



# Frequency Over Function: Raised Levels of CD127<sup>low/-</sup> Regulatory T cells in the Tumour Microenvironment Compared with the Periphery of Head and Neck Cancer Patients

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## Abstract

**Objective:** Regulatory T cells (Tregs) are known to infiltrate the tumour microenvironment of many cancers, including head and neck malignancies, and are thought to contribute to the host's impaired anti-tumour immune response. However, their immunosuppressive function remains poorly understood within the tumour microenvironment and this study aimed to address this.

**Methods:** The frequency and suppressive capacity of two CD4<sup>+</sup>CD127<sup>low/-</sup> Treg populations, separated on the basis of different levels of CD25 expression (CD25<sup>inter</sup> and CD25<sup>high</sup>), from the tumour/node microenvironment and peripheral circulation of newly-presenting head and neck squamous cell carcinoma patients (n=19), were assessed using multicolour flow cytometry.

**Results:** The proportion of Tregs (CD4<sup>+</sup>CD25<sup>high/inter</sup>CD127<sup>low/-</sup>) in the tumour/node microenvironment was significantly elevated compared with the peripheral circulation ( $p < 0.001$ ) and similar percentages were present in both the primary tumour and metastatic lymph node. The percentage of suppression induced by Tregs isolated from tumour associated nodes on the proliferation of nodal effector T cells was similar to that of peripheral Tregs on peripheral effector T cells. However, when the suppressive activity of both nodal and peripheral Tregs was compared on the same peripheral effectors, peripheral Tregs suppressed proliferation to a greater extent.

**Conclusion:** This work shows that the recruitment and percentages of tumour infiltrating Tregs are key factors in modulating the immune environment of head and neck tumours.

**Keywords:** CD127<sup>low/-</sup>; HNSCC; Regulatory T cells; Tumour infiltrating lymphocytes

## Abbreviations:

CFSE: Carboxyfluorescein Diacetate Succinimidyl Ester; Foxp3: Forkhead box transcription factor p3; HNSCC: Head and Neck Squamous Cell Carcinoma; IL: Interleukin; PBMC: Peripheral Blood Mononuclear Cells; TIL: Tumour Infiltrating Lymphocytes; Tregs: Regulatory T cells

## Introduction

Head and neck cancer is the sixth most common type of cancer worldwide [1] and encompasses a number of epithelial malignancies that develop from anatomically defined locations within the upper aerodigestive tract. Although united by general location and histology, head and neck squamous cell carcinomas (HNSCC) arise from different primary sites and vary in presentation, pathogenesis and prognosis. Although recent reports have shown improved prognosis and survival for head and neck tumours positive for the human papillomavirus [2,3], and despite advances in surgical and adjuvant chemoradiotherapy treatment strategies, the overall survival rate of

HNSCC patients has not significantly improved over the last 30 years. Treatment failure is almost always associated with locoregional recurrence and/or the development of distant metastases.

HNSCC patients are widely recognised as having a suppressed immune system thought to be mediated through a variety of mechanisms including, the presence of functionally defective circulating and tumour infiltrating lymphocytes that have enhanced levels of apoptosis [4,5] and increased suppression induced by regulatory T cells (Tregs) [6,7]. In addition, the tumour mass itself also appears to promote an immunosuppressive environment [8,9]. Whilst investigations in the peripheral circulation of cancer patients offers an indication of the effects the tumour is exerting upon the host's systemic immune system, direct examination of the tumour microenvironment provides a detailed insight into the localised anti-tumour immune responses implemented by the host and the immune evasion strategies employed by the tumour.

The tumour microenvironment consists of a complex network of epithelial cells, stroma, chemical secretions, vascular and lymphatic vessels, and a plethora of infiltrating immune cells, whose actions range from promotion to inhibition of tumour growth and progression [10]. The infiltration of Tregs into the tumour is thought to contribute to the host's impaired anti-tumour response and

promote an immunosuppressive environment, with the ability to suppress the function of antigen presenting cells and effector T cells [11]. Although the infiltration of Tregs has been assessed by many research groups in several different cancer types, including HNSCC, there are conflicting reports over whether the presence of Tregs are associated with better locoregional control [12], negative prognosis [13], or of no relevance at all [14], and requires further clarification if prognostic or therapeutic value is to be gained.

Assessing Tregs in the tumour microenvironment as a prognostic indicator has to date focussed solely on their frequency and several publications, including HNSCC studies, have observed an increased prevalence of Tregs infiltrating malignant tissue compared with the peripheral circulation of cancer patients [7,15-18]. However, when assessing the contribution of the Treg population their suppressive activity should also be investigated, as both frequency and function may contribute to how effective a host's anti-tumour response will be in combating the growth and progression of the tumour. Although tumour infiltrating Tregs have been shown to possess suppressive activity [19-22], only limited investigations exist, in human cancers, which have compared the suppressive activity of the circulating and infiltrating Treg populations [7,16,23]. To our knowledge this is the first study to use the CD4, CD25 and CD127 markers to examine both the frequency and function of tumour infiltrating and peripheral Tregs from newly-presenting HNSCC patients.

## Materials and Methods

### Patients and healthy donors

Characteristics	Number
<b>HNSCC patients sex and age</b>	
Male	16
Female	3
Mean age (years)	60
Age range (years)	41-84
<b>Sample site</b>	
Primary tumour	4
Tumour associated lymph node	15
<b>Tumour stage</b>	
Early (T1 and T2)	4
Advanced (T3 and T4)	12
Unknown	3
<b>Primary tumour site</b>	
Laryngeal	9
Oropharyngeal	6
Hypopharyngeal	2
Unknown	2

**Table 1:** Clinicopathological features of HNSCC patients whose tumour samples were assessed for Treg frequency and function.

Following ethical and NHS Trust approval (Yorkshire and the Humber research ethics committee; REC-10/H1304/7, HEY NHS Trust-R0988) and having obtained written informed consent, newly-presenting HNSCC patients that had not received previous radio- or chemotherapy prior to sample collection were recruited onto the study (n=19; Table 1). Patients who had received diagnosis or treatment for any other form of cancer, had active autoimmune or co-existing infectious disease were excluded from the study. The tissue specimens included 4 samples from the primary tumour site and 15 samples from tumour associated lymph nodes; 15 of the 19 patients recruited provided both blood and tissue for analysis.

### Isolation of peripheral blood mononuclear cells

A 50 ml venous blood sample was taken into a heparin coated syringe from each HNSCC patient prior to surgery. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using lymphocyte separation medium (PAA, Yeovil, UK), as described previously [24]. Isolated PBMC were re-suspended in freeze medium [foetal bovine serum (FBS; Biosera, East Sussex, UK) containing 10% v/v dimethyl sulfoxide (Sigma, Dorset, UK)] for cryopreservation and subsequent use in the assessment of Treg frequency and function.

### Isolation of tumour infiltrating lymphocytes

Freshly isolated tumour specimens were collected during resection surgery and transported to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM; PAA), supplemented with 10% (v/v) FBS (Biosera), 0.4 mM L-glutamine, penicillin/streptomycin (final concentrations: 0.1 U/ml and 0.1 mg/ml, respectively; PAA) and fungizone (2.5 µg/ml; Invitrogen, Paisley, UK). Following removal of fat, blood and necrotic areas, the HNSCC samples were minced into 1 mm<sup>3</sup> pieces using scalpels, whilst covered in medium. After an antibiotic wash (PBS plus fungizone and penicillin/streptomycin as above), the tumour fragments were dissociated for 2 hours at 37°C in medium containing 0.02% (w/v) DNase (type I; Roche Diagnostics, Burgess Hill, UK) and 0.02% (w/v) collagenase (type IV; Sigma) whilst under constant rotation (MACSmix<sup>TM</sup> Tube Rotator; Miltenyi Biotec, Bisley, UK). Following incubation the dissociated tumour fragments were washed three times in medium and cultured in 25 cm<sup>3</sup> culture flasks overnight at 37°C to enable the majority of fibroblasts, epithelial cells and monocytes to adhere to the plastic. The medium, containing tumour infiltrating lymphocytes (TIL), was then collected and cells were isolated by centrifugation for immediate use in fluorescent activated cell sorting to assess the suppressive function of Tregs, or were cryopreserved until analysis at a later date.

