Author's response to reviews

Title: TRAIL Death Receptor-4, Decoy Receptor-1 and Decoy Receptor-2 expression on CD8+ T cells correlate with the disease severity in patients with rheumatoid arthritis

Authors:

Atil Bisgin (abisgin@yahoo.com)
Ender Terzioglu (ender2504@gmail.com)
Cigdem Aydin (cigdemaydin@akdeniz.edu.tr)
Burcak Yoldas (burcakyol@yahoo.com)
Veli Yazisiz (drveliyazisiz60@hotmail.com)
Nilufer Balci (nilbalci@akdeniz.edu.tr)
Huseyin Bagci (hbagci@akdeniz.edu.tr)
Reginald M Gorczynski (reg.gorczynski@utoronto.ca)
Cezmi A Akdis (akdisac@siaf.unizh.ch)
Salih Sanlioglu (ssanlioglu@me.com)

Version: 3 Date: 3 June 2010

Author's response to reviews: see over
June 3, 2010

Editor in Chief of the BMC Musculoskeletal Disorders Journal

Ref: MS 1172304023373206

Dear Editor:

I would like to submit the revised manuscript entitled “TRAIL Death Receptor-4, Decoy Receptor-1 and Decoy Receptor-2 expression on CD8+ T cells correlate with the disease severity in patients with rheumatoid arthritis” for publication in the BMC Musculoskeletal Disorders journal. I have pasted below a response letter to reviewers’ comments and completed all the necessary assays suggested by the reviewers.

The data in the manuscript is original and the manuscript is not under consideration elsewhere. All authors have read and approved the final version of the manuscript, its content, and its submission to the BMC Musculoskeletal Disorders. Authors declare that they have no conflict of interest.

I am looking forward to hearing from you. Sincerely,

Prof. Dr. Salih Sanlioglu V.M.D., Ph.D.

The chair and the head of Department of Medical Genetics
The Director of the Human Gene Therapy Division
Akdeniz University Hospitals and Clinics

E mail: sanlioglu@akdeniz.edu.tr
Web: www.tsgct.org
Point by Point Reply to Comments for the Manuscript: MS 1172304023373206

Title: TRAIL Death Receptor-4, Decoy Receptor-1 and Decoy Receptor-2 expression on CD8+ T cells correlate with the disease severity in patients with rheumatoid arthritis

We thank the reviewers for their insight and helpful suggestions. Below are the comments from the reviewers and our responses to them.

Reviewer 1

Comment 1: The authors concluded in Fig. 1 that the CD4/CD8 ratios of RA patients were unchanged compared with the controls. However, the %CD4+ and %CD8+ cells were shown separately. The proper way to present the results will be to show the CD4/CD8 ratio for each individual in the sample pool.

Action taken: Upon the request of the reviewer Fig 1 has been changed accordingly as shown below.
Figure 1. Scatter dot plots of peripheral blood samples from 12 healthy control individuals and 20 RA patients showing the ratio of T cell subsets (CD4$^{+}$ and CD8$^{+}$) by flow cytometry.

Comment 2: There are a series of problems with the method and interpretation of results presented in Figs. 2 and 3. The authors’ results appear to show that RA patients express a
higher level of TRAIL and TRAIL receptor expression in CD8+ cells than in CD4+ cells. Assuming that the majority of CD4- cells in Fig. 2B are CD8+ cells, why did the authors not detect an increase in TRAIL receptor expression in the CD4- population? This raises the concern of whether the proper staining controls (e.g. isotype antibody controls should be used for every sample) have been used. The authors are reminded that it is not unusual that basal staining of any receptors differs greatly from one individual to the other.

**Action taken:** Every time we performed a flow cytometry assay for each patient, we included an isotype-matched control. Not to mention only one representative out of 20 assays was given in Figure 2B. Thus we replaced the Figure 2B with a new one representing another patient data to prevent any confusion. This particular example clearly demonstrates the presence of TRAIL and TRAIL receptor expressions in CD4- quadrant.

**Comment 3:** Even assuming that the FACS staining was done properly, there are still issues with the results in Figs 2-3. For example, the authors claimed that TRAIL receptors and TRAIL expression in CD4+ T-cells were increased in RA patients. However, CD4+ T-cell expression of these markers was negligible and thus this reviewer questions the biological significance of any modest increases observed by the authors. In Fig. 2C and 3C, how was the fold increase determined? Was it by mean fluorescence or by percentage? The percentages of cells in the FACS plots should be shown and the panels for RA patient and control should be labeled properly. The poor presentation of the data renders interpretation of these results difficult.
Response: We calculated individual fold change by exporting the FC data to an Excel spreadsheet. Then the fold change was revealed by the ratio of the measured value (percentage of positive cells) for the RA samples to the healthy control. As requested by the reviewer the percentage of cells are shown in Panels 2B and 3B and the plots are labeled clearly. Our data suggest that TRAIL and TRAIL receptor expression on CD4+ T cells were increased in RA patients compared to healthy controls. We do not have any data suggesting this subtle increase is negligible or important; we have no such claims in our manuscript. Our conclusion explicitly states that only the CD8+ T cell associated DR4, DcR1 and DcR2 expression levels correlated with DAS28 scores in patients with RA, implying that altered TRAIL receptor profiles on CD8+ T cell subsets rather than on CD4+ T cells is more important in terms of disease severity.

Comment 4: Could any increases in TRAIL or TRAIL receptor expression observed in RA patients be simply a consequence of T cell activation? The authors should examine the activation status of the T-cells (e.g. by staining with CD25, CD69 etc.). Also, how does the expression of TRAIL and TRAIL receptors compare with other more well-studied markers for RA (e.g. IL-17)? This is especially important given the small sample size (20) that the authors used in the study.

Action Taken:
T cell activation status of RA/control patients was analyzed as suggested and the following section was added to Results of the manuscript (Page 10):

**T cell activation status of newly diagnosed RA patients**

CD25 is one commonly used marker for recently activated T cells [1]. In order to document the activation status of T cells in RA patients; another set of flow cytometry assay was conducted. As shown in Table 3, the percentage of CD4^+CD25^+ T cells was statistically higher in RA patients compared to control individuals. However, as shown before both activated and regulatory T cells (T\textsubscript{reg}) can express CD25 marker on the cell surface [2]. To distinguish these two, FoxP3 staining was employed as described in Materials and Methods. No difference was noted in the amount of T\textsubscript{reg} (CD4^+CD25^+ FoxP3^+) between RA and control patients (Table 3).

**Table 3.** T cell activation marker profile and T\textsubscript{reg} status in RA versus healthy control patients.

<table>
<thead>
<tr>
<th>Cell Subsets</th>
<th>RA (% cell ± SD)</th>
<th>Control (% cell ± SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4^+ CD25^+</td>
<td>4.9 ± 2.9</td>
<td>3.1 ± 2.0</td>
<td>0.001</td>
</tr>
<tr>
<td>CD4^+ CD25^+ FoxP3^+</td>
<td>0.8 ± 1.9</td>
<td>0.6 ± 0.4</td>
<td>0.259</td>
</tr>
</tbody>
</table>
The following was added to the flow cytometry procedure in Methods section of the manuscript (Page 6, line 9 from the top):

Both the activation status of T cells (CD4⁺CD25⁺) and the amount of regulatory T cells (CD4⁺CD25⁺FoxP3⁺) present were revealed using CD25 ECD (Beckman Coulter, 6607112) and APC-anti-human FoxP3 (eBioscience, 17-4776-73) antibodies.

