Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer

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Background: We aimed to investigate the prognostic value of tumour-infiltrating lymphocytes' (TILs) expression in pretreatment specimens from patients with head and neck squamous cell carcinoma (HNSCC) treated with definitive chemoradiotherapy (CRT).

Methods: The prevalence of CD3 +, CD8 +, CD4 + and FOXP3 + TILs was assessed using immunohistochemistry in tumour tissue obtained from 101 patients before CRT and was correlated with clinicopathological characteristics as well as local failure-free (LFFS), distant metastases free (DMFS), progression-free (PFS) and overall survival (OS). Survival curves were measured using the Kaplan–Meier method, and differences in survival between the groups were estimated using the log-rank test. Prognostic effects of TIL subset density were determined using the Cox regression analysis.

Results: With a mean follow-up of 25 months (range, 2.3–63 months), OS at 2 years was 57.4% for the entire cohort. Patients with high immunohistochemical CD3 and CD8 expression had significantly increased OS (P = 0.024 and P = 0.028), PFS (P = 0.044 and P = 0.047) and DMFS (P = 0.021 and P = 0.026) but not LFFS (P = 0.90 and P = 0.104) in multivariate analysis that included predictive clinicopathologic factors, such as age, sex, T-stage, N-stage, tumour grading and localisation. Neither CD4 nor FOXP3 expression showed significance for the clinical outcome. The lower N-stage was associated with improved OS in the multivariate analysis (P = 0.049).

Conclusion: The positive correlation between a high number of infiltrating CD3 + and CD8 + cells and clinical outcome indicates that TILs may have a beneficial role in HNSCC patients and may serve as a biomarker to identify patients likely to benefit from definitive CRT.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world in developed countries (Argiris et al., 2008). Despite the improvement in local control and survival with the use of combined chemoradiotherapy (CRT), locoregional recurrence is encountered in ~ 30–40% of patients and distant metastases develop in 20–30% of HNSCC (Argiris et al., 2008). Locoregional and distal recurrence after definitive CRT constitute a major cause of morbidity and mortality in patients with HNSCC and has stimulated substantial efforts in identifying biological markers that predict patients at risk for disease recurrence (Begg, 2012).

The immune response to tumours is complex, involves the interaction of several cell types of the adaptive and the innate immune systems and has an important role in the progression of a variety of solid tumours (Bhurdwaj, 2007; Lesterhuis et al., 2011). The immune system promotes the elimination of tumour cells and control of tumour growth; however, cancers are characterised by a highly suppressive tumour microenvironment that hinders T-cell

Keywords: tumour-infiltrating lymphocytes; radiotherapy; head and neck cancer; prognostic value

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function (Bhardwaj, 2007). The presence of tumour-infiltrating lymphocytes (TILs), such as CD3+ and CD8+ cells, has been associated with improved outcome in patients with cancer (Gooden et al., 2011). Indeed, studies have revealed that a pre-existing immunologic response might enhance the effects of chemotherapy and radiotherapy (Grabenbauer et al., 2006; Kawai et al., 2008; Denkert et al., 2010).

Human papillomaviruses (HPV), mainly type 16, leads to tumorigenesis and is encountered in ~20–25% of the patients with HNSCC (Chaturvedi et al., 2011). p16INK4 is a marker of HPV that expresses E7 mRNA. HPV-positive patients present distinct clinicopathological features and are more radio- and chemosensitive compared with HPV-negative patients, possibly because of the presence of wild-type TP53 (Fakhry et al., 2008; Lassen et al., 2009; Ang et al., 2010; Rischin et al., 2010). Recent studies have examined the role of the immune system in HPV-associated HNSCC. One study demonstrated an increased T-cell response against the E7 oncoprotein epitope in the blood of HPV-positive patients, whereas another work revealed an increased T-cell proliferation in response to HPV E6 and E7 oncoproteins (Bontkes et al., 2000). Some studies have shown that strong CD8 expression is associated with better prognosis in HPV-positive patients (Nasman et al., 2012; Nordfors et al., 2013) but others have failed to reproduce this finding (Kong et al., 2009; Wansom et al., 2012).

Although previous reports have examined the role of TILs in HNSCC, the majority of the studies were characterised by small sample size and/or use of heterogeneous treatment schedules (Ginos et al., 2004; Le et al., 2005; Badoual et al., 2006). In the present work, we aimed to investigate the potential biologic importance of TILs in patients with HNSCC treated with primary CRT. We examined pretherapeutic core biopsy samples from a total of 101 patients with HNSCC treated with definitive CRT and investigated the protein expression of CD3+, CD8+, CD4+ and FOXP3+ TILs in tumour specimens. The correlation with clinical parameters and patient outcome was calculated in order to determine the potential prognostic role of TIL. Finally, we investigated the prognostic significance of the HPV surrogate p16INK4 (further denoted as HPV16) and also of TILs according to the HPV16.

PATIENTS AND METHODS

Patients. Between 2007 and 2010, a total of 101 patients with histologically proven advanced HNSCC were treated with definitive CRT at the Department of Radiotherapy and Oncology, University Hospital Frankfurt am Main. Pretreatment evaluation consisted of physical examination, pretherapeutic biopsy, CT/MRI scanning, chest X-ray, serum chemistry and complete blood count in all patients. Patients were staged according to the system adopted by the Union International Contre le Cancer (UICC) and the American Joint Committee on Cancer. Patients were included in this study according to the following criteria: histopathological confirmation of HNSCC; age between 18 and 75 years; sufficient haematological, liver and renal functions; absence of distant metastases and second cancer; curative intent.

