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ORIGINAL ARTICLE

Increased prevalence of tumour infiltrating immune cells in oropharyngeal tumours in comparison to other subsites: relationship to peripheral immunity

Victoria L. Green · Anna Michno · Nicholas D. Stafford · John Greenman

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Abstract
Background The nature of the tumour microenvironment immune response in head and neck cancer patients has an important role in tumour development and metastasis, but it is unknown if this differs between cancer subsites or whether it is related to the peripheral immune response.
Methods Immune cells (CD4, CD8, Foxp3) in head and neck squamous cell carcinoma tissue (HNSCC; $n = 66$), detected by immunohistochemistry, have been correlated with tumour subsite and immune cells in the peripheral circulation ($CD4^+CD25^{High}Foxp3^+$ Treg and $CD4^+$ T cells), identified using flow cytometry.
Results Oropharyngeal tumours had a greater number of infiltrating immune cells in both tumour and stroma compared with other subsites, but no difference was observed in the circulating levels. Immune cells in the stroma were positively related to those in the tumour with consistently higher levels in stroma. A strong relationship was found between the number of $CD4^+$ and $Foxp3^+$ cells but not between the number of $CD8^+$ and $Foxp3^+$ cells in the tumour. The number of $Foxp3^+$ cells within the tumour was positively correlated with the percentage of circulating $CD4^+CD25^{High}$ cells positive for Foxp3. Late stage laryngeal tumours showed a higher number of $Foxp3^+$ lymphocytes compared with early stage malignancies, and oropharyngeal tumours had more $CD4^+$ cells in node negative tumours compared with node positive ones.
Conclusion The level of immune cell infiltration in head and neck squamous cell carcinoma appears to be subsite dependent residing primarily in the stroma and is likely to be dependent on the peripheral immune response.

Keywords HNSCC · Oropharyngeal · T regulatory cells · Tumour microenvironment

V. L. Green · A. Michno · J. Greenman
Department of Biological Sciences, University of Hull, Hull HU6 7RX, UK

V. L. Green (✉)
Daisy Building Laboratories, Castle Hill Hospital, Hull HU16 5JQ, UK
e-mail: v.l.green@hull.ac.uk

N. D. Stafford
Hull York Medical School, University of Hull, Hull HU6 7RX, UK

Introduction

Head and neck cancer are the sixth most common solid tumour in the world accounting for approximately 5 % of all cancer incidences globally [1] and is a term used to group together a number of tumours arising from distinct locations (subsites; including nasal cavity, oral cavity, oropharynx, larynx and hypopharynx) within the upper aerodigestive tract. Histologically, 95 % of all head and neck cancers are squamous cell carcinomas (HNSCC), and although a subgroup of patients with tumours arising as a result of HPV16 infection tend to have an overall 3 year survival of 82.4 %, other patients with non-viral HNSCC still have a 5-year survival rate of only 57.1 % [2].

As is the case with many cancers, patients with head and neck cancer are generally immunosuppressed, having raised levels of T regulatory cells, as well as functionally defective circulating and tumour infiltrating lymphocytes that have enhanced levels of apoptosis [3]. However, good evidence exists to suggest that head and neck tumours invoke a host immune response to the over expression of tumour-associated antigens (TAAs) and the secretion of cytokines and

chemokines by the tumour, causing a leucocytic infiltrate into the tumour microenvironment [4, 5]. However, there is still debate over whether the presence of tumour infiltrating lymphocytes (TILs) is a positive or negative indicator of prognosis, and this could be dependent on the balance of the type of immune cells involved, specifically the ratio of immune suppressive T regulatory cells to immune stimulatory T effector cells, and/or the site of origin of the tumour [6–11].

Despite the immune response by the host, tumour regression/elimination is rarely observed without therapeutic intervention, due largely to various immune escape mechanisms employed by the tumour [12–14]. One such mechanism is thought to involve T regulatory cells (Tregs) which act to suppress the activity of $CD4^+$ and $CD8^+$ effector T cells ($CD4^+$ helper cells which promote phagocytosis by myeloid cells as well as antibody production by B cells, and $CD8^+$ cytotoxic cells which stimulate cell death) and also decrease antigen presentation and promote the immunosuppressive functions of dendritic cells, monocytes and macrophages together resulting in reduced anti-tumour immunity [15, 16]. Therefore, both cell number and the balance between regulatory and effector T cells are critical. Tregs have been reported to be elevated in the peripheral blood of HNSCC patients [17] where their presence has been related to early recurrence [18, 19], and they also accumulate in the microenvironment of HNSCC tumours, but their relationship to patient prognosis is not established [10, 20, 21].

To date, no single marker exists to identify naturally occurring Tregs (nTregs); however, they express high levels of the surface marker CD25 (α chain of IL2R) along with the forkhead box transcription factor (Foxp3) both of which are essential for nTreg maintenance, development and function [16]. In the current study, the presence of TILs, identified using single antibody immunohistochemistry (CD4, CD8, Foxp3), and immune cells in the peripheral circulation ($CD4^+$, $Foxp3^+$, $CD4^+CD25^{High}Foxp3^+$), determined using flow cytometry, of HNSCC patients were compared and related to HNSCC subsite. Finally, because the influence of the stroma (the tissue immediately surrounding the tumour cells) and its multiple cellular constituents (fibroblasts, glial, epithelial, fat, immune, vascular, smooth muscle) are increasingly thought to play a role in the development and progression of cancer [22–24], the specific location of the TILs in either the tumour nests or the stromal area of the resection specimen has been investigated.