Furthermore the following was added to the Discussion section of the manuscript (Page 12, last paragraph):

Based on our flow cytometry analysis, RA patients displayed higher levels of activated T cells compared to healthy controls. This finding is in accordance with previous studies demonstrating the presence of higher levels of CD4⁺CD25⁺ peripheral blood lymphocytes in RA patients compared to healthy individuals [3, 4]. However we did not observe any change in the level of CD4⁺CD25⁺ FoxP3⁺ T cells between RA and control groups. In accordance with previous studies, no relationship was found between disease activity and CD4⁺CD25⁺ or CD4⁺CD25⁺ FoxP3⁺ T cells in RA patients [5]. Whether any subtle increase in TRAIL or TRAIL receptor expression observed on CD4+ T cells in RA patients is simply a consequence of T cell activation remains to be clarified.

A note to reviewer: Considering the rise in TRAIL and TRAIL receptor expression levels and their correlation to disease activity; our discussion mainly concentrates on CD8+ T cells rather than CD4+ T cells. This does not mean that CD4+ T cells are less important, it just
means that for newly diagnosed RA patients TRAIL and TRAIL receptor composition on CD8+ T cells might be more important in terms of disease activity.

Reviewer 2

Comment 1: Percentage of expression ranging to 0-2% should not be considered “high expression” because the variability of the samples percentage, even comparing to control donors (no expression). The authors should explain that in CD4 positive cells, the expression of TRAIL and their receptor is barely perceptible or similar. It is not relevant if the data show a “35 fold increase in DcR1 expression” if you compare with a control sample without expression. The differences in the percentages should write in another way.

Response: As newly integrated into our discussion (Page 11 last paragraph) T cell lymphocytes of healthy individuals expressed low levels of TRAIL and TRAIL receptors on the cell surface. This does not mean the expression is absent. The fact that the level of expression was increased in RA patients does not mean that RA patients’ lymphocytes express high levels of TRAIL and TRAIL receptors. What we stated in our discussion is that both CD4+ and CD8+ T lymphocytes of RA patients displayed higher levels of TRAIL and its receptors on the cell surface compared to healthy control individuals.

Comment 2: The authors indicated in the text: “Representative FACS data” should be changed by “Representative FC data”. FACS meaning is Fluorescence Activated Cell Sorting. In this paper, only flow cytometry is done, not sorting.
Action Taken: FACS has been changed to FC.

Comment 3: The results shown in this paper indicated that TRAIL is not expressed in naïve T lymphocytes from healthy donors. Others group demonstrated that TRAIL is expressed in T lymphocytes. This point should be further discussed.

Response: Our data suggest that TRAIL is expressed but at low levels in T lymphocytes isolated from healthy volunteers. Most of the studies showed that freshly isolated PBT cells express very low levels of TRAIL on their surface and the expression of TRAIL could be induced in both CD4+ and CD8+ T cells.

Comment 4: Relating to DR4 and DR5 expression, Hasegawa (2004) did not observe DR4 and DR5 expression in naïve T lymphocytes from peripheral blood. By contrast, authors indicated DR5 expression. They should discuss this point.

Action Taken: Hasegawa et al. have reported that CD4+ T lymphocytes expressed none of the TRAIL receptors while only TRAIL-R4 (DcR2) expression was observed on CD8+ T lymphocytes [6]. Another study has demonstrated the expression of all TRAIL receptors in addition to TRAIL ligand on CD4+ T cells using flow cytometry [7]. Differences between these two studies were attributed to monoclonal antibody used. Thus the following sentence was added to the discussion section of our manuscript (Page 11, last paragraph).

“T cell lymphocytes of healthy control individuals expressed low levels of TRAIL and TRAIL receptors on the cell surface. While this is in accordance with a study conducted by Lu et al. [7], Hasegawa et al. have shown that only DcR2 expression but no other TRAIL receptor...
expression was detectable on CD8+ T cells [6]. Differences between these studies including ours could be attributed to differences in monoclonal antibodies used.”

REFERENCES:


