Reviewer's report

Title: 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

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Reviewer: Theresa Whiteside

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In this study, the authors wished to evaluate the distribution and prognostic significance of lymphocyte subsets (Foxp3+ Treg, CD4+ and CD8+ effector cells, CD68+ macrophages, CD20+ B cells) in four different tumor-related compartments (primary tumor, lymph node (LN) metastases, peritumoral area in metastatic LN and uninvolved draining LN) in 33 patients with oro- and hypo-pharyngeal squamous cell carcinomas. All patients were participants in a phase III trial of postoperative radiation vs. radiochemotherapy. Tissue microarrays were prepared for immunohistochemistry (198 cores), and images of photographed tissues were evaluated using the image analysis software. Numbers of infiltrating lymphocytes relative to 100 tumor cells or per image were also quantified using an image analysis system. The ratio of TIL/CD3+ T cells was used to compare the infiltration in the four compartments. Appropriate statistical analyses were used to relate the TIL data with OS or NED survival of the patients. Correlations between TIL infiltration in the four compartments were also determined.

This is a comprehensive and expertly performed analysis of distribution of various lymphocyte subsets in tumor and LN tissues of in a relatively small cohort of patients with HNC. The authors report some interesting differences in the distribution of lymphocyte subsets. CD20+ B cells were more numerous in metastatic LN than in primary tumors, and these higher B cell numbers predicted significantly better outcome. Higher numbers of CD8+ T cells were seen in uninvolved draining LN than in peritumoral areas of metastatic LN, and an increase in intraepithelial CD8+ T cells in metastatic LN was associated with a better although not significantly better NED survival. Foxp3+ Treg were equally frequent in all compartments, and an increase in Treg in the tumor or metastatic LN tended to predict improved NED survival. All of these correlations were of borderline significance or not statistically significant. The TIL/CD3+ cell ratio was decreased for CD8+, Granzyme B+ and Foxp3+ cells in LN compartments relative to tumor tissues and these ratios did not influence prognosis.

The authors correctly indicate in the Discussion that a controversy has long existed in respect to TIL numbers, localization, functional status and the role TIL play, if any, in cancer progression and prognosis. This study, although carefully performed and utilizing state-of-the-art methods, further adds to the existing confusion, without really explaining why the phenotypic and functional
heterogeneity of TIL exists and why it is important.

The in situ analyses of TIL are difficult to interpret, because it is the local microenvironment in the tumor which orchestrates TIL migration, localization, accumulation, function and survival. The local microenvironment (or “the tumor signature”) is obviously quite different in the tumor epithelium vs. stroma or in metastatic LN vs. uninvolved LN. The differences in the microenvironment exist not only between the compartments but also between individual patients. This microenvironment may be more or less immuno-suppressive, depending on the tumor and its aggressiveness. Since the biologic behavior, of individual tumors with the same histology and origin varies greatly, it is not unexpected that attributes of TIL also vary. The authors’ data in part provide support for this local diversity in human tumors and should be explained and discussed in this vein.

The major problem with this (and many other similar studies) is that tumors with different stage, grade, differentiation or site are lumped together for analysis. In this work we have tumors of tonsils, oro- and hypo-pharynx and of the oral cavity (Table 1). By the way, the Abstract (line 10) only mentions oro-and hypopharynx tumors. The text on p. 6 informs us that all patients who were entered in the trial had advanced (stages T3 and T4) disease with nodal involvement or extracapsular spread. According to Table 1, patients with all T stages were included, which does not fit with the entry criteria for the protocol, as stated on p. 6. So, the authors need to explain why there are these discrepancies between the text and Table 1. Their patient cohort appears to be very diverse. The appropriate TIL analysis would require grouping together a large number of patients who have tumors with the identical clinicopathologic criteria. This is, of course, would be quite difficult to arrange. But only then meaningful correlations could emerge between the immunologic tumor profiles and prognosis or survival.

The other perennial problem is that the patients whose tumors are examined for TIL are treated with various therapeutic regimens following surgery (e.g., radio vs. radiochemotherapy in this study). Therefore, the data for NED survival (it should be disease-free survival, DFS) or overall survival (OS; which is mentioned in the text but no data are presented) reflect responses to therapy and not natural tumor progression and further confuse potential interpretations and correlations. In this study the number of patients is too small, in any case, for any meaningful correlations of immunologic with survival data.

Last but not least, methodologic problems with sampling of tumors that are notoriously heterogenous, with immunostaining or with objective counting of the cells in situ create difficulties in data interpretation. In this paper, for example, no staining controls such as isotype controls for antibodies were specified in the methods. Without appropriate controls how can one be sure that their phenotyping is correct?? New data suggest that Foxp3 is not a marker of Treg, as it is present on activated CD4+ and CD8+ effector T cells and is even expressed in some tumor cells.

The data presentation is not optimal. In Figures 1A and 1B, the bar graphs should show means +/- SD. Figure 2, which presents DFS correlations with TIL
subgroups, is crowded and it is not clear why the survival curves originate at year 1.

The references are not current and do not adequately reflect the current state of immunologic knowledge about TIL.

**Level of interest:** An article of limited interest

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests.