Author's response to reviews

Title: Monocytes and the 38kDa-antigen of mycobacterium tuberculosis modulate natural killer cell activity and their cytolysis directed against ovarian cancer cell lines

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Version: 2 Date: 14 August 2012

Author's response to reviews: see over
REVISION OF BMC MS: 1722290197663418

Manuscript Title:

Old title: Modulation of natural killer cell-activity directed against ovarian cancer cell lines by the 38 kDa-antigen of mycobacterium tuberculosis

New title: Monocytes and the 38 kDa-antigen of mycobacterium tuberculosis modulate natural killer cell activity and their cytolysis directed against ovarian cancer cell lines

Dear Dr. Odunsi,

Thank you very much for reviewing our manuscript and for the opportunity to submit a revised version. We have thoroughly addressed all the comments and suggestions of the reviewers and incorporated the respective changes in the manuscript. Please find below our point-by-point reply.

We hope that our study will now fully meet the standards expected by the reviewers and will be suitable for publication in BMC Cancer.

We are looking forward to receiving your decision.

Sincerely,
Sven Brandau
GENERAL REMARKS:

– Both reviewers commented on the mechanism of NK activation in our system and more specifically on the role of soluble cytokines for NK activation. We thank the reviewers for raising this issue, because it prompted us to investigate the requirement for cell-cell contact and soluble cytokines during NK activation. Based on the previous set of data, we and the reviewers inferred that NK activation may occur via monocyte-derived cytokines such as IL-15 and IL-18. Trans-well experiments now clearly indicated that cell-cell contact rather than soluble cytokines are crucial for activation of NK cells by monocytes in our model.

– These new findings resulted in substantial changes and a re-organisation of the manuscript (see below). Because of this re-organisation, we do not indicate individual changes in the revised version.

– The title of the manuscript has been modified in order to better reflect the new data and the reviewers suggestions.

– The revised manuscript is now organized as follows:

Old fig 1 was deleted
Old fig 2 is now new fig 1 and contains new experimental data
Old fig 3 is now new fig 2 and contains new experimental data
New fig 3 shows transwell assays
Fig 4 was updated and now includes data on cytokine protein levels
Fig 5 is a newly added figure stimulated by a comment by Dr. Matsuzaki
Old fig 5 is now new fig 6.

DETAILED POINT BY POINT REPLY:

Reviewer (1 May 2012) Matsuzaki

General comment:
…….“The weakness of the study was the lack of new findings. Most observations were previously reported by others.”

Response: It is correct that previous studies investigated the anti-cancer activity of PstS-1 in tumours such as bladder cancer and melanoma. In this context the direct effect of PstS-1 on myeloid cells such as dendritic cells has also been investigated. In our study, we investigated the direct and indirect effect of immunostimulatory PstS-1 on NK cells and the consequences for cytolysis of ovarian cancer cells. We are not aware of previous reports on the direct or
indirect modulation of NK activity by PstS-1 (38 kDa antigen of mycobacterium tuberculosis). Also, to the best of our knowledge, no reports on the effect of monocytes on NK-mediated ADCC directed against EGFR-positive ovarian cancer cells are available at present.

Thus, although aspects of immunomodulatory mycobacterial antigens as well as effects of monocytes on anti-tumour activity of NK cells certainly have been previously reported by others and in other models, our studies consists of additional, previously not reported and therefore novel sets of data.

**Major Compulsory Revisions:**

**Ad 1.** Because there was no evidence that PstS-1 directly modified NK cell function, the title and several descriptions in the results are overstated for its effect on NK cells.

Response: We agree with the reviewer that from reading the title (without referring to the abstract), the reader could wrongly assume that the 38 kDa antigen directly modulates NK cells. We modified the title and the abstract to clarify the contribution of monocytes in our model.

**Ad 2.** PstS-1 has been reported to be recognized by T cells and to stimulate innate immune cells through TLR-2 and TLR-4. According to previous reports, NK cell does not express these TLRs nor recognize antigens that are recognized by T cells. It is recommended to describe why the authors attempted to test the direct effect of this reagent on NK cells.

Response: PstS-1 has been shown to stimulate monocytes and DCs via TLR-2 and TLR-4. Indeed, for many years the available literature suggested that NK cells do not express TLRs. However, recent data claimed that BCG directly stimulated NK cells in the absence of antigen-presenting cells [Marcenaro et al., 2008, Int Immunol], a process in which TLR-2 and TLR-5 on NK cells might be involved [Chalifour et al., 2004, Blood]. Potential direct effects of PstS-1 on NK cells have not been investigated so far. Thus, we wanted to test (and exclude) a direct activation of NK cells by our PstS-1 preparation and included some of those data into our manuscript.

**Ad 3.** PstS-1 appeared to enhance NK cell activation through monocyte activation. Data showing cytokine production from monocytes+PstS-1 in the absence of NK cells would be important in Figure 4 because IL-18, IL-15 and IL-12 are DC-derived cytokines as authors discussed whereas IFN-γ is dominantly produced by NK cells.

Response: As suggested by the reviewer we included data on stimulation and cytokine release of monocytes in the absence of NK cells (second section of “Results”). However, released cytokines do not seem to be the major mediators of NK activation in our system. This is indicated by new data (presented in new figure 3), which show that cell-cell contact rather than soluble cytokines is required for NK activation.

**Ad 4.** Authors concluded that PstS-1 contributed to full-activation of NK cells though it is not clear what the criterion was used for full-activation. Authors examined CD69 expression to evaluate NK cell activation. To study targeting of MIC-expressing ovarian tumors, analyses of NKG2D expression would be more important.
Response: We thank the reviewer for his comment and adopted his suggestion. We now show data on NKG2D expression of NK cells as an additional marker of NK activation. These data are depicted in the new figure 5 for CD16-positive (CD56 dim) and CD16-negative (CD56 bright) NK cells. Our data show that expression of NKG2D was induced by monocytes. However, CD69 but not NKG2D are induced by PstS-1 in NK-monocyte-co-cultures.

**Discretionary Revisions:**

**Ad 1.** Authors demonstrated that IFN-γ-production but not cytolytic function was enhanced by PstS-1 treatment. Because there are two distinct NK cell subsets (IFN-g producing CD56brightCD16- and cytolytic CD56dimCD16+) in human PBMC, investigating whether PstS-1 could activate CD56brightCD16- subset selectively would be interesting.

Response: This was an interesting suggestion and hypothesis, which we were happy to incorporate into our work. Results are depicted in new figure 5. Our results show that both subsets became activated in our system.

**Ad 2.** Authors use the same histogram data on Fig 1a/Fig 3a and Fig 1b/Fig 3b. Figure 1 and Figure 3 should be combined.

Response: During re-organisation of the manuscript and because of additional data, we deleted the old figure 1. Old figure 3 is now new figure 2 and contains additional experiments.

**Ad 3.** IFN-γ ELISA and CD69 expression were shown by two different ways; fold change in some figures, % or concentration in other figures. It would be better to show the data using the same unit.

Response: We now consistently show CD69 as (% positive cells) and IFN-γ protein as concentration (pg/mL).

**Reviewer (17 Feb 2012) Nishikawa**

**Major general comment:**
This study lacks critical experiments addressing the role of enhanced cytokine secretion by NK cells after stimulation with PstS-1 and monocytes compared with monocytes alone. The authors proposed the augmentation of T-cell function by the enhanced cytokines, but they do not show any experimental data. In addition, while they claim some clinical applications, they do not observe any enhanced tumor lytic activity by this strategy. As IFN-γ may induce the MHC class I expression, it may reduce NK cell killing capacity.

Response: We agree with the reviewer that induction of NK cytotoxicity is more pronounced compared with induction of IFN-γ in our system. Incorporation of additional donors revealed a statistically significant, but rather marginal increase in IFN-γ (figure 2b) induced by PstS-1. Induction of NK cytotoxicity was more pronounced, mainly mediated via monocytes and enhanced in the presence of EGFR-targeting antibodies (ADCC) (figure 2c, figure 6). When
co-cultures of NK and monocytes were stimulated with PstS-1, we observed an increase in IFN-γ and classical monokines such as IL-15 and IL-18 (figure 4). Subsequent experiments using trans-well assays clearly indicated that those soluble cytokines are not essential for NK activation in our system, which is rather mediated via cell-cell contact between accessory monocytes and NK cells (new figure 3).

**Specific points:**

Ad 1.): 1) In Fig. 3, expression of an activation marker CD69 was analyzed. It seems that both % of activated cells and CD69 expression was enhanced. It should be helpful to show the % positive cells and MFI of CD69 staining.

Response: Indeed, in some donors both MFI and % positive cells increase upon stimulation. In figure 3 of the first version an example is included, which shows donors with a high number of positive cells. However, in other donors we also observed a considerable number of cells remaining negative for CD69 (see figure at the bottom of this pbp-reply) resulting in distinct populations of CD69pos and CD69neg NK cells. For consistency within the manuscript and to also accommodate reviewer 1 (Matsuzaki, discretionary ad3), we now show % of positive cells in all panels throughout the manuscript.

Ad 2.): In Fig.4, the authors addressed cytokine secretion using real-time PCR. As mRNA expression sometimes does not reflect protein doses, cytokine assays at protein level (such as ELISA) should be included. In addition, while IL-15, IL-18 and IFN-γ secretion was augmented, it is necessary to examine which cytokine (s) is critical for the augmented NK activity by blocking assay.

Response: We thank the reviewer for this comment, as it stimulated experiments, which considerably improved the quality of our data sets. Based on the comment, we measured the expression of cytokines on the protein level. Data are depicted in the new figure 4b. The data show that the induction of mRNA for IL-18 is also reflected by an increase in protein secretion. However, no increase in protein secretion could be observed for IL-15 despite induction on the mRNA level. We next sought to analyze the role of soluble factors (including cytokines) in NK activation in our system using trans-well assays. Unexpectedly, we found that NK activation almost exclusively depends on cell-cell contact between monocytes and NK cells (new figure 3). These data argue against a role of cytokines during NK activation. Blocking experiments were still performed and showed no reduction of NK activity in the presence of inhibitory cytokine antibodies (data not included into the manuscript).
Illustration of CD69-neg (NK unstimulated in a) and distinct subsets of CD69-pos and CD69-neg NK (NK stimulated with monocytes in b) (related to specific point #1 of reviewer 2).