### Treg and effector T cell characterisation and isolation by flow cytometry

Tregs and effector T cells within cryopreserved PBMC and freshly isolated/cryopreserved TIL were labelled using the human regulatory T cell sorting kit (BD Biosciences, Oxford, UK). Lymphocytes (1 × 10<sup>4</sup>) were acquired and gated using forward and side scatter characteristics and, following doublet discrimination, a CD4 vs CD25 dot plot was created to determine the different levels of CD25 expression (CD25<sup>inter</sup> and CD25<sup>high</sup>) [6]; CD4<sup>+</sup> T cells exceeding the level of CD25 expression by CD4<sup>+</sup> T cells were defined as the CD4<sup>+</sup>CD25<sup>high</sup> population. Using these boundaries and the level of CD127 expression by CD4<sup>+</sup> lymphocytes both Tregs (CD4<sup>+</sup>CD25<sup>inter</sup>CD127<sup>low/-</sup> and CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low/-</sup>) and

effector T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+/+</sup>) were identified and isolated [6]. The prevalence of Tregs was expressed as a percentage of the total CD4<sup>+</sup> population (mean ± SEM).

### Suppression assay

The suppressive activity of isolated Tregs on the proliferation of autologous effector T cells was determined by a co-culture carboxyfluorescein diacetate succinimidyl ester (CFSE) assay, as described previously [6]. Briefly, effector T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+/+</sup>) were stained with 5 μM of CFSE (Sigma) and the labelling quenched with ice cold culture medium. Following incubation on ice for 5 minutes and washes with pre-warmed medium, the labelled effector T cells were co-cultured with Tregs (CD4<sup>+</sup>CD25<sup>inter</sup>CD127<sup>low/-</sup> and CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low/-</sup>) in 200 μl of culture medium at various ratios (range Treg:effector T cells; 1:1–1:10). Where possible the CFSE assay was performed with 5 × 10<sup>4</sup> effector cells cultured in each well of a 96 well round bottomed plate, however, when insufficient cells were isolated, the number of effector cells plated was successfully scaled down to 1 × 10<sup>4</sup>/well. Lymphocyte stimulation was provided by Human T-Activator CD3/CD28 dynabeads (Invitrogen) at a cell:bead ratio of 1:3 and 100 U/ml recombinant human IL-2 (AbD Serotec, Kidlington, UK).

Following four days of co-culture, the cells were harvested and the proliferation of the CFSE labelled effector T cells was determined using flow cytometry. All CFSE data was analysed using ModFit LT™ software (Verity Software House, Topsham, USA) to calculate the proliferation index. The percentages of suppression were determined based on the proliferation index for effector cells cultured alone (100% proliferation, 0% suppression) compared with the proliferation index of effector cells co-cultured with Tregs.

### Statistical analysis

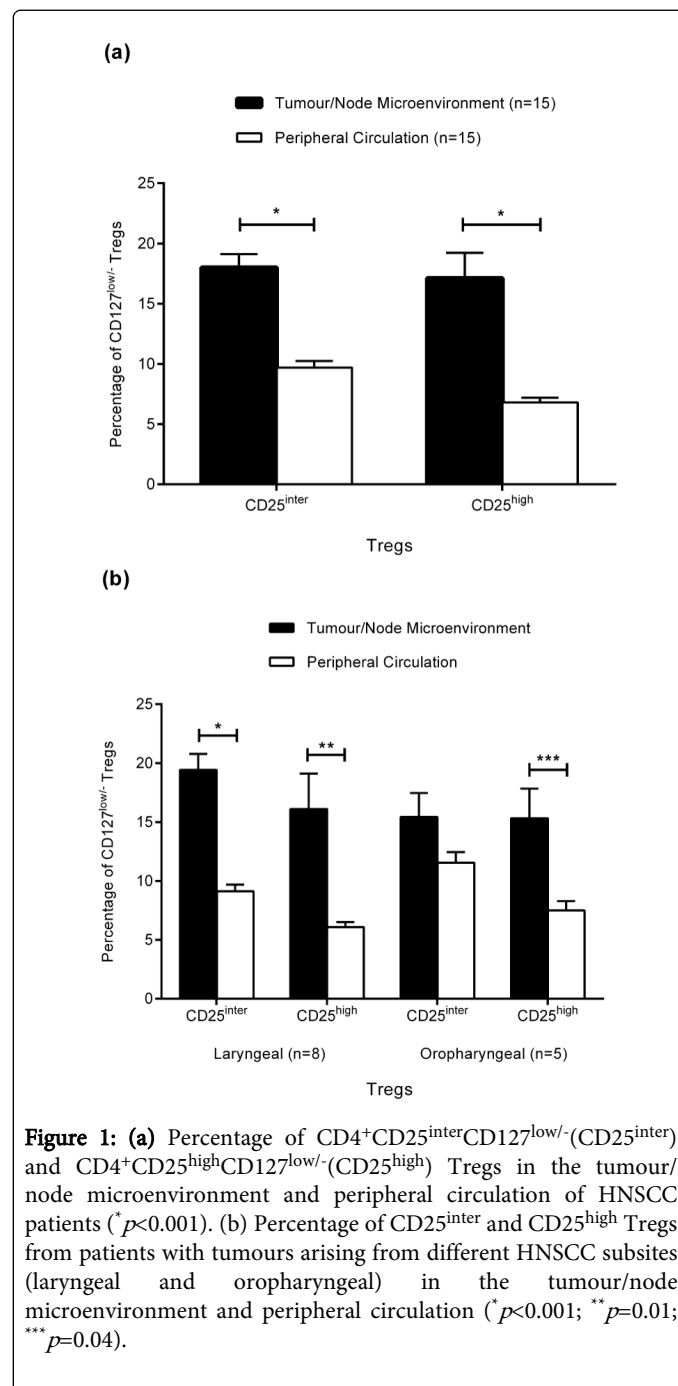
Statistical analysis was performed using SPSS version 19. The normality of the data was assessed by the Shapiro-Wilk test. Differences between independent data sets, with normal distribution, were analysed using the Student's unpaired T test with the assumption of equal variance assessed by the Levene's test. The Mann-Whitney U test was used for data sets without normal distribution. Differences between related data sets were analysed using the Student's paired T test and the Wilcoxon Signed Rank test for data sets normally or not normally distributed respectively. Values were considered significant when *p*<0.05.

