Title: The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time

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Author’s response to reviews: see over
Dear Editor Rachel Neilan,

Thank you and Dr. Lill-Tove Busund and Dr. Peter Bradding for the very thoughtful comments and suggestions. We are especially grateful to the reviewers for their time and efforts spent on reviewing our manuscript. We have addressed their comments as described below.

Reviewer: Lill-Tove Busund

Reviewer's report:
1. The authors have investigated the amount of tumor promoting macrophages (M2) and tumor inhibiting macrophages (M1) in tumor islets and tumor stroma in NSCLC in a short and a long survival group. They find M1 macrophage densities in tumor islet and stroma of the long survival group significantly higher than M1 macrophage densities in tumor islet and stroma of the short survival group. In a multivariate Cox proportional hazards analysis, M1 macrophage density in tumor islet was an independent predictor of patient's survival time. The overall methodology is state of the art.

Response: We appreciate the kind comment.

2. Macrophage polarization and activity in tumor biology is a very interesting and controversial field. The authors have used CD68 which is a rather unspecific antibody as it reacts with a diversity of cells of varying degree of activation. All antibodies are purchased from one and the same manufacturer. Since the findings are exclusively based on the specificity of these antibodies in a double staining kit, I would suggest redoing all the stains with at least one alternative antibody from alternative manufacturer for each marker.

Response: We agree with the reviewer that CD68 is not only expressed by macrophages. Given that the majority of literature still uses CD68 to describe macrophages and there are no better antibodies available, we feel that it is appropriate to follow this norm. We have added a discussion of the markers to highlight the limitation of this study (see the last paragraph in Discussion section). In fact, using CD68 as the marker, we have obtained consistent results from two independent studies on macrophages (reference #11, Yu N, Pu J, Pu Q, Che G, Zhang S, Liu L: Influence of Tumor Associated Macrophages Distribution on Prognosis of Non-small Cell Lung Cancer. Chin J Clin Thorac Cardiovasc Surg 2009, 16(1):44-47, and a revised manuscript # 4562464803117308 submitted to BMC Cancer).

The Zhongshan Goldenbridge Biotechnology Co., LTD., is the largest and most reliable distributor of antibodies in China. The primary antibodies used in this study were originally
produced by Zymed, now part of Invitrogen, Carlsbad, CA. These antibodies have been widely used by other investigators in the macrophage field. We now clarify this in our Methods section.

3. The pictures (Figure 1) are rather confusing. Picture a. shows aggregates of pycnatic white blood cells in the lumen of a bronchus. Picture b. shows double stained (?) aggregates of cells/nuclei. I would suggest new pictures demonstrating with higher magnification single cells, double stained M1 and M2 cells in tumor islets and tumor stroma, respectively.

Response: We now add new pictures taken at x1000 magnification for Fig. 1a and 1b, to focus on the double stained M1 and M2 macrophages in the tumor islets.

Reviewer: Peter Bradding

Reviewer's report:
This study has examined the distribution of CD68+ macrophages expressing the M1 marker HLA-DR and the M2 marker CD163 in the tumour islets and stroma of patients with NSCLC. Overall the study demonstrates that increased numbers of HLA-DR+ macrophages in the tumour islets and stroma are associated with improved survival. The study supports the view that “cytotoxic” M1 macrophages are involved in and tumour immune response.

Minor essential revisions:

1. There has been previous work in this area (Ohri et al) which is discussed in detail in the discussion but should also be acknowledged in the introduction. The results of the two studies give a similar message although there are some differences which are discussed satisfactorily.

Response: We now describe the work of Ohri and colleagues in the Background introduction (see last sentence in the 2nd paragraph).

2. Clarify whether formal ethical approval in place for this research?

Response: This research was approved by the Institutional Review Board of West China Hospital, Sichuan University. We now add this statement at the beginning of Methods section.

3. Clarify how were the patients chosen for this study? Was there any potential for bias?

Response: We chose the patients based on the inclusion criteria: (1) follow-up data were complete; and (2) paraffin blocks were available; and (3) without pre-operative chemotherapy or radiotherapy. All of the cases that satisfied the inclusion criteria were included in this study. We now add this information in the Methods section.
We are not sure if there is any potential bias that may be caused by the representativeness of the included population, as there were some cases that were excluded due to unavailability of paraffin blocks or follow-up data. Given the relatively large sample size, we believe such sampling bias, if any, is minimal.

We have also corrected some linguistic issues (see change of title; change “islet” into “islets”; adding “the”…).