Materials and methods

Patients

Subsequent to obtaining ethical approval (South Humber local research ethics committee; LREC-05/Q1105/55, HEY NHS Trust R0220), patients with newly presenting HNSCC were recruited ($n = 66$) onto the study between August 2008 and November 2010. Exclusion criteria included previous diagnosis and treatment for any other form of cancer, active autoimmune or co-existing infectious disease and previous radio- or chemotherapy. Tumour samples included 6 oral cavity (anterior tongue, floor of mouth, palate, lip), 20 oropharyngeal (tongue base, tonsil), 32 laryngeal, 3 hypopharyngeal, 1 parotid and 4 samples from unknown subsites (Table 1). Peripheral blood was also collected from 22 patients undergoing surgery for non-cancer related illnesses (age range 23–62; mean age 43).

Table 1 Clinicopathological features of HNSCC patients

Tumour site	*n*	Age	Sex		*T* stage		Node status	
		Median (range)	F	M	*T1/T2*	*T3/T4*	*N0*	*N+*
Larynx	32	65 (41–92)	5	27	16	16	23	9[†]
Oropharynx	20	57 (30–67)	4	16	11	8[a]	4	16
Oral cavity	6	61 (51–69)	2	4	3	3	3	3
Hypopharynx	3	56 (50–63)	0	3	0	3	1	2
Parotid	1	85	0	1	1	0	1	0
Unknown	4	65 (57–67)**	1[b]	2	[c]		[b]	3
Total	66	62 (30–92)	12	53	31	30	32	33

[†] Significantly less nodal involvement in laryngeal tumours compared to other subsites

[a] One oropharyngeal tumour was of unknown *T* stage

[b] One unknown primary tumour was of unknown age, sex and nodal status

[c] All of the unknown primary tumours were of unknown *T* stage

Immunohistochemistry

Following ethical approval (Hull & East Riding Research Ethics Committee 10/H1304/8, HEY NHS Trust R0986), formalin fixed and paraffin-embedded sections of tumour tissue from 66 patients (Table 1) were cut into 5-μm sections and routinely mounted on to glass slides by Hull Royal Infirmary Pathology Department. Normal tonsil was used as a positive control for immunohistochemistry following standard procedures. The sections were dewaxed in three changes of Histoclear II (National Diagnostics, Hull, UK), before being rehydrated through graded alcohols. Endogenous peroxidase activity was blocked using 3 % hydrogen peroxide (v/v; Sigma-Aldrich, Dorset, UK) in methanol for 15 min. The sections were rinsed in tap water and boiled in a citrate-based antigen unmasking solution (pH6; Vector Laboratories, Peterborough, UK), using a pressure cooker, for 3 min. The sections were rinsed in Tris-buffered saline (TBS, pH7.6) and placed in a Sequenza^{TM} slide rack, before blocking of avidin and biotin binding sites as directed by the

manufacturer (avidin/biotin blocking kit Vector Laboratories). Immunostaining was then achieved using the Universal Vectastain® Elite kit (Vector Laboratories). Sections were incubated with a 100-fold dilution of normal horse serum in TBS for 20 min, followed by titrated concentrations of monoclonal mouse anti-human primary antibodies for 1 h at room temperature (Foxp3, clone 236A/E7, Abcam, Cambridge, UK, 1:200 dilution; CD4, clone 4B12 and CD8, clone 4B11, Novocastra™, Leica Biosystems, Newcastle, UK, 1:40 dilutions). Following washing with TBS, sections were incubated with biotinylated universal secondary antibody for 30 min, followed by a streptavidin/biotin detection system linked to horse radish peroxidase for a further 30 min. Positive staining was visualised using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) for 5 min. Subsequent to counterstaining with Harris Hematoxylin (Sigma-Aldrich), sections were dehydrated through increasing concentrations of alcohol and three changes of Histoclear II, before being mounted using Histomount (National Diagnostics). Negative controls were performed on tonsil tissue using equivalent concentrations of isotype control reagents.

Quantification of immunostaining

Positive immunostaining was identified in both the tumour-associated stroma and the tumour nests using a light microscope under 400× magnification. Photomicrographs of five representative fields from each tissue area (tumour and stroma) were taken and scored using Image J software by two independent assessors (VG, AM). The nuclear localisation of Foxp3 made positive cells in both areas clearly identifiable and enumerable (Fig. 1). Although the number of CD4 and CD8 cells within the tumour nest was quantifiable, the cytoplasmic location of both CD4 and CD8 made the numerous positive cells in the stroma difficult to count (Fig. 1); therefore, the following coded scoring system was applied: 0 = negative, 1 = 1–25 % positive, 2 = 26–50 % positive, 3 = 51–75 % positive, 4 = 76–100 % positive. In addition ratios of immunostaining were determined for Foxp3/CD4, Foxp3/CD8 and CD4/CD8 within both the tumour and the stromal areas.

Isolation of peripheral blood mononuclear cells (PBMC)

A 50-ml venous blood sample was taken from each patient into a heparin-coated syringe prior to surgery, and peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation, as described previously [25]. Isolated PBMC were counted using trypan blue exclusion, and the concentration of PBMC was adjusted to 2×10^7 cells/ml by re-suspension in an appropriate volume of freeze medium (foetal bovine serum plus 10 %[v/v] dimethyl sulphoxide) and cryo-preserved in 1 ml aliquots for subsequent phenotyping of circulating Treg cells.

Flow cytometry

Fluorescent-conjugated mouse anti-human monoclonal antibodies were all purchased from BD Biosciences (Oxford, UK) and following titration used for flow cytometric determination of Tregs: anti-CD4-PerCP (clone SK3), anti-CD25-APC (clone M-A251) and anti-Foxp3-AF488 (clone 259D/C7). Corresponding isotype controls and intracellular staining buffer were also purchased from BD Biosciences.

