 1997.
40. Fujii-Maeda, S, Kajiwara K, Ikizawa K, Shinazawa M, Yu B, Koga T, Furue M and Yanagihara Y: Reciprocal regulation of thymus and activation-regulated chemokine/macrophage-derived chemokine production by interleukin (IL)-4/IL-13 and interferon-gamma in HaCaT keratinocytes is mediated by alternations in E-cadherin distribution. *J Invest Dermatol* 122: 20-28, 2004.
41. Ziegler SF: FOXP3: not just for regulatory T cells anymore. *Eur J Immunol* 37: 21-23, 2007.
42. Purwar R, Werfel T and Wittmann M: IL-13-stimulated human keratinocytes preferentially attract CD4⁺CCR4⁺ T cells: possible role in atopic dermatitis. *J Invest Dermatol* 126: 1043-1051, 2006.
43. Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D and Mantovani A: Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* 92: 2668-2671, 1998.
44. Bergmann C, Strauss L, Wang Y, Szczepanski MJ, Lang S, Johnson JT and Whiteside TL: T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease. *Clin Cancer Res* 14: 3706-3715, 2008.
45. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnajic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen Y, Ohtani H, Old LJ and Odunsi K: Intraepithelial CD8⁺ tumor-infiltrating lymphocytes and a high CD8⁺/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 102: 18538-18543, 2005.
46. Petersen RP, Campa MJ, Sperlazza J, Conlon D, Joshi MB, Harpole DH Jr and Patz EF Jr: Tumor infiltrating Foxp3⁺ regulatory T cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 107: 2866-2872, 2006.
47. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL and Banham AH: Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 24: 5373-5380, 2006.
48. Hori S: Rethinking the molecular definition of regulatory T cells. *Eur J Immunol* 38: 928-930, 2008.
49. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN and Whiteside TL: Characteristics of CD4⁺CD25⁺ regulatory T cells in the peripheral circulation of patients with head and neck cancer. *Br J Cancer* 92: 913-920, 2005.
50. D'Ambrosio D, Iellem A, Bonecchi R, Mazzeo D, Sozzani S, Mantovani A and Sinigaglia F: Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol* 161: 5111-5115, 1998.
51. Shimizu J, Yamazaki S, Takahashi T, Ishida Y and Sakaguchi S: Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3: 135-142, 2002.
52. Nocentini G, Ronchetti S, Cuzzocrea S and Riccardi C: GITR/GITRL: more than an effector T cell co-stimulatory system. *Eur J Immunol* 37: 1165-1169, 2007.
53. Nocentini G and Riccardi C: GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. *Eur J Immunol* 35: 1016-1022, 2005.
54. Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, Ayroldi E and Riccardi C: GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. *Eur J Immunol* 34: 613-622, 2004.
55. Vulcano M, Albanesi C, Stoppacciaro A, Bagnati R, D'Amico G, Struyf S, Transidico P, Bonecchi R, Del Prete A, Allavena P, Ruco LP, Chiabrando C, Girolomoni G, Mantovani A and Sozzani S: Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur J Immunol* 31: 812-822, 2001.
56. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A and Sinigaglia F: Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 187: 129-134, 1998.
57. Andrew DP, Chang MS, McNinch J, Wathen ST, Rihaneh M, Tseng J, Spellberg JP and Elias CG 3rd: STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. *J Immunol* 161: 5027-5038, 1998.
58. Echigo T, Hasegawa M, Shimada Y, Inaoki M, Takehara K and Sato S: Both Th1 and Th2 chemokines are elevated in sera of patients with autoimmune blistering diseases. *Arch Dermatol Res* 298: 38-45, 2006.
59. Mariani M, Lang R, Binda E, Panina-Bordignon P and D'Ambrosio D: Dominance of CCL22 over CCL17 in induction of chemokine receptor CCR4 desensitization and internalization on human Th2 cells. *Eur J Immunol* 34: 231-240, 2004.
60. Jakubzick C, Wen H, Matsukawa A, Keller M, Kunkel SL and Hogaboam CM: Role of CCR4 ligands, CCL17 and CCL22, during *Schistosoma mansoni* egg-induced pulmonary granuloma formation in mice. *Am J Pathol* 165: 1211-1221, 2004.
61. Ishida T and Ueda R: CCR4 as a novel molecular target for immunotherapy of cancer. *Cancer Sci* 98: 1139-1146, 2006.
62. Yamashita U and Kuroda E: Regulation of macrophage-derived chemokine (MDC, CCL22) production. *Crit Rev Immunol* 22: 105-114, 2002.
63. Pries R and Wollenberg B: Cytokines in head and neck cancer. *Cytokine Growth Factor Rev* 17: 141-146, 2006.
64. Pries R, Thiel A, Brocks C and Wollenberg B: Secretion of tumor-promoting and immune suppressive cytokines by cell lines of head and neck squamous cell carcinoma. *In Vivo* 20: 45-48, 2006.