## Results

### Prevalence of Tregs in the tumour microenvironment and peripheral circulation of HNSCC patients

To compare the prevalence of Tregs between the tumour microenvironment and the peripheral circulation, tumour samples from both the primary tumour site and tumour associated lymph nodes were analysed alongside the patient's corresponding PBMC sample. The proportion of CD4<sup>+</sup> lymphocytes with the CD127<sup>low/-</sup> Treg phenotype, irrespective of the level of CD25 expression, was significantly elevated in the tumour/node microenvironment compared with that in the peripheral circulation (Figure 1a). This significant increase in the tumour/node microenvironment was maintained in the patients with laryngeal tumours when the patient cohort was divided by tumour subsite (Figure 1b), but only reached

significance for the CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low/-</sup> (termed CD25<sup>high</sup>) Tregs in patients with oropharyngeal cancers (Figure 1b). The trend was maintained for patients with cancer of the hypopharynx however, due to the low number of samples (n=2) this subsite was not statistically assessed.



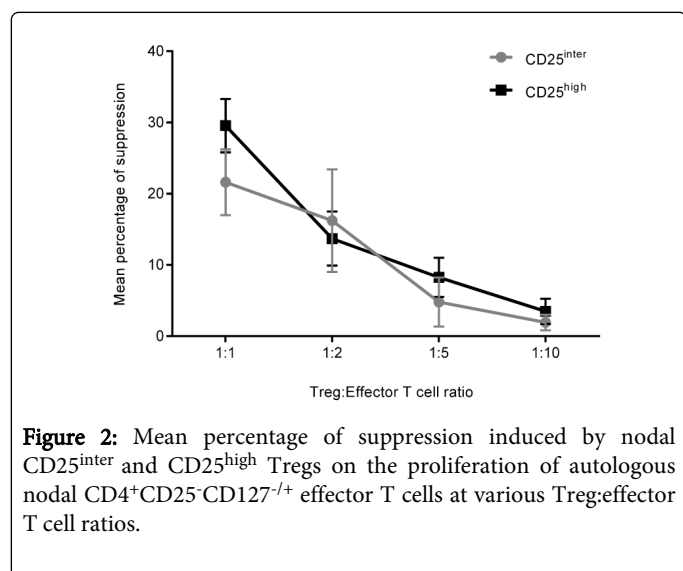
There was no difference in the proportion of CD4<sup>+</sup>CD25<sup>inter</sup>CD127<sup>low/-</sup> (termed CD25<sup>inter</sup>) and CD25<sup>high</sup> Tregs infiltrating the primary tumour tissue (n=4) compared with the tumour associated lymph nodes (n=15; Table 2), and no significant difference in the percentages of infiltrating CD25<sup>inter</sup> and CD25<sup>high</sup> populations was observed between the laryngeal (n=9) and oropharyngeal tumours (n=6).

Treg phenotype	Primary tumour tissue (n=4)	Tumour associated lymph node (n=15)	p value
CD25 <sup>inter</sup>	18.7 ± 1.7	16.9 ± 1.2	0.97
CD25 <sup>high</sup>	21.2 ± 7.1	19.1 ± 2.0	0.65
Mean percentage ± SEM			

**Table 2:** Frequency of Tregs in the tumour microenvironment of HNSCC patients.

### Suppressive activity of CD127<sup>low/-</sup> Tregs in the tumour microenvironment of HNSCC patients

Previous results have shown that the level of suppression induced by peripheral CD25<sup>inter</sup> and CD25<sup>high</sup> Tregs on peripheral effector T cell proliferation is ratio dependent [6]. The same was true in the current study when Tregs and effector T cells, both isolated from the tumour associated nodes, were incubated together at various ratios (Figure 2). Due to the size of the primary tumour biopsies it was only possible to isolate sufficient Tregs to establish a 1:1 Treg:effector T cell ratio. This condition was used for statistical analysis of differences between various cell populations and clinicopathological parameters. When the suppressive activity of Tregs isolated from tumour associated lymph nodes was related to tumour subsite, no significant differences in the level of suppression were observed between the laryngeal and oropharyngeal cohorts, irrespective of the Treg population being analysed (data not shown). Relationships with other clinical parameters (tumour stage, nodal status) were not examined as the majority of samples were late stage and node positive.



**Figure 2:** Mean percentage of suppression induced by nodal CD25<sup>inter</sup> and CD25<sup>high</sup> Tregs on the proliferation of autologous nodal CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>-/+</sup> effector T cells at various Treg:effector T cell ratios.

### Suppressive activity of CD127<sup>low/-</sup> Tregs in the tumour microenvironment compared with Tregs from the peripheral circulation of HNSCC patients

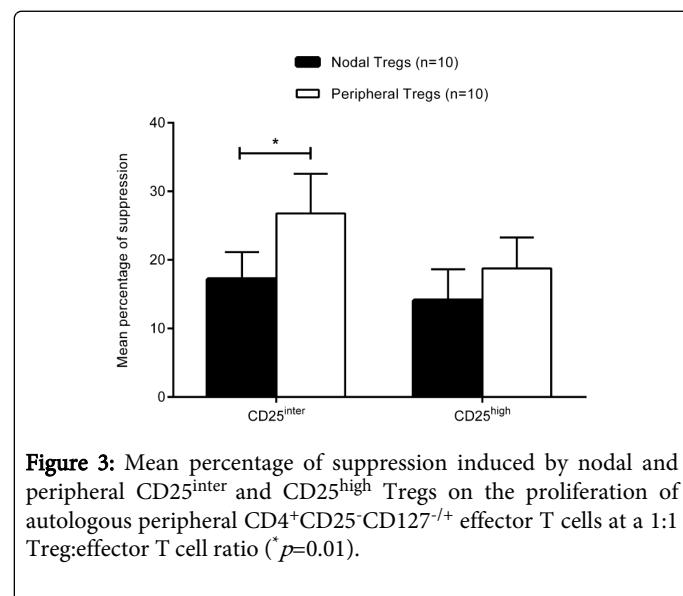
No difference was observed in the basal proliferation index of effector T cells isolated from the tissue microenvironment (6.1 ± 0.6) compared with that of those from the peripheral circulation (6.8 ± 0.6) meaning that direct comparisons could be made.

Both CD25<sup>inter</sup> and CD25<sup>high</sup> Tregs isolated from tumour associated nodes induced similar levels of suppression on autologous nodal effector T cells (24.3 ± 5.0 and 27.1 ± 4.0%, respectively) when compared with peripheral Tregs on peripheral effector T cells from the same patients (26.8 ± 5.8 and 18.8 ± 4.5%, respectively). Similar levels of suppression were also observed by the Tregs obtained from the primary tumour samples on their tumour effector counterparts, however, due to the low number of samples (n=2) the data could not be statistically assessed.