Treatment protocol and follow-up. All patients received a 3D-conformal RT planning and immobilisation using thermoplastic masks. In addition, CT and MRI scans were performed for target volume delineation. All patients prophylactically received a feeding tube before the initiation of CRT. Patients were treated with definitive CRT using ≥6-MeV energy photon beams that were administered once daily with standard fractionation of 1.8–2 Gy to a total dose of 70–72 Gy. During the second half of the radiotherapy (after 30 Gy in 2 Gy fractions was reached), a hyperfractionated accelerated fractionation schedule (1.4–1.5 Gy twice daily) was applied. Four patients received a different RT fractionation schedule. Two cycles of chemotherapy were applied on days 1–5, and 29–33 consisting of 5-FU (600 mg·m⁻²·per day) as a continuous 120-h intravenous infusion and Cisplatin (20 mg·m⁻²·per day) as short intravenous infusion. In patients with poor kidney function (<60 ml·h⁻¹·creatinine clearance), carboplatin or mitomycin were administered instead of cisplatin. Chemotherapy dose was reduced during the second cycle in patients who developed leucopenia (leucocyte count <3×10⁹·l⁻¹) and/or thrombocytopenia (platelet count<100 000·nl⁻¹). All patient cases were presented at the Head and Neck Cancer interdisciplinary in-house tumour board. Definitive CRT was applied in the following cases: (1) patients with functionally inoperable tumours (≥T3 stage); (2) patients considered inoperable because of poor general health status; and (3) patients who have electively opted for CRT instead of surgery. Follow-up examinations were first performed 6 weeks after the completion of CRT and thereafter at 3-month intervals using clinical examination, endoscopy and CT/MRI imaging. A panendoscopy with biopsy was performed in patients that presented suspicious and/or inconclusive radiologic and clinical findings in the follow-up examination. Patient characteristics are shown in Supplementary Table 1.

Immunohistochemical evaluation of CD3, CD8, CD4 and FOXP3. Formalin-fixed, paraffin-embedded (FFPE) blocks of all patients included in the study were obtained from the Pathology Department, University Hospital Frankfurt archives. Immunohistochemical staining of CD3, CD8, CD4 and FOXP3 was performed with a horseradish-peroxidase technique using a DAKO Autostainer Link 48 (DAKO, Hamburg, Germany) at the Pathology Department. Antigen retrieval was carried out by the pretreatment of microscope slides (Star Frost, Engelbrecht, Germany) with an Epitope Retrieval Solution (Trilog, Cell Marque, Rocklin, CA, USA) for 30 min. Following that, staining was conducted with standardised Dako EnVision FLEX Peroxidase Blocking reagent (K800, DAKO) and polyclonal antibodies for CD3, CD8, CD4 (dilution 1:50; Dako) and FOXP3 (dilution 1:100; Sigma, Munich, Germany) following incubation for 120 min at room temperature. Next, dextran polymer-conjugated horseradish-peroxidase and 3,3′-diaminobenzidine (DAB) chromogen were used for visualisation followed by counterstaining with hematoxylin solution (Gill 3, Sigma). Negative control slides in the absence of primary antibodies were included for each staining.

To determine HPV status, immunohistochemical staining of p16INK4a protein (HPV16) was performed on cancer paraffin-embedded tissue using a CINtec histology Kit (Roche, Heidelberg, Germany) according to the manufacturer’s recommendations using a DAKO Autostainer Link 48 (DAKO).

Immunohistochemistry scoring. TIL scoring of tumours was performed semiquantitatively by measuring the densities of CD3+, CD8+, CD4+ and FOXP3+ cells as previously described (Dahlin et al., 2011): (1) no, or sporadic cells; (2) moderate numbers of cells; (3) abundant occurrence of cells; and (4) highly abundant occurrence of cells. TILs were evaluated in the following three different areas of the tumour: the intraepithelial compartment (cells within tumour cell nests); the stroma (cells within the intratumoural stroma) and the tumour periphery (cells localised in tumour periphery). Three random fields were examined, whereas necrotic areas were excluded from the measurements. Sections were examined by two observers (E Fokas, P Balempas). In case of discrepancy, a final decision was made upon further examination of the slides in a microscope based on consensus by the investigators. Discrepancy occurred in seven cases. The total score for CD3, CD8, CD4 and FOXP3 was
measured as the sum of the individual scores from the three tumour areas (intra-epithelial compartment, stroma and tumour periphery), respectively. The total score ranged from 3 to 12, and the median value was used as a cutoff point to separate the patient cohort into two groups with either low or high CD3 +, CD8 +, CD4 + and FOXP3 + expressions. Furthermore, the ratios of CD3 and CD8 to both CD4 and FOXP3 (CD3:CD4; CD8:CD4; CD3:FOXP3; and CD8:FOXP3 ratio, respectively) were calculated for each individual tumour based on the cutoff value of each TIL marker, and their prognostic significance on clinical end points was examined. In addition, we assessed the prognostic role of the TIL score for each of the three different tumour areas (intra-epithelial compartment, stroma and tumour periphery). For that purpose, the median TIL score of each area was measured and the cutoff point was used to separate the cohort into two groups with either low or high TIL score. We also validated the semiquantitative scoring of TIL expression using a quantitative approach. For that purpose, the median number of cells, as assessed in three randomised ×10 magnification fields per sample, was used as a cutoff point to separate the patient cohort into two groups with either low or high TILs cell number.

