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Interleukin-38 promotes skin tumorigenesis in an IL-1Rrp2-dependent manner

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1st Editorial Decision **14th Sep 2021**

Dear Dr. Zhou,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here. Very importantly, the manuscript needs to be re-organised and proof-read by a native speaker!

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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See also our guide for figure preparation:
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments were performed, if these were biological or technical replicates, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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9) Please also note our reference format:
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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) Please add content to the 'Author contributions' and 'Acknowledgements' sections.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This report investigates the pro-tumorigenic role of IL-38 in skin tumorigenesis. They show that human cSCC and DMBA-TPA-induced skin tumors in mice have decreased level of IL-38 expression. However, keratinocyte-specific IL-38 deletion in mice suppressed skin tumor development. Moreover, the authors also provide role of IL-38 on proliferation and skin cancer hyperplasia both in vivo and in vitro. Finally, IL-38/IL-1Rrp2 mediated pathways activate JNK/AP1 signaling to promote pro-inflammatory cytokines expression and skin tumor development. Thus, the authors demonstrate that IL-38 has an important role and can be a therapeutic target in skin tumorigenesis.

IL-38 belongs to the IL-1 cytokine family and IL-36 cytokine group as well. Accordingly, previous publications have shown that IL-38 acts as an antagonist of IL-36 cytokine function (PMID: 22315422). That has been proposed as a reason why IL-38 can have an anti-inflammatory function by blocking IL-36 signaling. On the other hand, IL-38 is a newly discovered cytokine in inflammatory condition like allergic asthma and rheumatoid arthritis, and there are less known about IL-38 function in cancer. IL-38 mediated signaling in skin tumor development is an interesting paradigm and has novelty, but some points are still questionable about IL-38 anti-inflammatory function and expression level in skin cancer development.

Major points:

Figure 1: IL-38 showed different level between normal skin and cSCC. But if there is lower expression of IL-38 in both human and mouse skin tumor, how does it act to promote skin cancer development? It is hard to understand even-though they describe in discussion. Please see below comment about Figure 6. In addition, this work suggests that blocking IL-38 could have preventive effect but not a therapeutic impact as the cytokine is lost in tumors - this point needs to be discussed in the paper.

Is IL-38 the only cytokine from IL-1 family that shows differential expression in skin cancer compared to normal skin in mouse and human? The known roles of other IL-1-like cytokine, like IL-18 and IL-33, in skin cancer needs to be discussed. Also, can authors determine the levels of these other cytokines in their human and mouse skin/tumor samples shown in Figure 1?

Figure 2: Authors have demonstrated that IL-38 is decreased in DMBA/TPA model. Considering that IL-38 can potentially be expressed by cells other than keratinocytes, what is IL-38 expression level in the skin/tumor of IL-38 f/f versus K14-Cre, IL-38 f/f after DMBA/TPA treatment? Do authors see complete loss of IL-38 from skin lysate by deleting the cytokine expression in the keratinocytes? How about from DMBA/TPA-induced tumors comparing of IL-38 f/f to K14-Cre, IL-38 f/f? It is important to know if levels in K14-Cre, IL-38 f/f tumors are even lower than IL-38 f/f to support some degree of role for IL-38 in tumor progression? This especially critical as authors argue K14-Cre, IL-38 f/f have more invasive tumors but based on Figure 1 data, it is assumed that IL-38 is already lost in early tumors of WT mice.

Figure 3: what is IL-38 expression level in normal keratinocyte cell lines compared to SCC cell lines, not only A431 cells? Is there a similar pattern as seen In vivo? If SCC cell lines have lower IL-38 expression, how does knockdown of IL-38 reveal cytokine expression changes? Or SCC cell lines highly express IL-38??

For example, there are dramatic changes in the level of Cxcl1 and Tnf-alpha with/without knockdown of IL-38 in figure 3. But there are only smaller changes of Cxcl1 and Tnf-alpha in figure 6 and 7. Does this reflect the heterogenicity in the efficiency of IL-38 knockdown in different experiment?? The data suggest that the SCC cell line used has high IL-38 expression unlike the findings in vivo

IL-38 also induces anti-inflammatory genes (PMID: 22315422), how about the expression of this set of genes in the cell line upon IL-38 induction versus knockdown?

