Author's response to reviews

Title: Targeting of human interleukin-12B by small hairpin RNAs in xenografted psoriatic skin

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Author's response to reviews: see over
To
Dr J. A. Le Good,
Senior Scientific Editor,
BMC-series journals

Dear Dr. Le Good

We thank you and BMC Dermatology for considering our manuscript ‘Targeting of human interleukin-12B by small hairpin RNAs in xenografted psoriatic skin’. We were pleased to receive your reply and the reports concerning our manuscript including both positive responses and some relevant constructive criticism from both reviewers. We thank the reviewers for comments and suggestions and have aimed at revising the manuscript accordingly with careful consideration of all comments from the reviewers. As a result, we have included further experimentation, and the manuscript has been quite significantly modified and, we think, improved relative to the original version. We have now included experimental evidence that systemic administration of anti-IL12B monoclonal antibodies leads to amelioration of the psoriasis phenotype in the xenograft transplantation model, demonstrating the applicability of this animal model in studies of IL12B-targeted RNAi therapy. Also, immunohistological analyses of shRNA-treated skin have now been performed. The manuscript has been revised accordingly. Please find attached a revised version of the manuscript in which all new or revised text pieces are highlighted (red font).

Reviewer #1 (Nestle)

**Ad 1. Targeting of IL12B mRNA for treatment of psoriasis.** The reviewer is correct that we with this work are taking some of the very early steps towards development of RNA-directed therapies for psoriasis. In our hands, knockdown of IL12B mRNA does not ameliorate the psoriasis phenotype. Although this finding may stand out as negative data, we have previously seen consistent improvement of the phenotype by targeting TNF-α mRNA in the same model. In this perspective, we believe that our findings provide important information as a basis for comparison of different cytokine targets in the model.

**Ad 2. Discussion of the suitability of lentiviral vectors for the treatment of humans.** We do not envision at this stage that lentiviral vectors are suitable for treatment of inflammatory skin disorders. Nevertheless, provided the high lentiviral transfer efficiency in skin, such vectors allow efficient shRNA delivery in skin and therefore represent a unique tool in proof-of-principle studies of cytokine targeting by RNA interference. We believe that this point has been made already towards the end of the manuscript (last paragraph of Discussion, p. 26, lines 6-8 from bottom). Given the fact that lentiviral vectors are here used primarily as a delivery tool for studies of skin inflammation, we did not find it relevant to include a more general discussion of the use of lentiviral vectors in humans, for example in relation to the current clinical use of such vectors in treatment of hematopoietic stem cells.
Ad 3. Problems with the clinical psoriasis scoring.

(i) Subjective score: Treatment with lentiviral vectors encoding shIL-12B or shirrelevant were given on day 0 and given only once. After treatment all mice were coded and randomly placed in new cages prior to scoring; this procedure was performed by an independent person. In this way the treatment of the individual mouse was blinded to the observer throughout the study (as described at p. 13 middle section). All scores were evaluated by the same observer.

(ii) A slight decrease in the semi-quantitative clinical psoriasis score was observed in the negative control treated group post treatment. This is usually the case when we employ this psoriasis xenograft transplantation model (see ref. 7 for an example). Others have carried out an additional injection of activated autologous PBMCs during their study to “maintain” the clinical psoriatic phenotype. We did not inject additional PBMCs in the model we employed, as the treatment window – the difference between the negative control treated group and the tested group – usually presents a difference large enough to detect significant treatment effects. Also, when evaluating the histological sections, evaluated approximately 5 weeks after transplantation (1-2 weeks healing + 3 weeks treatment), we observe no significant reduction in the epidermal thickness measured in transplanted lesional psoriatic skin as compared to non-transplanted lesional psoriatic skin.

Ad 4. Presentation of histology. Based on both reviewers’ comments, we decided to carry out a panel of histological stainings. As suggested, histology is now presented; a new panel has been included in Figure 5 (figure 5e) addressing issues of keratinocyte proliferation and lymphocyte expansion. To address keratinocyte proliferation, we have included a ki-67 immuno-histochemical staining where similar expression patterns of ki-67 in the shIL-12b and the negative control treated groups were observed, indicating no effect of treatment on the proliferation status of the keratinocytes. Likewise, immunohistochemical staining for CD4 and CD8 positive lymphocytes revealed that a reduction in the lymphocytic infiltrate in the shIL-12b treated grafts could not be detected within the 3-week time frame that was used in the experiment. The immunohistological findings are described at p. 21 (bottom) and p. 22 (top).