Following that, we analysed the expression of HPV16 and assessed the clinical outcome according to HPV16 immunoreactivity (positive vs negative). We also analysed p16 immunoreactivity by considering both the percentage of positive cells and the intensity of staining. For that purpose, the staining intensity was scored as: 1 (+), 2 (+) (moderate) and 3 (+) (intense). The fraction of tumour cells with p16 positivity was assigned to the following: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (>75%). To minimise the interobserver variability, scoring was performed by two independent investigators without the knowledge of the clinicopathological data. The percentage of positive tumour cells and the staining intensity were then multiplied to produce an individual labelling score for each case, ranging from 0 (no positive tumour cells) to 12 (>75% of tumour cells with intense staining).

Finally, we examined the prognostic value of the four TIL markers separately in HPV16-positive- and HPV16-negative patients. Images were acquired using ×10 and ×40 magnifications.

Statistical analysis. The differences between categorical variables were assessed by the Fisher’s exact test. Overall survival (OS) and time to recurrence were calculated from the date of CRT—beginning to the day of death and recurrences, respectively. Patients without tumour recurrence were censored at the last follow-up contact. Progression-free survival (PFS) was defined as the time between the start of therapy and tumour relapse (loccregional recurrence and/or distant metastases) or death because of non-tumour related causes. Local failure-free survival (LFFS) was defined as the time from the start of CRT to the first local tumour detection after CRT (that is, non-complete response), local tumour recurrence after complete response or death from any cause. Distant metastasis-free survival (DMFS) was assessed separately. A P < 0.05 was considered statistically significant. Survival curves were plotted according to the Kaplan–Meier method using SPSS 19 for Windows (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses were performed using the log-rank (Mantel–Cox) test and the Cox proportional hazard models, respectively.

RESULTS

TIL immunostaining on pretreatment biopsies. The immunohistochemistry characteristics and labelling scores are shown in Table 1. As a dichotomous variable, CD3 expression was defined as ‘low’ (weighted score ≤ 6) in 60 patients (59.4%) and ‘high’ (weighted score > 6) in 26 patients (25.8%). The median score for both CD4 and FOXP3 was 4. Immunohistochemical analysis revealed ‘low’ CD4 score in 58 (56.9%) patients and ‘high’ in 43 (42.2%) patients, whereas FOXP3 expression was ‘low’ in 97 (95.1%) and ‘high’ in 4 (3.9%) patients. As shown in Table 2, patients with lower CD8 expression presented a significantly higher incidence of N2c-3 stage – that is, advanced lymphadenopathy (CD8 weighted score ≤ 6 vs > 6: 57 vs 31%; P = 0.035), indicating a more aggressive disease. We did not find any other significant relationship between TILs’ expression and clinicopathologic parameters (Table 2). Moreover, we validated the semiquantitative scoring of TILs expression in a representative patient subgroup (n = 30) by using a quantitative method based on the median number of cells per tumour field (×10 magnification). CD3 and CD8 retained significance for all four clinical end points (OS, PFS, LFFS and DMFS), whereas neither FOXP3 nor CD4 was significantly correlated with improved outcome. The results are shown in Supplementary Table 2.

TIL immunostaining and treatment response. With a mean follow-up of 25 months (range, 2.3–63 months), OS at 2 years was 57.4% for the entire cohort. In univariate analysis, patients with high CD3 expression had a significantly superior OS (low vs high CD3: mean 30.3 vs 46.4 months; P = 0.002), PFS (low vs high CD3: mean 26.1 vs 42.7 months; P = 0.002), LFFS (low vs high CD3: mean 26.9 vs 42.7 months; P = 0.003) and DMFS (low vs high CD3: mean 28.6 vs 46 months; P = 0.001) (Figure 1A–D and Table 3). Similarly to CD3, high expression of CD8 correlated with better outcome. Indeed, patients with high CD8 expression had a significantly superior OS (low vs high CD8: mean 31.2 vs 50.1 months; P = 0.002), PFS (low vs high CD8: mean 28.4 vs 45.3 months; P = 0.005), LFFS (low vs high CD8: mean 29.2 vs 45.3 months; P = 0.008) and DMFS (low vs high CD8: mean 30.0 vs 50.3 months; P = 0.002) (Figure 1A–D and Table 3). Neither CD4 nor FOXP3 expression was significantly associated with any of the clinical end points (OS, PFS, LFFS and DMFS) in the univariate analysis (Table 3). Figure 2 illustrates the representative examples of low and high intra-epithelial CD3 and CD8 expressions.

Of note, the N-stage was the only clinicopathological parameter that reached statistical significance for a clinical end point (OS, P = 0.036) (Table 3). Multivariate analysis was performed including the four TIL markers and all clinicopathological factors (Table 3). In the Cox model, high CD3 expression was confirmed as an independent prognostic parameter for OS (P = 0.024), PFS (P = 0.044) and DMFS (P = 0.021), whereas no significance was found for LFFS (P = 0.90). Similarly, high CD8 expression retained prognostic significance for OS (P = 0.028), PFS (P = 0.047) and DMFS (P = 0.026) but lost significance for LFFS (P = 0.104). We did not observe any significance for either CD4 or FOXP3 in the multivariate analysis. Early N-stage was associated with improved OS in the multivariate analysis (P = 0.046). A trend towards improved DMFS was noticed for lower N-stage in the multivariate analysis (P = 0.060) (Table 3).

### Table 1. Results of CD3, CD8, CD4 and FOXP3 immunohistochemistry

| TIL markers | CD3 | CD8 | CD4 | FOXP3 |
|-------------|-----|-----|-----|-------|
| Dichotomized labelling score* | ≤ 6 vs > 6 | ≤ 6 vs > 6 | ≤ 4 vs > 4 | ≤ 4 vs > 4 |
| Low score | 60 (59.4) | 75 (74.2) | 58 (56.9) | 76 (74.5) |
| High score | 41 (40.6) | 26 (25.8) | 43 (42.2) | 25 (24.5) |

Abbreviation: AbTILs = tumour-infiltrating lymphocytes.

* Dichotomised labelling (low vs high score) based on the median value of TILs’ expression.
In addition, we investigated the impact of TIL expression, according to the three separate tumour compartments (tumour periphery, tumour stroma and tumour cells; Supplementary Table 3, Supplementary Figures 1–4). The density of all four TIL markers was usually heterogenous within a tumour section and varied among individual tumours. Indeed, in some cases strong infiltration of only the stroma and/or tumour cell/periphery compartment. We found differences in prognostic significance of TIL markers, depending on the separate compartments, failed to identify significance for any of the clinical end points (Supplementary Table 3; Supplementary Figures 1–4).

Similarly, we did not observe any significance for any of the TIL markers, usually heterogenous within a tumour section and varied among individual tumours. Indeed, in some cases strong infiltration of only the stroma and/or tumour cell/periphery compartment. We found differences in prognostic significance of TIL markers, depending on the separate compartments, failed to identify significance for any of the clinical end points (Supplementary Table 3; Supplementary Figures 1–4).

Following that, we investigated the impact of HPV16 on clinical outcome and the prognostic value of the four TIL markers both in HPV16-positive- and HPV16-negative patients (Supplementary Table 4).
Figure 1. Prognostic role of CD3 and CD8 in the outcome of patients with head and neck squamous cell carcinoma after definitive chemoradiotherapy. (A) OS; (B) PFS; (C) LFFS; (D) and DMFS according to pretreatment CD3 and CD8 expression (low CD3 and CD8 expression: weighted score ≤ 6; high CD3 and CD8 expression: weighted score > 6; the cutoff score was based on the median value).
Table 3. Univariate and multivariate analyses of prognostic factors in patients with HNSCC

|            | Univariate | Multivariate |
|------------|------------|--------------|
|            | P-value    | HR | 95% CI | P-value |
| OS         |            |    |        |         |
| CD3 + (low/high) | 0.002  | 0.429 | 0.206 | 0.895 | 0.024 |
| CD8 + (low/high) | 0.002  | 0.359 | 0.130 | 0.990 | 0.028 |
| CD4 + (low/high) | 0.604  | 1.536 | 0.555 | 4.246 | 0.408 |
| FOXP3 + (low/high) | 0.878  | 1.040 | 0.227 | 4.765 | 0.960 |
| Grade (G1/2/G3) | 0.783  | 0.911 | 0.334 | 2.324 | 0.316 |
| N-stage (N0-1/N2a/b/N2c-N3) | 0.036  | 1.324 | 1.004 | 1.746 | 0.046 |
| T-stage (T1-2/T3-4) | 0.178  | 0.396 | 0.162 | 0.970 | 0.162 |
| Tumour localisation | 0.658  | 0.915 | 0.623 | 1.344 | 0.649 |
| Age (<61/≥61) | 0.102  | 1.414 | 0.762 | 2.624 | 0.272 |
| Sex (male/female) | 0.911  | 0.785 | 0.353 | 1.745 | 0.553 |
| PFS         |            |    |        |         |
| CD3 + (low/high) | 0.002  | 0.494 | 0.248 | 0.982 | 0.044 |
| CD8 + (low/high) | 0.005  | 0.464 | 0.198 | 1.087 | 0.047 |
| CD4 + (low/high) | 0.365  | 1.118 | 0.403 | 3.098 | 0.831 |
| FOXP3 + (low/high) | 0.810  | 0.719 | 0.154 | 3.368 | 0.675 |
| Grade (G1/2/G3) | 0.637  | 0.977 | 0.309 | 1.875 | 0.944 |
| N-stage (N0-1/N2a/b/N2c-N3) | 0.160  | 1.176 | 0.926 | 1.493 | 0.184 |
| T-stage (T1-2/T3-4) | 0.282  | 0.462 | 0.181 | 1.179 | 0.106 |
| Tumour localisation | 0.587  | 0.896 | 0.428 | 1.277 | 0.542 |
| Age (<61/≥61) | 0.260  | 1.006 | 0.541 | 1.870 | 0.986 |
| Sex (male/female) | 0.753  | 0.951 | 0.417 | 2.169 | 0.905 |
| LFSS        |            |    |        |         |
| CD3 + (low/high) | 0.003  | 0.547 | 0.272 | 1.100 | 0.090 |
| CD8 + (low/high) | 0.005  | 0.491 | 0.208 | 1.158 | 0.104 |
| CD4 + (low/high) | 0.400  | 1.211 | 0.437 | 3.359 | 0.713 |
| FOXP3 + (low/high) | 0.599  | 0.726 | 0.153 | 3.436 | 0.686 |
| Grade (G1/2/G3) | 0.646  | 0.768 | 0.386 | 1.528 | 0.452 |
| N-stage (N0-1/N2a/b/N2c-N3) | 0.082  | 1.248 | 0.971 | 1.604 | 0.084 |
| T-stage (T1-2/T3-4) | 0.266  | 0.434 | 0.173 | 1.087 | 0.075 |
| Tumour localisation | 0.509  | 0.928 | 0.646 | 1.334 | 0.687 |
| Age (<61/61) | 0.121  | 1.344 | 0.734 | 2.458 | 0.338 |
| Sex (male/female) | 0.626  | 0.849 | 0.381 | 1.893 | 0.690 |
| DMFS        |            |    |        |         |
| CD3 + (low/high) | 0.001  | 0.432 | 0.211 | 0.880 | 0.021 |
| CD8 + (low/high) | 0.002  | 0.314 | 0.113 | 0.872 | 0.026 |
| CD4 + (low/high) | 0.440  | 1.444 | 0.526 | 3.962 | 0.475 |
| FOXP3 + (low/high) | 0.992  | 0.804 | 0.175 | 3.698 | 0.780 |
| Grade (G1/2/G3) | 0.822  | 0.839 | 0.411 | 1.712 | 0.629 |
| N-stage (N0-1/N2a/b/N2c-N3) | 0.079  | 1.295 | 0.990 | 1.695 | 0.060 |
| T-stage (T1-2/T3-4) | 0.187  | 0.378 | 0.146 | 0.980 | 0.145 |
| Tumour localisation | 0.785  | 0.922 | 0.625 | 1.361 | 0.684 |
| Age (<61/61) | 0.151  | 1.088 | 0.577 | 2.053 | 0.795 |
| Sex (male/female) | 0.949  | 1.144 | 0.494 | 2.649 | 0.753 |