Following thawing, PBMC was washed twice in PBS-BSA-Azide (PBS pH 7.4; 0.1 % w/v bovine serum albumin; 10nM NaN_3; Sigma, Dorset, UK), and the number and viability of cells were assessed using trypan blue exclusion. PBMC samples (1×10^6 cells) were incubated with surface marker antibodies (CD4-PerCP, CD25-APC) for 30 min in the dark, before being washed in PBS buffer. Where necessary, the cells were fixed and permeabilised using buffers as directed (BD Biosciences) and incubated with the intracellular antibody Foxp3 for 30 min in the dark, before further washes and analysis by flow cytometry (FACS Calibur™ equipped with Cell Quest Pro V software; BD Biosciences).

Acquisition and analysis gates were restricted to the lymphocyte population identified by their forward (FSC) and side scatter (SSC) characteristics and between 1×10^4 and 1×10^5 lymphocytes were acquired (Fig. 2a). The lymphocyte population was used to create a dot plot of CD4 versus CD25, and the $CD4^+CD25^{High}$ population was gated with reference to both the negative control and to the negative CD4 population (Fig. 2b, c). A histogram was plotted using this gated population for the FL-1 channel to determine the percentage of $CD4^+CD25^{High}Foxp3^+$ cells (Fig. 2e). From these plots, values for the percentage of $CD4^+$, $Foxp3^+$ and $CD4^+CD25^{High}Foxp3^+$ cells within the total lymphocyte population were determined along with the percentage of $CD4^+CD25^{High}$ cells which was positive for Foxp3.

Statistical analysis

The results were analysed using SPSS version 18 (SPSS Inc, Chicago, USA), and missing data were excluded from each analysis. The level of agreement between the immunohistochemical assessments of the two independent observers was determined using either the Bland–Altman method (continuous variables) or the Kappa measurement (coded variables). Associations between tumour site and *T* stage (early, *T*1/*T*2; late, *T*3/*T*4), nodal status (node negative[*N*0]; node positive [*N*+]), sex (M/F) or age ($\leq$60; >60) were determined using Fisher's exact test.

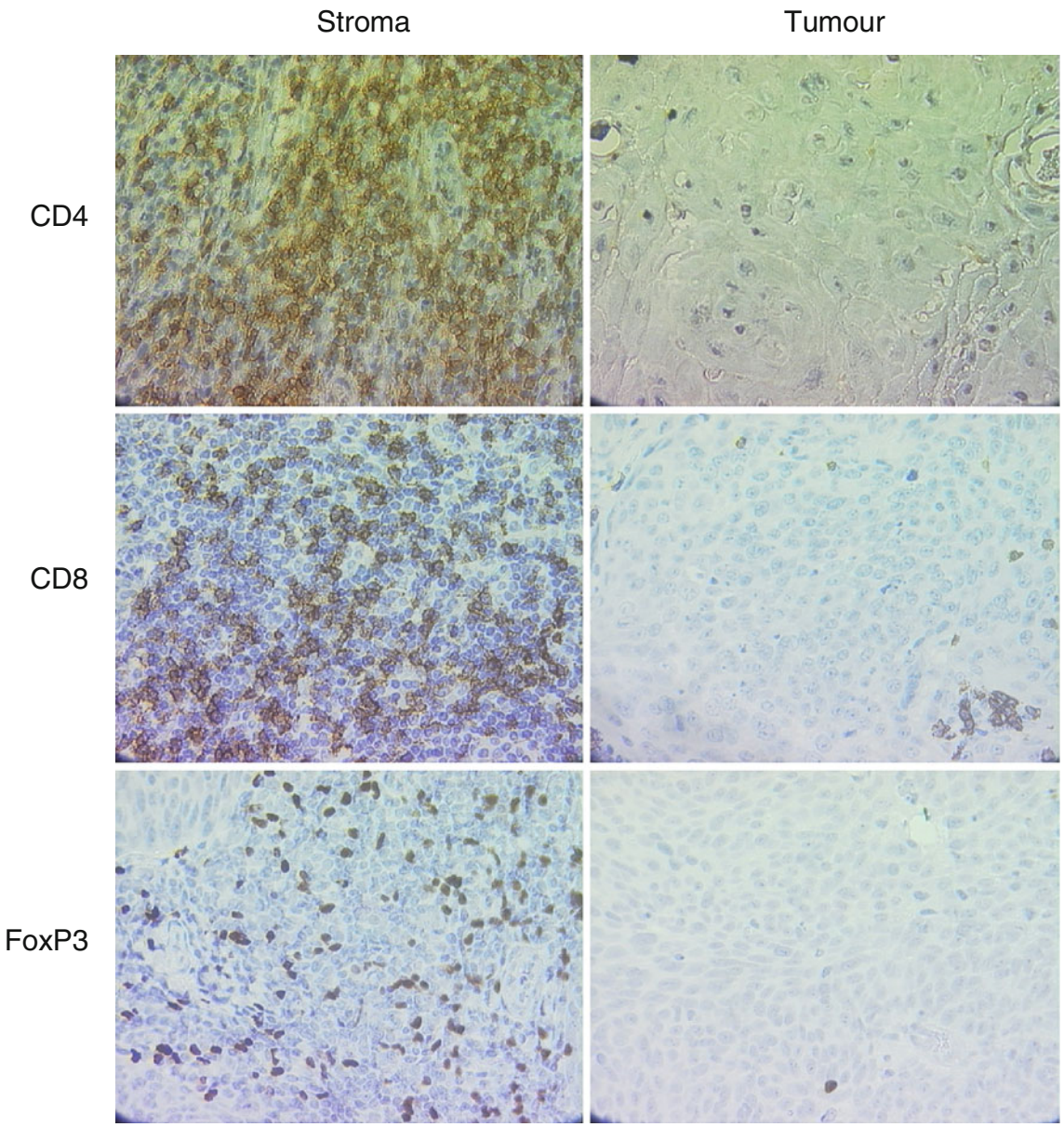

Fig. 1 Localisation of immune cells in head and neck tumour tissue following immunohistochemistry of paraffin-embedded tissue sections. Magnification ×400, unstained nuclei are identified by hematoxylin blue counterstain; immune cells are identified by cytoplasmic (CD4 and CD8) or nuclear (FoxP3) brown staining stromal, and tumour areas were sourced from the same paraffin section

