Author’s response to reviews

Title: Mycophenolate Mofetil Modulates Adhesion Receptors of the beta1 Integrin-Family on Tumor Cells: Impact on Tumor Recurrence and Malignancy

Authors:

Tobias Engl (engl@em.uni-frankfurt.de)
Jasmina Makarevic (jmakarevic@air-net.de)
Borna Relja (jmakarevic@air-net.de)
Iyad Natsheh (blaheta@em.uni-frankfurt.de)
Iris Muller (Iris.Mueller@em.uni-frankfurt.de)
Wolf-Dietrich Beecken (beecken@em.uni-frankfurt.de)
Dietger Jonas (beecken@em.uni-frankfurt.de)
Roman A Blaheta (blaheta@em.uni-frankfurt.de)

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To whom it may concern,

we are very happy about your positive answer. We have now changed the manuscript according to the suggestions of the referee and hope that the manuscript will meet your satisfaction. You will now find the revised manuscript, figures and our answers to the referee comments.

Sincerely yours,

PD Dr. phil. nat. R. Blaheta
**Answers to the referee’s comments**

1. The authors are not consistent in their use of terms to describe integrins. For example, a4b1 is described both as VLA-4 and CD49d. In addition, since this paper is being published for the cancer community, it would be much clearer to consistently use the heterodimer description of the integrin (e.g. a4b1, a5b1) which will make the paper easier to follow for those in the cancer community (the cancer community almost never uses the CD nomenclature for integrins).

**Our answer:** We changed the text accordingly and used the heterodimer description throughout the text.

2. What was the rationale behind the choice of doses for MMF?

**Our answer:** Based on own and other studies, significant effects of MMF on cell cultures have been observed within the range of 0.1-1 µM. Most notably, 0.1 and 1 µM reflect the clinical relevant dosage of MMF. We have now added this point in “Materials and methods”, chapter “Mycophenolate Mofetil (MMF)”: **Tumor cells were pretreated with clinically relevant concentrations of 0.1 µM and 1 µM MMF** (Roche Bioscience, Grenzach-Wyhlen, Germany).

3. The re-differentiation of DU-145 cells to the low-invasive phenotype is mentioned several times and this is incorrect for several reasons. There is not necessarily a correlation between decreased adhesion and decreased invasiveness (in fact, in many cell lines, it is the opposite). Also, DU145 cells are poorly invasive to start so it is not clear what the change in integrin expression might mean in this cell line.

**Our answer:** We absolutely agree that cell adhesion and transendothelial penetration reveal different programs in the tumor invasion cascade and might not happen in parallel. We now took care of this and changed several text phrases to replace the term “invasion” by “adhesion”:

Abstract: We conclude that MMF possesses distinct anti-tumoral properties, particularly in colon and prostate carcinoma cells. Adhesion blockage of HT-29 cells was due to the loss of
alpha3beta1 and alpha6beta1 surface expression, which might contribute to a reduced invasive behaviour of this tumor entity. The enhancement of integrin beta1-subtypes observed in DU-145 cells possibly causes re-differentiation towards a low-invasive phenotype.

Discussion, p11, second para: From a clinical viewpoint, distinct adhesion-blocking properties of MMF (former: invasion-blocking properties) ….

Discussion, p12, second para: …. surface expression directly contributes to the reduced adhesive behaviour (former: invasive behaviour) of HT-29 cells, b) Enhancement of integrin beta1 subtypes might cause re-differentiation of DU-145 cells towards a low-adhesive phenotype (former: low-invasive phenotype).

The role of integrin subtypes and splice variants in tumor development and malignancy is still not clear. Recently, differentiation inducing activities on tumor cells have been ascribed to MMF by upregulating integrin related structures (CD11b) on the tumor cell surface (Inai K et al. Leuk Res 2000; 24: 761-768). Evans and coworkers demonstrated that introduction of a wild-type beta 1 integrin subunit into SCC4 tumor cells stimulates differentiation by activating integrin signaling pathways that control differentiation (J Cell Biol 2003; 160: 589-596; see also: Fornaro et al. Matrix Biology 1997, 16: 185-193). Bello-DeOcampo and coworkers (ref. 20 in the manuscript) have pointed out that beta1 integrin receptors are required for cellular differentiation, and a loss correlates with de-differentiation.

Nevertheless, there is still no evidence that this might be true for prostate tumor cells as well. Hypothetically, beta1 upregulation might either activate differentiation inducing signals or selectively inhibit tumor-promoting pathways. We therefore added in “Discussion”, page 12, last para: However, it still remains to be determined if MMF indeed acts as a differentiation inducing drug in prostate tumor cells. Beside the hypothesis that beta1 upregulation might activate differentiation inducing signals, selective inhibition of tumor-promoting pathways should also taken into consideration.

4. Do the authors have any explanation for the bi-phasic effects of MMF on adhesion and the expression of integrins?
Our answer: Currently, there is now clear idea why MMF causes integrin up-regulation in one tumor entity but down-regulation in another entity, both coupled with reduced tumor cell adhesiveness. Presumably, HT-29 and DU-145 tumor cells might be equipped with different enzyme systems, the intracellular signaling cascade might be activated differentially in colon versus prostate tumor cells, or sensitivity of specific pathways to MMF differs between both tumor types. We have now added this thought in “Discussion”, p13, first para: There is still no clear concept why MMF causes integrin up-regulation in one tumor entity but down-regulation in another entity, both coupled with reduced tumor cell adhesiveness. Presumably, HT-29 and DU-145 tumor cells might be equipped with different enzyme systems, the intracellular signaling cascade might be activated differentially in colon versus prostate tumor cells, or sensitivity of specific pathways to MMF might differ between both tumor types.

5. The authors do describe their statistical analysis methods but significantly different groups are not marked on the graphs or stated in the figure legends or results. This needs to be done. In addition, it is preferable to state the exact p value for the groups that are different rather than just stating that they are all p<0.05.

Our answer: Statistical significance are now marked in the graphs. Furthermore, exact p values are now given in the text.