Abbreviations: CI = confidence interval; DMFS = distant metastasis-free survival; HNSCC = head neck squamous cell carcinoma; HR = hazard ratio; LFSS = local failure-free survival; OS = overall survival; PFS = progression-free survival. Significant results have been marked in bold.

In the present study, HNSCC patients whose tumours were densely infiltrated by CD3+ and CD8+ T cells had a significantly longer OS, PFS and DMFS compared with patients whose tumours were poorly infiltrated. This finding was independent of clinicopathological parameters with a predictive role in this disease. Neither CD4 nor FOXP3 expression was significantly associated with any of the clinical end points.

The CD3 antigen is a protein complex comprising the T-cell receptor and has been commonly used as a T-cell marker (Gooden et al, 2011). The presence of higher numbers of CD3+ TILs has been associated with an improved outcome in different tumour types including colorectal cancer, breast, oesophageal, ovariian and anal cancer (Grabenbauer et al, 2006; Nedergaard et al, 2007; Denkert et al, 2010; Ducray et al, 2010; Dahlin et al, 2011; Gooden et al, 2011). Hence, our results are in accordance with previous observations.

High peritumoural lymphocytic infiltration has been associated with lower tumour stage and less invasive growth also in patients with HNSCC (Ginos et al, 2004; Rajjoub et al, 2007; Pretschner et al, 2009). However, these studies were characterised by low patient number and/or inhomogeneous treatment regimens (Ginos et al, 2004; Badoual et al, 2006; Rajjoub et al, 2007; Distel et al, 2009). Strengths of the present work rely on the fact that it includes a relatively large cohort of patients (n = 101) treated homogeneously with CRT in a single centre. Whiteshie and colleagues (Reichert et al, 2002) have shown that patients with HNSCC present defects in circulating T-cell signalling and tumour-directed cell killing, and TIL often undergoes apoptosis in tumours that are mediated by the Fas/Fasl pathway. Le et al (2005) found a strong inverse correlation between the hypoxia-related marker galectin-1 and CD3 staining in patients with HNSCC, providing evidence on how hypoxia can help tumours in evading immune surveillance. In that work, patients with high infiltration of CD3+ cells presented better clinical outcome, similarly to our observations. However, treatment was heterogenous, as 80 patients received chemoradiation and 21 patients were treated with surgery + radiation. In addition, no information with regard to the chemotherapy drugs in the chemoradiation treatment arm was provided (Le et al, 2005).

CD8 is a glycoprotein that constitutes a heterodimer of alpha and beta chains that are covalently linked by a disulphide bond (Gooden et al, 2011; Lieberman et al, 2001). A single immunoglobulin-like domain is contained to each dimer chain that is linked to the cell membrane via a polypeptide segment. CD8 has a key role in immune defense, as it serves as a coreceptor for the T-cell receptor (Gooden et al, 2011; Lieberman et al, 2001). Simultaneous
binding of CD8 and T-cell receptor to the major histocompatibility complex class I molecule enhances profoundly the cytotoxic capability of T cells in killing tumour cells (Lieberman et al, 2001). Accordingly, a favourable outcome has been demonstrated for tumours with high tumour infiltration by CD8+ cells in patients with breast, colorectal, ovarian, renal and lung cancer, which is in agreement with our observations. With regard to HNSCC, Pretscher et al (2009) detected a reduced number of CD8+ cells in metastatic cervical lymph nodes. Distel et al (2009) reported better survival in 62 patients with low-risk HNSCC and high CD8 expression, after treatment with primary surgery followed by external radiotherapy. Ogino et al (2006) revealed that patients with laryngeal cancer and low CD8+ T cell infiltration had poor survival. Moreover, this constituted a heterogenous group of HNSCC patients, as 38 (60%) of the 63 patients were treated with surgery and only the remaining 25 (40%) received radiotherapy (Ogino et al, 2006), which makes interpretation of the results difficult.