To determine whether the close proximity of the tumour microenvironment influences the susceptibility of the effector T cell population to Treg induced suppression, Tregs isolated from the tumour associated lymph nodes were co-cultured with autologous effector T cells isolated from both the tumour infiltrated node and the peripheral circulation. Irrespective of the Treg population analysed (CD25<sup>inter</sup> and CD25<sup>high</sup>) the co-culture of nodal Tregs with nodal effector T cells produced higher percentages of suppression compared with the incubation of nodal Tregs with peripheral effectors, however these differences did not quite reach significance (Table 3).

Nodal population	Treg	Co-culture with nodal effector T cells (n=10)	Co-culture with peripheral effector T cells (n=10)	p value
CD25 <sup>inter</sup>		24.3 ± 5.0	17.2 ± 4.0	0.15
CD25 <sup>high</sup>		27.1 ± 4.0	14.1 ± 4.6	0.09
Mean percentage ± SEM				

**Table 3:** Comparing the percentage of suppression induced by nodal Tregs on the proliferation of autologous nodal and peripheral effector T cells in HNSCC patients.



**Figure 3:** Mean percentage of suppression induced by nodal and peripheral CD25<sup>inter</sup> and CD25<sup>high</sup> Tregs on the proliferation of autologous peripheral CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>-/+</sup> effector T cells at a 1:1 Treg:effector T cell ratio (\*p=0.01).

To determine the suppressive function of Tregs isolated from tumour associated lymph nodes and those from the peripheral circulation, Tregs from the two different environments were each co-cultured with autologous peripheral effector T cells. It was demonstrated that, regardless of the level of CD25 expression, the percentages of suppression induced by peripheral Tregs was always greater than nodal Tregs. This difference reached significance for the



CD25<sup>inter</sup> Treg population (Figure 3). Due to insufficient immune cell numbers being available from the biopsies the suppressive function of **nodal** and **peripheral** Tregs on the proliferation of tumour infiltrated lymph **node** effector T cells could not be determined.

## Discussion

Tumour infiltrating Tregs have been identified in numerous cancer types, however their role in tumour immunity and their use as a prognostic determinant remains to be clarified [10,12-14]. The aim of the current study was to understand better the Treg population infiltrating the head and neck tumour microenvironment and the influence of the tumour on these T cells, by assessing their frequency and suppressive activity in comparison to those found in the periphery. To the authors knowledge this is the first study to examine both the frequency and function of CD127<sup>low/-</sup> Tregs infiltrating the tumour microenvironment of newly-presenting HNSCC patients. In addition the suppressive activity of Tregs, of any phenotype, from tumour associated lymph nodes has not previously been assessed in HNSCC.

The proportion of lymphocytes which displayed the Treg phenotype (CD25<sup>inter</sup> or CD25<sup>high</sup>) was similar in both the tumour infiltrated lymph nodes and the primary tumour of HNSCC patients, and no significant difference was found between subsites. This is in agreement with Pretscher et al. who reported similar frequencies of Foxp3<sup>+</sup> (forkhead box transcription factor p3) Tregs infiltrating the primary tumour and metastatic lymph nodes of HNSCC patients using immunohistochemistry, implying that the metastasised tumour recruits Tregs in a similar manner to the primary tumour [25].

Irrespective of whether the Tregs expressed high or intermediate levels of CD25, their frequency was significantly elevated in the tumour/node microenvironment of HNSCC patients compared with that in the peripheral circulation of the same patient. When studying subsites, the laryngeal cohort showed this trend for both CD25<sup>inter</sup> and CD25<sup>high</sup> Tregs, whereas in samples from oropharyngeal patients only the CD25<sup>high</sup> tumour Tregs remained statistically raised compared with those in the peripheral circulation. Similar studies in gastric and hepatocellular cancer patients also found significantly elevated proportions of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup>CD49d<sup>-</sup> Tregs respectively, in TIL compared with the peripheral circulation [18,26]. In head and neck cancer patients Strauss and colleagues also reported a significantly higher level of CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the tumour microenvironment (n=15) compared with the peripheral circulation (n=14) of patients, despite only five of the fifteen patients providing matched tumour and PBMC samples [7]. The matching of the tumour and peripheral samples used in the current study helped to minimise any potential influence of clinicopathological features.