Following determination that the majority of parameters under investigation were not normally distributed (D'Agostino & Pearson omnibus normality test), the relationship between immunohistochemical parameters or circulating Treg levels with site was analysed using the Kruskal–Wallis test and that with *T* stage, nodal status, sex and age were analysed with the Mann–Whitney *U* test for unrelated samples.

Relationships between the levels of immunohistochemical parameters in the stroma and those in the tumour were determined by simple regression using a univariate general linear model if the variables were continuous or with Spearman's correlation if one or more of the variables was categorical. The simple regression model was also used to compare the relationship between circulating immune cells and the immunohistochemical parameters in both the tumour and the stroma, using both individual and multiple immunohistochemical parameters for comparison with the circulating cells. The continuous variables were used as covariates or dependant factors, and the coded parameters (CD4 and CD8 stroma) were used as fixed factors in the model. To determine whether there was more staining in the stroma

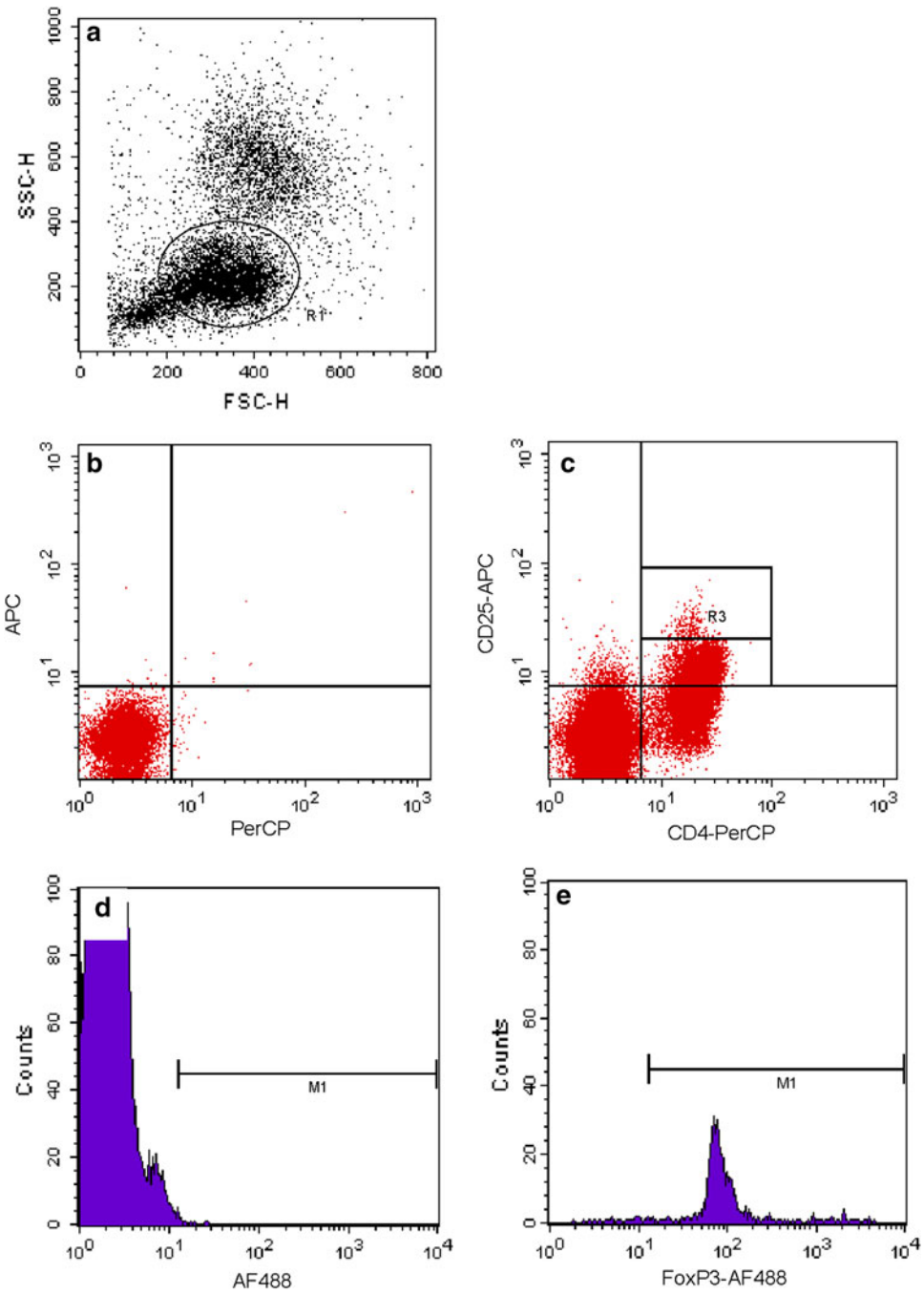

Fig. 2 Flow cytometry plots following acquisition of PBMC showing **a** the gated lymphocyte population [R1] isolated with reference to forward scatter [FSC-H] and side scatter [SSC-H] characteristics, **b** a dot plot of the gated lymphocytes labelled with PerCP and APC isotype control antibodies, **c** a dot plot of the gated lymphocytes labelled with anti-CD4-PerCP and anti-CD25-APC antibodies, showing the quadrant set on the isotype control and the gate [R3] depicting the $CD4^{+}CD25^{High}$ population, **d** a histogram of gated $CD4^{+}CD25^{High}$ cells [R3] labelled with AF488 isotype control antibody, the marker M1 encompasses <1 % of cells and **e** a histogram showing the proportion of $CD4^{+}CD25^{High}$[R3] cells labelled with the anti-$FoxP3^{+}$-AF488 antibody, M1 copied from the isotype control plot

compared with that in the tumour for the same parameter, Wilcoxon's signed rank test for related samples was used.