Interestingly, we found differences in the prognostic value of CD3+ and CD8+ TILs that varied according to the tumour compartment. Indeed, high expression of CD3+ T cells in tumour cells correlated with better outcome, whereas high CD3 infiltration of tumour stroma or periphery did not affect outcome. High stromal CD8 infiltration was a positive prognostic factor for all four clinical end points, whereas high tumour cell CD8 expression correlated only with better OS and DMFS, and high tumour periphery CD8 expression correlated only with better PFS and LFFS. Several studies have observed differences in the prognostic value of TIL expression that varied according to the tumour compartment, similar to our work (Nedergaard et al, 2007; Distel et al, 2009; Pretscher et al, 2009; Denkert et al, 2010; Dahlin et al, 2011). CD4+ T cells respond to antigens presented by HLA class II proteins to generate effective antitumour immune responses by promoting proliferation of CD8+ cytotoxic T lymphocytes (Whiteside, 2012). In addition, T regulatory cells (Tregs; defined as Foxp3+ lymphocytes) infiltrate tumours following stimulation by inflammatory chemokines and macrophages and can lead to the suppression of effective immunosurveillance (Whiteside, 2012); however, this effect appears to tumour histology-dependent. The large meta-analysis by Gooden et al (2011) failed to detect a significant association between either CD4 or FOXP3 expression and clinical outcome. In HNSCC, the role of CD4 and FOXP3 remains controversial as mixed findings have been reported. Distel et al (2009) found no correlation between CD4 or FOXP3 expression and clinical outcome of patients with head and neck cancer, whereas Badoual et al (2006) reported better locoregional control and longer survival in 51 HNSCC patients with high CD4 and FOXP3 expression. We did not observe any correlation for either CD4 or FOXP3 and the clinical outcome in our series.

Several mechanisms have been proposed regarding tumour–immune interaction in response to cancer treatment in malignant tumours (Denkert et al, 2010; Deschoolmeester et al, 2010; Dahlin et al, 2011; Gooden et al, 2011). These investigations have hypothesised that the pretreatment of host immune response may augment the potential of cancer therapies to eliminate cancer cells. This hypothesis is strongly supported by our analysis. We are tempted to speculate that the killing of tumour cells by CRT might release tumour-associated antigens and chemokines that stimulate an immune response against the tumour cells – that is, will be highly potent in patients with immunosensitization already present before the initiation of cancer treatment. Moreover, the significant correlation of high CD3 and CD8 expression with better DMFS could indicate the presence of a systemic immunosurveillance mechanism that suppresses the development of micrometastases in distant organs. Milas and colleagues (Stone et al, 1979) have previously demonstrated reduced tumour response to radiotherapy in mice that lacked a normal T-cell repertoire. In a preclinical study, Burnette et al (2011) have previously shown that the efficacy of radiotherapy largely relies upon the induction of interferon-dependent innate and adaptive immunity. This effect was mediated by CD8, as the depletion of CD8+ T cells drastically reduced treatment efficacy, whereas alteration of CD4 did not affect tumour growth (Burnette et al, 2011). Strategies that induce T-cell activation, for instance by using an antibody targeting the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) have been previously explored in combination with radiotherapy (Formenti and Demaria, 2013). In two carcinoma models growing in syngeneic mice, the addition of anti-CTLA-4 significantly and dose-dependently enhanced response to radiotherapy that resulted in complete regression of the majority of irradiated tumours.

Figure 2. Representative examples of low and high CD3 and CD8 expression in head and neck squamous cell carcinoma samples, as indicated. Magnification, ×1.
Stimulation of T cells via the inhibition of programmed-death-1 (PD-1), a surface receptor that leads to immunosuppression via the blockade of CD8 cell motility (Zinselmeyer et al., 2013), significantly enhanced radiotherapy response in an orthotopic model of glioblastoma (Zeng et al., 2013). Illidge and colleagues (Dovedi et al., 2013) demonstrated that CD8 + T-cell-mediated tumour eradication in experimental mouse models of T- and B-cell lymphomas following treatment with the Toll-like receptor 7 agonist, R848 in combination with radiation therapy. Hence, the use of immunotherapies that promote T-cell activation in combination with radiotherapy might be useful in patients with low CD8 expression in their pretherapeutic biopsy specimens to maximise irradiation outcome.

Liu and colleagues (Nordfors et al., 2013) did not detect any difference in TIL expression or prognostic impact by the HPV status in HNSCC patients. Nasman et al. (2012) found that high CD8 expression was correlated to a good clinical outcome in both HPV-positive- and HPV-negative patients. Similar findings were reported from Nordfors et al. (2013), and the authors hypothesised that the increased expression of CD8 + TILs could contribute to the better response of HPV-positive patients to therapy. In contrast, Wansom et al. (2012) did not detect any difference in TIL expression or prognostic impact by the HPV status, which is in line to our observations. Kong et al. (2009) reported the significance for high counts of CD3 + T cells only in HPV-negative tumours, similarly to our work. In addition, mechanisms other than T-cell infiltration, such as gene upregulation in the TP53 pathway that results in increased tumour-cell killing (Kimpel et al., 2013) and differential expression of cell cycle-related genes (Pyone et al., 2007), are known to contribute to the better response of HPV-positive tumours to radiotherapy. Hence, this issue remains controversial, and more studies with higher number of patients, preferably treated within randomized trials, are required to better elucidate the expression pattern and prognostic significance of TILs in HPV-positive- and HPV-negative patients.

Common risk factors for HNSCC include smoking and alcohol history and HPV infection. Aviles-Jurado and Leon (2013) have excluded. The median follow-up in our study (25 months; range, 2.3–63 months) is also relatively short, as studies with a follow-up of several years in HNSCC have been previously reported (Lassen et al., 2009; Ang et al., 2010). Moreover, our observations warrant validation in large, independent cohorts.

