Author’s response to reviews

Title: The efficacy of tumor debulking surgery is improved by adjuvant immunotherapy using imiquimod and anti-CD40

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Version: 3 Date: 12 November 2014

Author’s response to reviews: see over
Dear Dr Eyad Elkord,

We sincerely thank the reviewers for their consideration of our manuscript. Here we have provided our point-by-point response to address each of the reviewers’ concerns. We also provide an updated version of the manuscript (v2) including changes highlighted in yellow.

Reviewer: Michael Kershaw

- Tumor experiments in Figure 2 show that combination of surgery with either Imiquimod or Imiquimod+anti-CD40 provide a higher survival benefit than surgery alone. However, are these treatments statistically different? One of the main concerns of these results is that despite a general trend of better results obtained when using surgery with immunotherapy (Imiquimod +anti-CD40), it is not clear whether combination with Imiquimod +anti-CD40 is better than combination with Imiquimod alone. Confirmation of these differences would clearly show that this combination is really needed.

The statistics indicated on the graph of Figure 2 were calculated by comparing debulking surgery alone [D] to either debulk + IMQ [D + I] and debulk + IMQ + anti-CD40 [D + I + aCD40], to highlight that the addition of adjuvant treatment is beneficial over debulking surgery alone.

When [D + I + aCD40] is compared to [D + I] the p value drops to 0.066, so the addition of anti-CD40 to the treatment regimen did not reach statistical significance in terms of improving survival. However, statistical differences between these groups can be seen in Figures 4, 5 and 6 where the addition of anti-CD40 [D + I + aCD40] produced significant changes in CD4 and CD8 T cells compared to debulk + IMQ alone [D + I].

- Keeping with the last comment, results in Figure 3 do not show an improvement when using surgery with Imiquimod+anti-CD40 as compared with surgery with Imiquimod.

Similarly, the triple combination exhibits significant advantage over debulk alone. However, when compared to double therapy (either D + I or D + anti-CD40), the triple combination did not elicit a statistically significant improvement. Despite this, it is clear that target cell killing is elevated in the
tumour draining LN as a result of triple combination therapy (~45% compared to ~25% [D + I] and ~20% [D + aCD40]).

- Finally, analyses of total and activated CD4 and CD8 T cells in lymphoid organs and tumors do not show the best results for surgery with Imiquimod+anti-CD40. Indeed, it is interesting that in previous comments surgery with Imiquimod was the second best treatment whereas in this case surgery with anti-CD40 seems to be better than surgery with Imiquimod. These results should be discussed in order to better understand the immune mechanisms responsible for the antitumor effect.

The reviewer is correct to point out that, in terms of CD4 and CD8 T cells, there is no apparent advantage with triple combination over surgery + anti-CD40, and that the effect of surgery + anti-CD40 is greater than surgery + IMQ. The immune-modulatory effect of anti-CD40 on T cells is clearly evident in the lymphoid organs at the time of analysis. One possible explanation is that the effect of IMQ on DC licensing is occurring at the site of administration (tumor) and may not be detectable as a change in T cell numbers or activation, whereas anti-CD40 is administered systemically and the effect is more likely to be manifest in lymph nodes and spleen.

- Since combination including surgery with Imiquimod+antiCD40 is suggested as the best treatment, depletion experiments to characterize the role of CD8 T cells should be done in this setting, which would be more informative than experiments done using surgery with Imiquimod.

This is a valid point by the reviewer. While we haven’t included it in the present study with surgical debulk, we have demonstrated in a previous study, using CD4 and CD8 T cell depletion, that the effective combination of IMQ and anti-CD40 was critically reliant on CD8 T cells (Broomfield 2009).

- Authors have previously reported (Broomfield et al Cancer Res 2005) that a combination of 75% debulking surgery with gemcitabine+antiCD40 has a survival efficacy of 70%, whereas in the present manuscript they reach a 30-40 % survival. Given these results, which is the advantage of using the current strategy?

Gemcitabine is an immunogenic chemotherapy that synergises well with immunotherapy to treat mesothelioma tumours in vivo. While gemcitabine can be used as second-line chemotherapy in mesothelioma patients, it may not be applicable in other cancer settings. The current strategy aims to stimulate the patient’s immune response and hence would not be limited to mesothelioma. Imiquimod treatment also has the potential for local, targeted administration and lowered levels of toxicity, which in some situations may be a preferred alternative to chemotherapy.

- Is Aldara cream directly applied i.t.? Also, authors indicate the advantage of i.t. administration due to surgery. However, in their model, Imiquimod is administered repeatedly. How feasible would this be in a clinical setting?

Our study was conducted in a subcutaneous tumor model, hence IMQ was injected directly into the tumour. The dosing regimen (6x daily) was based on our previous study which showed that the immunostimulatory effect of IMQ required continuing administration. Due to the i.t. delivery route we were restricted in the amount that could be injected in a single bolus, requiring repeated injections.