Results

Patient demographics

The most abundant subtypes of HNSCC tumour were the larynx group ($n = 32$) and the oropharynx tumours ($n = 20$; Table 1). For all tumour subsites, there was a greater incidence in males compared with females. There was no statistical difference in the distribution of early (*T*1/*T*2) or late (*T*3/*T*4) stage tumours between subsites; however, there was significantly less nodal involvement in the larynx group compared with other tumour subsites ($p = 0.001$). The median age of the whole group of HNSCC patients was 62 (range 30–92), with the larynx group containing significantly more patients over the age of 60 ($p = 0.003$).

Immunohistochemical staining of TILs

Immunohistochemical staining was counted and scored by two independent observers, and a Kappa value of 0.77 and 0.95 for $CD4^+$ and $CD8^+$ cells in the stroma, respectively, was obtained, and the Bland–Altman analysis for the continuous variables gave similarly good inter-observer relationships with the majority of values falling within the 95 % confidence intervals.

Significantly more staining was found for all three immune cell markers in the tumour-associated stroma compared with that in the tumour ($p < 0.05$), and regression analysis/Spearman's correlations identified significant positive relationships, in the whole patient cohort, between the CD4 in the tumour and the CD4 in the stroma ($p < 0.05$; Fig. 3a); the CD8 in the tumour and CD8 in the stroma ($p = 0.001$; Fig. 3b), and the relationship between Foxp3 in the tumour and Foxp3 in the stroma approached significance ($p = 0.056$; Fig. 3c).

Within the separate stroma and tumour areas, it was found that there was a positive relationship between CD4 and Foxp3 ($p < 0.05$) as well as between CD4 and CD8 ($p = 0.004$ and 0.038), and positive correlations between CD4 and Foxp3 as well as between CD4 and CD8 were also found across the tumour/stromal areas (CD4 stroma vs. Foxp3 tumour $p = 0.001$; Foxp3 stroma vs. CD4 tumour $p = 0.029$; CD4 stroma vs. CD4 tumour $p = 0.003$; CD4 tumour vs. CD8 stroma $p = 0.006$). Notably, no relationship was found between Foxp3 and CD8 in or between either the tumour or the stromal areas.

Dividing the sample group into individual subsites maintained the positive relationship between CD4 in the tumour and stroma in both the larynx ($p < 0.05$) and the oropharynx group ($p = 0.003$) and that between CD4 and