Ad 5. Is IL-12b mRNA a relevant target molecule in the model?. We understand the reviewers concern, as a model, by nature, does not resemble all aspects of a disease phenotype. To consolidate the importance of IL-12b in the psoriasis xenograft transplantation model, we have now included experimental data demonstrating the effect of treating transplanted lesional psoriasis skin in the model by injecting ustekinumab, the monoclonal antibody targeting IL-12b protein, approved in the clinic for treating psoriasis. In the model, ustekinumab treatment significantly reduced both the semi-quantitative clinical psoriasis score and the epidermal thickness. Many treatments approved in the clinic (e.g. steroids, vit D, and CsA) have demonstrated similar treatment effects in the psoriasis xenograft transplantation model. These findings are presented in a new Figure 1, and the first section of the manuscript describes the applicability of ustekinumab administration in the model (p. 16). Altogether, these data support that IL12B is an appropriate target in the xenograft model and that the model is relevant in studies of IL12B-directed RNA interference.
**Ad 6. Combining two control groups.** No significant difference was found between shIRrelevant and untreated skin grafts when comparing semi-quantitative clinical psoriasis scores for the duration of the experiment and the epidermal thickness, as stated on p. 21, lines 5-8. Also, we have included in the text that a similar degree of psorasisiform papillae, Monroe’s abscess, vessel formation and parakeratosis was observed in the two groups (p. 21, lines 7-8). Concerning IL-12B mRNA measurements and immunohistochemical staining, only grafts receiving shRNA encoding lentivirus were included in the assay which is stated in the figure legend of Figure 5d and 5e.

**Ad 7. Inclusion of shTNFα as a control.** We acknowledge that the shRNA targeting TNFα would have been an appropriate control in the study, but since the study requires the use of limited available keratome skin biopsies from psoriasis patients, this control was omitted.

**Response to minor comments**

**Ad 1. The use of only two MOI units in the study does not constitute a proper dose response experiment.** We agree that use of two vector concentrations does not constitute a proper dose response experiments. However, this experiment was not meant as a dose response experiment since the dose-dependency of the RNAi response is very well established in the literature (see e.g. Siolas et. al., Nature Biotech. 2005 or Kim et. al., Nature Biotech. 2005). The experiment with low MOI (single lentiviral insertion) was included to provide evidence that shRNA expression from the lowest possible MOI (a single lentiviral insertion) is capable of reducing the intracellular levels of IL12B mRNA.

**Ad 2. During the initial whole mouse luc experiments (figure 3) it is unclear whether normal or psoriatic skin was used. There is no mention in the text of figure 3a being separate from figure 3b).** We apologize for the lack of clarity concerning this experiment. To clarify that normal skin was used in the luciferase whole mouse luc experiment, the term ‘normal human skin’ has been used instead of ‘human skin’ (p. 20, line 6 and p. 36, line 3 from bottom).

**Ad 3. Figure 4b. The authors suggest a ‘tendency’. This phraseology is too ambiguous and data should be interpreted on face value.** Face values have now been included in the ‘Results’ section describing the findings presented in Figure 4b (Figure 5b in the revised manuscript), and the term ‘tendency’ removed (p. 21, second paragraph).

**Ad 4. Comments concerning angle of sectioning.** We agree that measuring epidermal thickness is not a simple task and care in this procedure must be taken. In the present manuscript, we employed an in vitro technique based on microscopic visualization. It is well known that the proportions of the tissue samples are distorted in the preparation process due to contraction of the skin following biopsy and in the process of dehydration and subsequent infiltration with paraffin of tissue samples for standard light microscopy. To minimize the preparation artifacts in this study great care was taken to ensure that vertical sections were made. Consequently, embedded tissue was reoriented if dermal papilla were observed not to be vertically aligned which is indicative of vertical sectioning. Nevertheless, some variation in the angle of sectioning cannot be excluded. Other in vivo methods are being developed that may be relevant to employ in future studies such as high resolution ultra sound (HRU), confocal laser microscopy, multiphoton laser
tomography, optical coherence tomography (OCT), and magnetic resonance imaging (MRI) where preparation artifacts may be omitted.

Ad 5. Regarding “Betnovat at day 14”. No, the reduction is not significant (p = 0.052, Mann Whitney test).

Ad 6. Regarding Figure 5. Figure 5 (Figure 6 in the revised manuscript) was intended solely for the discussion with the purpose of comparing the present study targeting IL12B to results obtained in our previous publication targeting TNFα. The data concerning IL12B is already presented in the results section and, hence, figure 6 does not contain new data from the current study. The figure has been maintained as a part of the discussion to illustrate the important difference between TNF-α- and IL12B directed RNAi.

Ad 7. Knockdown in primary keratinocytes or dendritic cells. We agree that it would be interesting to investigate cytokine knockdown primary cells and possibly in vivo in skin-homing antigen-presenting cells. For the present work, however, knockdown of IL12B has not been pursued in primary keratinocytes or dendritic cells, since we reasoned that shRNA potency should not be dependent on cell type. Since several cell types in the skin produce IL12B, we chose to test shRNA potency in vitro in a common cell line made for the purpose (HeLa-IL12B) and then confirm IL12B knockdown in the in vivo settings in the xenotransplanted psoriatic skin.

Ad 8. Regarding ‘over-interpretation of the results’. The discussion section has been revised accordingly and now also include a discussion of the results describing the effects of ustekinumab treatment in the model (p. 24, lines 9-12).

Ad 9. The effects of shRNAs on IL12 and IL23. We have not made any attempts to investigate the differential effect of IL12B knockdown on the levels of IL12 and IL23. Although the clear rationale was that both cytokines would be affected by the treatment, we acknowledge that this would be an interesting study that could also shed light on the differential effect and pathogenic contribution of each of the two cytokines. Our focus here has been to investigate the effect of knocking out a common subunit of the two cytokines.

Ad 10. Missing legends and error bars. In all figures containing multi-barred graphs we have now included legends explaining white and black bars, respectively, in the figure. The experiment shown in figure 3b of the revised manuscript has been repeated in triplicates and only for a single MOI, since the dual MOI study in figure 3a already confirmed an MOI-dependent knockdown and also that a single lentiviral integration was sufficient to mediate IL12B knockdown. Error bars have now been included in figure 3b.
Ad 11. Measurement of IL-12b over a time course. The timepoint in figure 5d is stated in the legend (p. 37, line 4 from bottom). Biopsies from the xenografted psoriatic skin injected with shRNA-encoding lentiviral vectors were acquired at treatment endpoint three weeks post-transduction and evaluated for IL-12B gene expression by qRT-PCR. Measuring IL-12b over a time course would require a new experimental setup including several mice for each relevant time point. As the procedure of taking a biopsy inflicts a wound healing process it would not seem optimal to obtain more than one biopsy per time point per mouse. Investigating the expression of IL-12b over a time course would of course be interesting, but the number of experimental animals was of critical concern in the design of the experimental setup.

Reviewer #2 (Zeeuwen)

Ad 1. Regarding genetic factors. We agree that some of the genetic factors contributing to the susceptibility for psoriasis should be touched upon in the introduction. Polymorphisms in IL-12 and IL-23R have now been mentioned (p. 4, lines 7-8 from bottom).

Ad 2. Immunohistochemistry. As mentioned above, we decided to perform and include immunohistochemistry staining for relevant markers in shRNA-treated skin (described at p. 21, bottom). Figure 5e has now been included in the manuscript showing immune-histochemical staining for SKALP/elafin, hBD2, Ki-67, CD4, and CD8. These data strengthen the conclusion that IL12B-directed RNA targeting does not ameliorate the psoriasis phenotype within the 3-week window of the experiment.

Ad 3. Positive control. We have now included experimental data demonstrating the potency of ustekinumab treatment in the model (new figure 1). Based on these findings, we agree that ustekinumab would have been an obvious positive control for shRNA-based treatment. However, we sought in the shRNA experiment to utilize a control that was topically applied and therefore would be expected to serve as a control for local treatment rather than systemic. We have maintained Betnovat treatment as a positive control treatment for our shRNA studies.

Ad 4. Why do already published siRNAs not work as shRNAs? Based on our current knowledge it is not possible at this stage to explain why previously published target sequences did not work in our hands. However, it is expected that synthetic siRNAs may not necessarily perform as transcribed shRNAs and vice versa. Hence, other parameters including expression, structural folding, and processing may affect shRNA efficacy, whereas yet other parameters like transfection efficiency and RNA stability may affect siRNA functionality. A brief discussion of these aspects has now been included in the ‘Discussion’ (p. 23, end of second paragraph).

Ad 5. Regarding inclusion of immunohistochemical pictures in figure 5. As previously mentioned, we intended by including figure 5 (figure 6 in the revised manuscript) to provide a comparison of RNA-directed treatments targeting IL12B and TNF-α. Therefore, although data-based this figure has been included in the discussion to ease a comparison of the two strategies and not to provide a detailed phenotypic comparison
of the two approaches. As indicated, due to limitations on the patient-derived material, it has not been possible for us to repeat a full series of experiments including TNFα shRNAs as a positive control.

Response to minor comments

All minor revisions suggested by reviewer #2 have been acknowledged and appropriate alterations have been made to the manuscript. Revised sentences or text sections are highlighted with red font in the revised manuscript.

We are grateful to the reviewers for thorough and critical handling of our manuscript and believe that the reviewer’s comments have been seriously evaluated and utilized to improve the manuscript significantly. We are looking forward to receiving a decision on the revised manuscript.

Sincerely,

Jacob Giehm Mikkelsen, PhD
Corresponding author