In summary, CD3 + and CD8 + TIL represent strong markers to identify a subgroup of HNSCC patients with higher probability of disease progression and shorter survival after definitive CRT. The use of TIL markers, such as CD3 and CD8, to predict recurrence and cancer-related death is of particular clinical interest in advanced stage HNSCC, as high-risk patients may potentially benefit from the implementation of novel immunotherapy to stimulate T cells in combination with CRT.

**REFERENCES**

Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axlrod R, Silverman CC, Redmond KP, Gillison ML (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 363(1): 24–35.

Argiris A, Karamouzis MV, Raben D, Ferris RL (2008) Head and neck cancer. Lancet 371(9625): 1695–1709.

Aviles-Jurado FX, Leon X (2013) Prognostic factors in head and neck squamous cell carcinoma: comparison of CHAID decision trees technology and Cox analysis. Head Neck 35(6): 877–883.

Badoual C, Hans S, Rodriguez J, Peyrad S, Klein C, Aguezenay NeH, Mosseri V, Laccoureye O, Bruneval P, Friman WH, Brasnu DF, Tartour E (2006) Prognostic value of tumour-infiltrating CD4 + T-cell subpopulations in head and neck cancers. Clin Cancer Res 12(2): 465–472.

Begg AC (2012) Predicting recurrence after radiotherapy in head and neck cancer. Semin Radiat Oncol 22(2): 108–118.

Bhardwaj N (2007) Harnessing the immune system to treat cancer. J Clin Invest 117(5): 1130–1136.

Boontkes HJ, De Gruijl TD, van den Muyzenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Schep R, Stacey SN, Duggan-Keen MF, Stern PL, Man S, Borysiwetz LK, Walboomers JM (2000) Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. Int J Cancer 88(1): 92–98.

Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, Fu YX, Auh SL (2011) The efficacy of radiotherapy relies upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res 71(7): 2488–2496.

Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Alkekrsue S, Anderson WF, Rosenberg PS, Gillison ML (2011) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol 29(32): 4294–4301.

Dahlin AM, Henriksson ML, Van Guelpen B, Lijenhagen L, Roberg A, Rutegard J, Palmqvist R (2011) Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. Mod Pathol 24(5): 671–682.

Dijkstra C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, von Tornne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28(1): 105–113.

Deshodhmeeter V, Baay M, Van Marck E, Weyer J, Vermeulen P, Lardon F, Vermorken JB (2010) Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. BMC Immunol 11: 19.

Dewyngaert JK, Babb JS, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axlrod R, Silverman CC, Redmond KP, Gillison ML (2009) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol 29(32): 4294–4301.

Dewyngaert JK, Babb JS, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axlrod R, Silverman CC, Redmond KP, Gillison ML (2009) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol 29(32): 4294–4301.

Dexert LV, Fickenscher R, Dietel K, Hung A, Iro H, Zenk J, Nkenke E, Argiris A, Karamouzis MV, Raben D, Ferris RL (2008) Head and neck cancer. Lancet 371(9625): 1695–1709.

Dovedi SJ, Melis MH, Wilkinson RW, Adlard AL, Stratford IJ, Honeychurch J, Distel LV, Fickenscher R, Dietel K, Hung A, Iro H, Zenk J, Nkenke E, Argiris A, Karamouzis MV, Raben D, Ferris RL (2008) Head and neck cancer. Lancet 371(9625): 1695–1709.

Dovedi SJ, Melis MH, Wilkinson RW, Adlard AL, Stratford IJ, Honeychurch J, Distel LV, Fickenscher R, Dietel K, Hung A, Iro H, Zenk J, Nkenke E, Argiris A, Karamouzis MV, Raben D, Ferris RL (2008) Head and neck cancer. Lancet 371(9625): 1695–1709.

Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28(1): 105–113.

Fakhry C, Lipp A, Ferris RL, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E, Schild SE, Kemeny NE, Timlin DJ, Schuchter LM, Morgenstern MB, Alexander J, Mankoff DA, Huang H, Helson L, O’Rourke J, Phillips G, Bohlke K, Gilchrist J, Petersen KA, Perera A, Vokes E, Keshock K, Seiwert T, Hitt M, Seidman AD, Eshleman J, Leary K, Vokes E,
Rickman D, Thomas E, Delattre JY, Honnorat J, Sanson M, Berger F (2010) An ANOCEF genomic and transcriptomic microarray study of the response to radiotherapy or to alkylating first-line chemotherapy in glioblastoma patients. Mol Cancer 9: 234.

Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 100(4): 261–269.

Formenti SC, Demaria S (2013) Combining radiotherapy and cancer immunotherapy: a paradigm shift. J Natl Cancer Inst 105(4): 256–265.

Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker M, Pambuccian SE, Ondrey FG, Adams GL, Gaffney PM (2004) Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res 64(1): 55–63.

Gooden MJ, De Bock GH, Jeffers N, Daemen T, Nijman HW (2011) The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 105(1): 93–103.

Grabenbauer GG, Lahmer G, Distel LV, Grabenbauer GW, Wittlinger M, Buettner M, Pretsch D, Distel L, Niedobitek G (2009) Distribution of immune cells in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 74(2): 535–561.

Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong AC, Harari PM (2013) Enhanced radiation sensitivity in HPV-positive head and neck cancer. Cancer Res 73(13): 4791–4800.

Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A, Kudoh S, Ochiai A (2008) Predominant infiltration of macrophages and CD8(+ ) T Cells in cancer nests is a significant predictor of survival in stage IV non-small cell lung cancer. Cancer 113(6): 1387–1395.