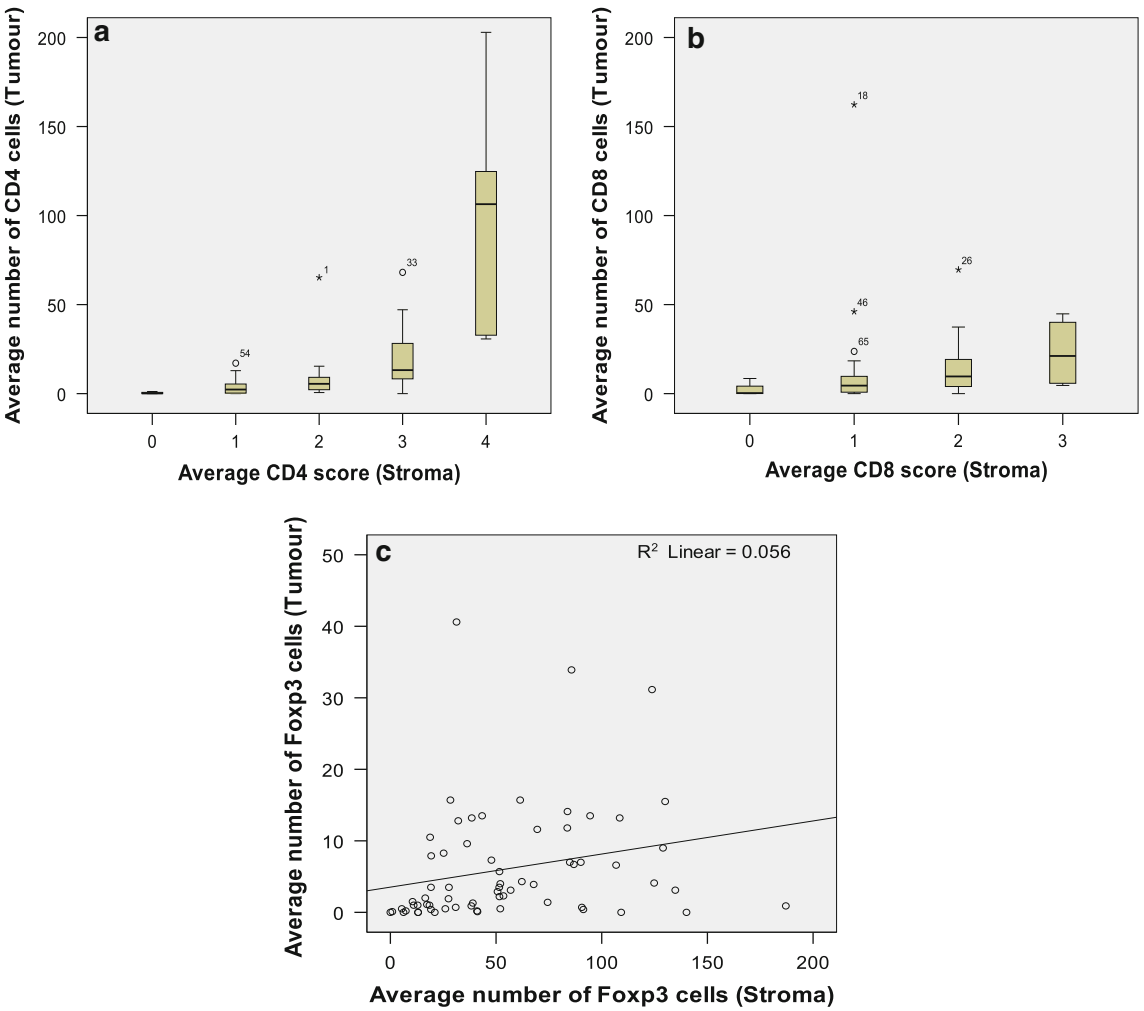

Fig. 3 Plots showing the relationship between immunohistochemical staining in the stroma and the tumour of all HNSCC samples for **a** CD4 ($p < 0.05$), **b** CD8 ($p = 0.001$) (Spearman's correlation) and **c** Foxp3 ($p = 0.056$) (univariate general linear model regression analysis). CD4 and CD8 stroma were coded variables 0–4 with 0 being no staining and 4 being 76–100 % staining. CD8 did not reach a score of 4. Outliers shown by *symbols* and numbers

Foxp3 in the stroma of laryngeal tumours ($p = 0.005$) and CD4 and CD8 in the tumour of laryngeal samples ($p = 0.003$).

The highest proportion of all studied immune cells was found in the stroma and the tumour of the oropharynx subset, and the difference between the oropharynx and the larynx was significant for Foxp3 and CD4 in the tumour and the CD4 and CD8 in the stroma ($p \leq 0.005$; Fig. 4). In addition, the difference between the CD4 in the stroma of the oropharynx group and the hypopharynx group was also significant. The positivity in the oropharynx group was higher than in the oral cavity group, which had similar levels to the larynx group (Fig. 4), but this difference was not significant probably due to the relatively small numbers of oral cavity samples ($n = 6$).

In both the oropharyngeal tumour and the stroma, the number of $CD4^+$ cells was greater than the number of $CD8^+$ cells. This was reflected when the ratios of Foxp3/CD8, Foxp3/CD4 and CD4/CD8 were considered; unsurprisingly, the ratio of CD4/CD8 in the tumour area was again significantly higher in the oropharyngeal tumours compared with the laryngeal tumours ($p = 0.045$; Fig. 4).

Flow cytometric determination of circulating lymphocytes

No significant differences were observed between tumour subsites for the percentage of total circulating $CD4^+$ cells, $Foxp3^+$ cells and Tregs ($CD4^+CD25^{High}Foxp3^+$) within the gated lymphocyte population, or for the percentage of gated $CD4^+CD25^{High}$ cells positive for Foxp3 (Kruskall–Wallis for independent samples; Table 2). In addition, no relationship was found between any of the circulating immune cells measured and the clinicopathological parameters of *T* stage, nodal status, age or sex (Man–Whitney *U*, unrelated samples) when the group was considered as a whole. However, when individual subsites were considered, significantly higher levels of the total number of lymphocytes positive for Foxp3 in the circulation of laryngeal patients with late stage tumours were observed (21.7 % ± 6.9) compared to those with early stage tumours (7.6 % ± 2.6; $p = 0.05$), and a significantly higher percentage of the total number of lymphocytes positive for CD4 in the circulation of patients with node negative oropharyngeal tumours (35.4 % ± 3.9) was found compared to node positive ones (21.2 % ± 2.1; $p = 0.014$).

Of the twenty-two healthy volunteer samples analysed, only sixteen had sufficient sample quality to provide a full set of data (Table 2). The values obtained from these samples were highly variable, and no significant differences were observed between healthy volunteers and HNSCC patients.