The significant increase of Tregs in TIL compared with that in the peripheral circulation has also been reported in several other cancer types [15-17], and suggests that the tumour may be recruiting or expanding the suppressive Treg population, both mechanisms having been demonstrated in murine and human studies, along with the induction of Tregs from naïve precursors [27-30]. One attractive theory is that the elevated frequency of Tregs observed in tumour associated lymph nodes could arise from the expansion of lymph node resident Tregs [31]. However, a significant correlation between the degree of CCL22 and/or CCL17 positive cells and the frequency of Tregs in the tumour microenvironment of breast, oesophageal and

gastric cancer patients has been reported [30,32,33], supporting the evidence that chemokines are involved in the recruitment of the Treg population to the primary tumour [19,32,33].

In addition to the frequency of Tregs their level of suppressive activity is likely to contribute to overall immune suppression. During the quest to find a unique marker to identify Tregs, it has been reported that those expressing high levels of the IL-2 receptor (CD4<sup>+</sup>CD25<sup>high</sup>) had the capacity to inhibit effector T cell proliferation compared with T cells expressing intermediate/low levels of CD25 [34]. The current study demonstrated that CD127<sup>low/-</sup> Tregs, with both intermediate and high levels of CD25 expression, isolated from either tumour associated lymph nodes or the primary tumour site of HNSCC patients had an equivalent ability to suppress the proliferation of the classic CD4<sup>+</sup>CD25<sup>-</sup> effector T cell population, frequently used by other research groups [7,16,21].

No significant differences were observed between the level of suppression induced by nodal infiltrating Tregs and peripheral Tregs on the proliferation of effector T cell populations derived from the same compartment. This is in agreement with Chi et al. who found no significant differences between the regulatory function of Tregs isolated from the PBMC (n=10) and TIL (n=10) of bladder carcinoma patients when co-cultured with their autologous effector T cells at a 1:1 ratio [16].

Although it was hypothesised that Tregs isolated from the tumour microenvironment would induce a significantly greater level of suppression as a result of direct influences from the tumour to promote mechanisms to thwart the host's anti-tumour immune response, the current study suggests that in fact the peripheral Tregs (particularly those with the CD25<sup>inter</sup> phenotype) had the greater suppressive activity than nodal Tregs when the level of suppression was measured on autologous peripheral effector T cells. This is in contrast to a recent study by Jie et al. who demonstrated, in only a single patient, that peripheral and tumour infiltrating Tregs (CD4<sup>+</sup>CD25<sup>+</sup>) induced similar levels of suppression on effector T cell proliferation when incubated at a 1:1 ratio, and in a second patient the suppressive activity of the tumour derived Tregs was greater [23].

The fact that there was a trend towards a higher level of suppression of the nodal effectors by the nodal Tregs, as compared with their suppression induced on peripheral effectors would suggest that the tumour microenvironment causes the infiltrating effector T cells to become more susceptible to the actions of the tumour Tregs. There was no significant difference between the proliferation index of effector cells from the tumour and those from the periphery and so this was a real observed effect.

In summary, Tregs are significantly elevated in the tumour microenvironment of newly-presenting HNSCC patients compared with their peripheral circulation, regardless of primary tumour site. For the first time CD127<sup>low/-</sup> Tregs have been identified and isolated from the TIL of head and neck cancer patients and it has been demonstrated that the level of suppression induced by tumour/node infiltrating Tregs and peripheral Tregs on the proliferation of their effector T cell populations from the same compartment was similar. However, when the suppressive activity of both peripheral and nodal Tregs was studied on peripheral effector T cells, infiltrating Tregs were observed to be less suppressive compared with peripheral Tregs despite their close proximity to the suppressive tumour microenvironment. However, it is likely that there is an overall greater level of suppression induced by Tregs in the tumour

microenvironment compared with the peripheral circulation due to both the elevation of the proportion of Tregs infiltrating the tumour and other cytokine influences acting directly on T effector cells [35].

This study together with other recent work has highlighted the complexity of Treg function. Thus for research in this area to make a significant impact on patient outcome, well powered investigations, using precisely defined cell populations are needed.

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SD and VLG performed the experiments and drafted the manuscript, NDS provided both clinical samples and advice, VLG and JG designed the study; all authors reviewed and approved the final manuscript. SD was supported by a University of Hull studentship. We would like to thank Mr Jose and other members of the head and neck surgical team in Hull for consenting the patients and collection of peripheral blood samples.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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