It is envisioned that in the clinical setting a higher single dose could be administered. Alternatively, mesothelioma patients may receive an indwelling catheter to drain pleural fluid, which may provide a portal for repeated drug administration directly into the pleural cavity. Lipid sustained release implants may also represent a possibility for continuous drug administration after initial implantation i.e. after surgery (Myschik et al., 2008 Pharmazie).

- Experiments in Supplementary Figure 1B-C do not add any information, since all these groups (except Imiquimod alone, whose effect has been already reported in previous publications) are included in Figure 2. Moreover, the surgery+Imiquimod group shown in Supplementary Fig 1B-C and Supplementary 2 is exactly the same.

Supplementary Figure 1 has been removed, with Suppl. Fig 2 remaining (now renamed as Suppl Figure 1).

Reviewer: William J Murphy
This study shows that anti-CD40 and IMQ can work together and promote CD8 T cell responses at primary tumor sites. The immune stimulatory and anti-tumor effects of these approaches have been well documented. While CD8 T cell effects are shown it is proposed (and likely) that IMQ works on DCs (as does also anti-CD40) but this needs to be shown.

We have not thoroughly investigated the specific effects of imiquimod (and anti-CD40) on intratumoral DCs in this study. The main focus of our approach was to highlight the possibility of adjuvant treatment following surgical resection, in situations where resection would be incomplete (due to tumour bulk/accessibility, occult residual tumour, or distant metastases). We have chosen immune-modulating agents that are readily available in the clinic, with known mechanism of activity on the major players of the immune response, namely DCs and CD8 T cells in the case of solid tumors. While we agree it would be important to elucidate the specific roles of these drugs in licensing DCs, ultimately we have provided evidence of T cell activation and enhanced CTL responses induced by the combination therapy and demonstrated the efficacy of the combined approach, which was the goal of this study. Further studies will likely address the contribution of both IMQ and anti-CD40 towards DC function in this setting.

The authors also need to demonstrate effects on Tregs as other groups have indicated that CD40 stimulation may inhibit them.

There are relatively few studies describing the effects of anti-CD40 on Tregs, particularly in tumor immunology. The study by Weiss et al., (PNAS 2009) describes the combination of anti-CD40 and IL-2 is able to reduce intratumoral Tregs, despite increasing levels in the spleen. However, either agent alone did not affect splenic or tumor Treg numbers. Pan et al., (Cancer Research 2009) showed that agonist anti-CD40 antibody can prevent tumor-specific Treg development and T-cell tolerance.

In our own study, we found that the percentage of CD4+ cells was decreased in the spleen and tumour compared to untreated mice. Interestingly, there were less CD4+ cells in the triple combination compared to debulk + anti-CD40 only in the dLN and tumor, but the reverse was true in the spleen. The study by Broomfield et al., (J Immunol 2009) showed CD4 depletion of mice treated with IMQ/anti-CD40 exhibited similar responses to IMQ-only. Based on these observations, it is indeed possible that anti-CD40 (+IMQ) could have had an effect on Tregs. It would be interesting to confirm this in future studies, and BALB/c.Foxp.dtr mice (allowing specific Foxp3+ Treg depletion) could be used to elucidate this.

Effects on draining LN cell phenotype and numbers are important.

We agree to the importance of the draining LN in observing the anti-tumor response brought about as a result of effective treatment. Here, we have focused on CD4 and CD8 T cell proportions and activation status (ICOS expression), as well as target cell killing in response to treatment, which we believe provide the most insight for this study. Additional information on the proliferation status of these cells, or whether they express CTLA-4 or PD-1 for example, would give further insight to the kinetics of the T cell response induced by these treatments. The tumor antigen (HA)- specificity of these cells could also be elucidated by HA dextramer staining if future studies were to be conducted, but was outside the scope of this study.

It is important to state whether control injections were given for the IMQ it experiments and that simple it injections can cause immune and inflammatory stimulation.

This is a valid point and we have now amended the manuscript (Results section, p9) to include the following wording:

Intratumoral injections can potentially cause immune and inflammatory stimulation, however we have shown in previous studies that i.t. administration of saline does not affect subcutaneous tumor outgrowth, nor cause the release of type I IFNs or promote antigen-specific immune responses in our model [10]. Control i.t. injections were therefore not included in this study.
- The figure legends need more detail on the treatment regimen.

Figure legends have been updated with more details of dose, route and timing of administration for treatment with IMQ, anti-CD40 and CD4/CD8 depleting antibodies.

- Other groups (ie Murphy et al and studies by Wiltrout et al) have used CD40 stimulation in a renal cell/nephrectomy model and these should be cited.

The work of Murphy and Wiltrout is now referenced in the discussion, Reference [27]:

Indeed, a preclinical surgical study using the renal cell carcinoma model has shown that anti-CD40 may be successfully combined with IL-2 to orchestrate effective DC and CD8 T cell response against distal tumours [27].

Yours sincerely,

[Signature]

Andrea Khong