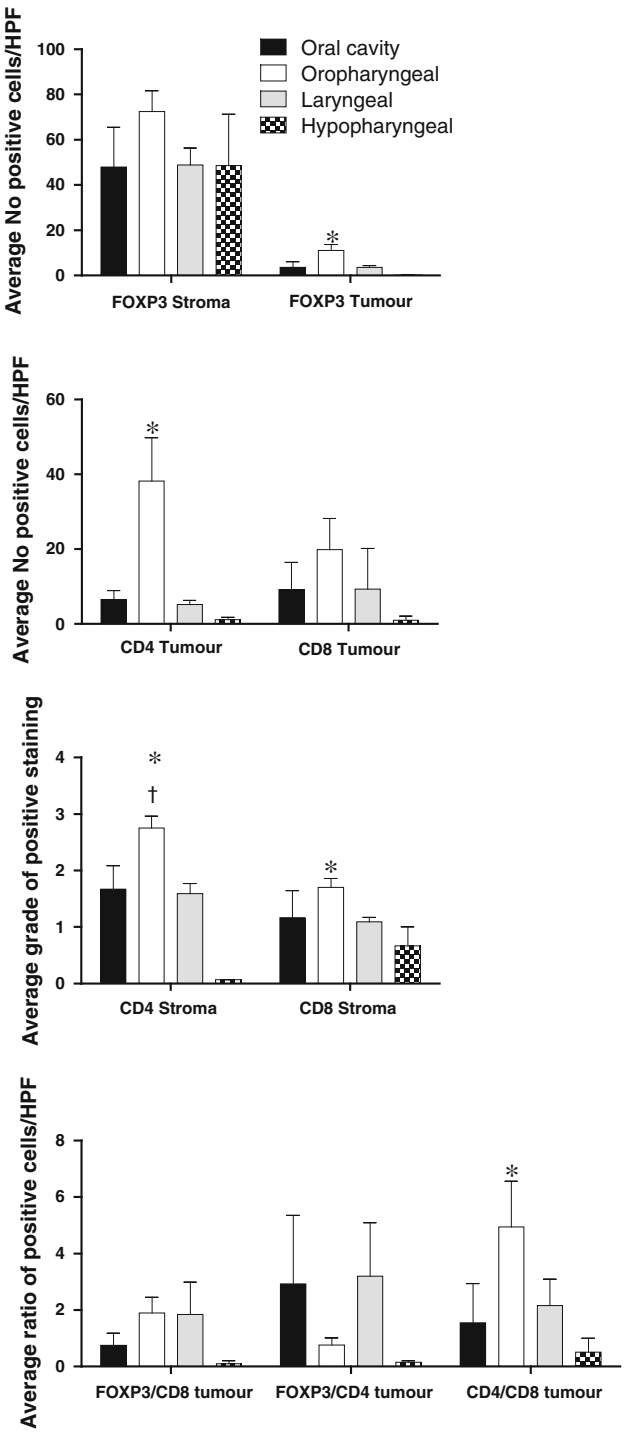

Fig. 4 Relationship of the immunohistological parameters CD4, CD8 and Foxp3 in both tumour and stromal areas with subsite of the tumour (Mean ± SEM). CD4 and CD8 in the stroma were scored from 0 to 4, whereas individual cells were counted for all the other parameters. Significance between * oropharyngeal and laryngeal and †oropharyngeal and hypopharyngeal groups ($p < 0.05$; Kruskall–Wallis test for independent samples). HPF = High powered fields (×400)

Table 2 Percentage of different immune cells in the circulation of patients with different forms of HNSCC

Cell type	Oral cavity ($n = 6$)	Oropharyngeal ($n = 20$)	Laryngeal ($n = 32$)	Hypopharyngeal ($n = 3$)	Healthy controls ($n = 16$)
[a]$CD4^+$	24.2 ± 3.0	24.1 ± 2.2	29.7 ± 2.1	27.0 ± 12.1	29.1 ± 9.57
[a]$Foxp3^+$	18.2 ± 8.2	9.7 ± 3.6	14.7 ± 3.8	22.0 ± 8.8	16.1 ± 19.3
[a]$CD4^+CD25^{High}Foxp3^+$	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.6
[b]$Foxp3^+(CD4^+CD25^{high})$	51.7 ± 14.2	45.0 ± 6.3	45.2 ± 5.0	62.9 ± 1.6	45.2 ± 25.6

[a] Percentage of cells within the gated lymphocyte population (Mean ± SD)

[b] Percentage of $CD4^+CD25^{High}$ cells which are positive for Foxp3 (Mean ± SD)

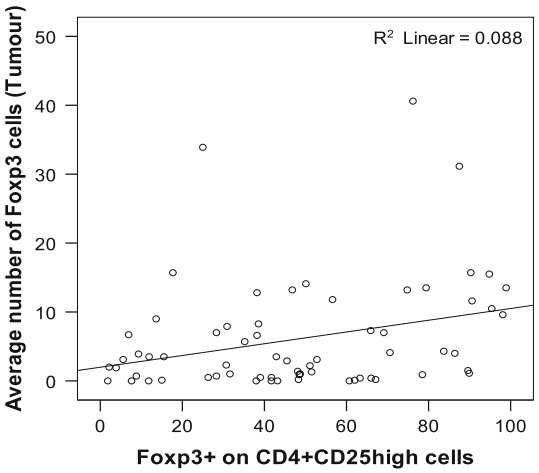

Fig. 5 Relationship between immunohistochemical staining of Foxp3 in the tumour microenvironment of HNSCC and the percentage of circulating $CD4^+CD25^{High}$ cells which were positive for Foxp3 in the same patient ($p = 0.016$). Significance calculated using univariate general linear model regression analysis

Relationship between circulating lymphocytes and TILs

When the whole HNSCC group was analysed, a significantly positive relationship ($p = 0.016$, Fig. 5) was found between immunohistochemical staining for Foxp3 in the tumour and the circulating levels of $CD4^+CD25^{High}$ cells positive for Foxp3. This relationship was less strong when Foxp3 in the stroma was considered in addition to that in the tumour ($p = 0.031$). There were no other significant relationships between the circulating immune cells and those in the tumour or the stroma.

Analysis of the individual subsites showed no significant relationships between the immunohistochemical parameters and the circulating immune cells in the oropharynx group. However, in the larynx group, Foxp3 staining in the tumour was significantly related to the percentage of circulating Foxp3 positive $CD4^+CD25^{High}$ cells ($p = 0.013$) and again this significance decreased when the staining in the stroma was included as a variable ($p = 0.025$). Other relationships observed in the larynx group which were not seen in the whole HNSCC group included a significant relationship between the number of Foxp3 positive cells in the tumour and the total percentage of circulating PBMC positive for Foxp3 ($p = 0.007$), and this significance was maintained when the Foxp3 staining in the stroma was included as a variable ($p = 0.006$).

Discussion

Tregs have been hypothesized to play a role in immune evasion initiated by the tumour [15, 16]. However, higher numbers of peripheral Tregs are not always related to worse prognosis [17, 19]. Therefore, the presence of intratumoural Tregs and the balance of these with T helper and T cytotoxic cells are more likely to be of relevance to immune evasion [26, 27].

Although commonly grouped together, HNSCC comprises malignancies which arise from functionally distinct subsites, whose aetiology, incidence and patient prognosis differ significantly [28]. Previous studies have investigated TILs in HNSCC [10, 20, 21, 29]; however, any differences in TILs between tumour subsites has not been clearly demonstrated. The current study encompassed sufficiently large cohorts of oropharyngeal ($n = 20$) and laryngeal tumours ($n = 32$) to allow comparisons of TILs between these subsites. The number of TILs (CD4, CD8, Foxp3) and the ratio of CD4/CD8 cells were found to be significantly higher in the microenvironment of the oropharynx tumours compared with that of the laryngeal cohort which may be explained by the different aetiology of the tumours; traditionally, the main risk factors for the development of HNSCC were smoking and alcohol consumption; however, more recently HPV infection has been shown increasingly to be a causative agent [30], especially for oropharyngeal tumours [31, 32]. Interestingly, although the incidence of HPV positive oropharyngeal tumours is increasing, especially in the younger male population [33], these patients have a better prognosis following treatment [2]. Various theories for the increased survival of these patients have been postulated and include the elevated presence of immune cells in response to the HPV antigens [34], which could be supported by the higher number of TILs found in the oropharyngeal tumours of the current study. However,

the HPV status of patients in the current cohort is unknown. In contrast, no differences in the circulating populations of Tregs were observed between subsites which is in agreement with Strauss et al. [35], again supporting the idea that the differences in immune cells may be influenced locally by the tumour.

The role of the tumour-associated stroma in the progression of malignancies is becoming increasingly apparent [22–24, 36] and may relate to the prognosis of the patient [37]. Consequently, the location of immune cells within the tumour microenvironment was also determined. Immune cells in the tumour were positively correlated to those in the tumour-associated stroma for all of the markers investigated, with the levels consistently higher in the stroma, suggesting that immune cells congregate in surrounding stromal tissue before migrating into the tumour nests, and/or or they interact with the stroma to exert their effects. Unsurprisingly, positive correlations were evident between the $CD4^+$ and $CD8^+$ cells, which usually exist in a 2:1 ratio in the periphery, and between $CD4^+$ and $Foxp3^+$ cells, because the majority of $Foxp3^+$ cells is also $CD4^+$. Although Foxp3 is expressed by $CD8^+$ Tregs which form a minor, less suppressive fraction of Tregs in the periphery [38] and by some activated T cells and tumour cells themselves [39, 40], no relationship was found between Foxp3 and CD8. This indicates that the Foxp3 found in the tumour microenvironment of HNSCC is associated with $CD4^+$ Tregs.

By using flow cytometry, the percentage of circulating $CD4^+CD25^{High}$ cells from HNSCC patients expressing Foxp3 in the current study was found to be substantial (45–63 %) although less than the >80 % determined by Strauss et al. [35, 41]. The reasons for this difference may lie with the slightly different gating strategies employed, in that the cut-off point for the $CD25^{High}$ population is determined differently and the fact that some of the patients from the Strauss studies had undergone previous therapy, which may affect the circulating immune cells. In addition, the larger cohort of patients included in the present study is likely to provide a more robust assessment of the immune parameters investigated ($n = 61$ vs. $n = 35$).

Although no differences in circulating lymphocyte populations were observed between subsites and clinicopathological parameters, there was a higher percentage of lymphocytes positive for Foxp3 in the peripheral circulation of patients with later stage laryngeal tumours compared to those with early stage tumours. If Treg numbers in advanced tumours also result in enhanced suppression of the anti-tumour immune response, this may be a reason why these tumours are able to progress. However, whether the tumour has progressed as a result of increased Treg activity or whether the advanced stage tumour stimulates peripheral Treg expansion is unknown. A significantly higher percentage of $CD4^+$ cells in the circulation of node negative oropharyngeal tumours compared with node positive ones was observed, which was not accompanied by increased $Foxp3^+$ cells, suggesting these cells are likely to be Thelper cells rather than Tregs. These Thelper cells may have been mobilised against tumour-associated antigens or HPV infection, which is a common risk factor for these particular tumours, and their correlation with lack of nodal spread could indicate that they are eliciting an effective immune response [34].

Tregs expressing chemokine receptors have been demonstrated to migrate towards chemokines such as CCL22 expressed in the tumour microenvironment [42, 43]. However, the relationship between the levels of circulating immune cells and those in the HNSCC malignancies has not been fully investigated. Gasparoto et al. [44] studied the phenotype and suppressive activity of Treg in both the periphery and the tumour in a small group of oropharyngeal patients ($n = 12$), and although their phenotypes were different, both circulating and infiltrating Tregs inhibited allogenic T cell proliferation. In addition, Strauss et al. [45] demonstrated that the proportion and suppressive activity of Tregs were greater in TILs compared with that in the peripheral circulation of HNSCC patients ($n = 15$); however, in both these studies, the patient cohort was relatively small, and no direct correlation between the levels of Treg in the periphery and that in the tumour was made. The number of $Foxp3^+$ cells in the tumour tissue from the whole patient cohort of the current study was positively correlated with the percentage of circulating $CD4^+CD25^{High}$ cells positive for Foxp3, and this relationship was maintained in the laryngeal cohort when considered alone. Also in the laryngeal patients, the total percentage of $Foxp3^+$ lymphocytes in the circulation was positively correlated with the number of $Foxp3^+$ cells in the tumour tissue, providing further evidence for the theory of Treg recruitment into the tumour from the peripheral circulation.

In conclusion, the degree of lymphocyte infiltration into HNSCC tumours appears to be subsite specific and is likely to be influenced by the differing microenvironments and the stage of the tumour. This observed difference in levels of TILs between subsites should be considered if targeted immunotherapy is to be used clinically, as the initial immune cell complement will have a major impact on the potential outcome.

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Conflict of interest The authors declare that they have no conflict of interest.

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