Kimple RJ, Smith MA, Blitzer GC, Torres AD, Martin JA, Yang RZ, Peet CR, Lorenz LD, Nickel KP, Klingelhoett JM, Lambert PF, Harari PM (2013) Effect of HPV-associated p16INK4A expression on response to radiotherapy or to alkylating first-line chemotherapy in papillomavirus-positive head and neck squamous cell carcinoma. Clin Cancer Res 12(11 Pt 1): 3355–3360.

Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, Koong AC, Pourmand N, Le QT (2009) The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 74(2): 535–561.

Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alner JG, Overgaard J, Alsner J, Overgaard M (2001) Dressed to kill? A comparison of the interaction between squamous cell carcinoma and cytotoxic T cells but not regulatory T cells predict outcome in anal carcinomas. J Clin Oncol 19(5): 1515–1520.

Liem J, Shankar P, Manjunath N, Andersson J (2001) Infiltrating lymphocytes and human papillomavirus-16–associated oropharyngeal cancer. Laryngoscope 111(15): 2090–2095.

Lesterhuis WJ, Haanen JB, Punt CJ (2011) Cancer immunotherapy–revisited. Semin Cancer Biol 21(6): e38711.

Le QT, Shi G, Cao H, Nelson DW, Wang Y, Chen EY, Zhao S, Kong C, Richardson D, O’Byrne KJ, Giaccia AJ, Koong AC (2005) Galectin-1: a marker of tumor cell antigenicity and a potential target for manipulation in cancer treatment. J Clin Oncol 23(35): 8932–8941.

Lesterhuis WJ, Haanen JB, Punt CJ (2011) Cancer immunotherapy revisited. Nat Rev Drug Discov 10(8): 591–600.

Lieberman J, Shankar P, Manjunath N, Andersson J (2001) Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. Blood 98(6): 1667–1677.

Nasman A, Romaniet M, Nordfors C, Grun N, Johannson H, Hammarstedt L, Marklund L, Munck-Wikland E, Dalianis T, Ramqvist T (2012) Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. PLoS One 7(6): e38711.

Nedergaard BS, Ladelkari M, Thomsen HF, Nyengaard JR, Nielsen K (2007) Low density of CD3+, CD4+ and CD8+ cells is associated with increased risk of relapse in squamous cell cervical cancer. Br J Cancer 97(8): 1135–1138.

Nordfors C, Grun N, Tertipis N, Ahrlund-Richter A, Haeggblom L, Sivars L, Du J, Nyberg T, Marklund L, Munck-Wikland E, Nasman A, Ramqvist T, Dalianis T (2013) CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. Eur J Cancer 49: 2522–2530.

Ogino T, Shigyo H, Ishii H, Katayama N, Miyokawa N, Harabuchi Y, Ferrone S (2006) HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker. Cancer Res 66(18): 9281–9289.

Pretsch D, Distel LV, Grabenbauer GG, Wittlinger M, Buettner M, Niedobitek G (2009) Distribution of immune cells in head and neck cancer: CD8+ T-cells and CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro- and hypopharyngeal carcinoma. BMC Cancer 9: 292.

Pyeon D, Newton MA, Lambert PF, den Boon JA, Sengupta S, Marsit CJ, Woodworth CD, Connor JP, Haugen TH, Smith EM, Kelsey KT, Turek LP, Ahlquist P (2007) Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head and cervical cancers. Cancer Res 67(10): 4605–4619.

Rajouj S, Bashar SR, Einhorn E, Cohen MC, Marvel MD, Sewell DA (2007) Prognostic significance of tumor-infiltrating lymphocytes in oropharyngeal cancer. Eur J Oral Oncol 43(3): 506–511.

Reichert TE, Strauss L, Wagner EM, Gooding W, Whiteside TL (2002) Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. Clin Cancer Res 8(10): 3137–3145.

Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ, Solomon B, Choi J, O’Sullivan B, Kenny LM, McArthur GA (2010) Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol 28(7): 4142–4148.

Shi W, Kato H, Perez-Ordonez B, Pintilie M, Huang S, Hui A, O’Sullivan B, Waldron J, Cummings B, Kim J, Ringash J, Dawson LA, Gullane P, Silliman R, Gillison M, Liu FF (2009) Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. J Clin Oncol 27(36): 6213–6221.

Stone HB, Peters LJ, Milas L (1979) Effect of host immune capability on radiocurability and subsequent transplantability of a murine fibrosarcoma. J Natl Cancer Inst 63(5): 1229–1235.

Wansom D, Light E, Thomas D, Worden F, Prince M, Urba S, Chepeha D, Kumar B, Cordell K, Eisbruch A, Taylor J, Moyer J, Bradford C, D’Silva N, Carey T, McHugh J, Wolf G (2012) Infiltrating lymphocytes and human papillomavirus-16–associated oropharyngeal cancer. Laryngoscope 122(1): 121–127.

Whiteside TL (2012) What are regulatory T cells (Treg) regulating in cancer and why? Semin Cancer Biol 22(4): 327–334.

Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, Durham N, Meyer C, Harris TJ, Albensio E, Pradilla G, Ford E, Wong J, Hammers HJ, Mathios D, Tyler B, Brem H, Tran PT, Pardoll D, Drake CG, Lim M (2013) Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. Int J Radiat Oncol Biol Phys 86(2): 343–349.

Zinzelmeyer BH, Heydari S, Sacristan C, Nayak D, Cammer M, Herz J, Cheng X, Davis SJ, Dustin ML, McGavren DB (2013) PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. J Exp Med 210(4): 757–774.

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