Figure 6: IL-38 promotes skin tumorigenesis even though IL-38 is lost in cSCC - what is IL-1Rrp2 receptor expression in skin versus tumor? If this receptor expression is also changed, it may help explain the complex of role of IL-38 in skin tumorigenesis. IL-38 can also bind to IL-36 receptor, how about IL-36 receptor expression as a control in skin and tumor.

Minor points:

- Revise picture to show a clearer IL-38 positive staining in figure 1C
- The labels in Figure 1 are off (no G label in the figure itself...)
- Add IL-38 expression level in Figure 3C and D.
- Hard to see Ki67 staining in IHC, could change to immunofluorescent-stained image with Ki67.
- Add IL-38 western blot data in Figure 4I and J.
- Revise description of Figure 5G in manuscript.

Referee #2:

In this study, the authors elucidate a role for interleukin-38, a member of the IL-1 family, in chemically-induced skin

carcinogenesis. They demonstrate that IL-38 is downregulated in cutaneous squamous cell carcinomas and use keratinocyte-specific IL-38-deficient mice to show that less tumours arise in these mice relative to controls. Additionally, a lower conversion rate to SCCs was observed in IL-38-deficient mice. The authors go on to show that IL-38 promotes the infiltration of immune cells in the skin upon DMBA-TPA treatment and demonstrate that IL-38 can drive cell proliferation. In in vitro studies, the authors show that IL-38 binds to IL-1Rp2 and this binding induces inflammatory mediators and cell proliferation.

Major concerns:
The manuscript needs to be edited to a major extent, as many sentences are incomprehensible. The analyses of the in vivo studies are difficult to interpret, as figure legends often do not correspond with what is shown in graphs. Many claims are not sufficiently backed up by the data.

Quantification of IL-38 staining in Figure 1 is of poor quality. This Reviewer does not see any IL-38 staining in mouse tissues. These stainings should be performed by immunofluorescence (with the addition of appropriate controls).
Panels are not properly indicated in figure legend 1: panel f shows immunoblotting of IL-38; there is no panel G.

If the authors want to claim that there is no significant effect on epidermal barrier as is now stated on page 3, they need to perform epidermal barrier functionality assays, such as assessment of transepidermal water loss, dye permeability assays,...

In figure 2e: the graph is labelled as percentage of tumour-free survival, what is meant by this? Did animals die during the protocol? Or do the authors show percentage of mice that are tumour-free? The percentage of mice that develop tumours should be shown for the entire length of the promotion phase. This panel is not described in figure legend.

The authors claim that 12.94% of tumours in control mice develop into SCCs. This is extremely high for mice on C57Bl6 background. How did the authors investigate conversion? The local micro-invasive foci that the authors show in Fig2f are not clear and do not convincingly point to malignancy. The authors should perform Keratin-8 staining to back up this claim.

In Figure 3 the authors show flow cytometry data as percentage of total live cells. In the corresponding figure legends they claim these graphs show percentage of total immune cells. It is not clear what is shown here, hence the data are hard to interpret. Also, flow cytometry gating strategy is missing and amount of immune cells have not been standardized for the skin surface area that was digested. How long were these mice treated with DMBA/TPA? Which antibodies were used to distinguish different immune cell populations? This is essential information to assess the quality of these data and is sorely lacking from the manuscript.

The authors claim that IL-38 affects cell proliferation and migration in a SCC cancer cell line. However, with the scratch-wound assay, proliferation and migration can not be interpreted separately. If the authors want to make claims about migration potential, they have to incubate the cells with mitomycin to rule out any proliferation effects.

Referee #3:

In this study, the authors show a role of IL-38 cytokine in promoting skin tumorigenesis, through a signaling pathway involving the IL-1Rrp2 receptor and downstream activation of JNK/AP1.
The authors first illustrated decreased IL-38 expression in both human cSCC and a DMBA/TPA murine model of cSCC. Using a keratinocyte-specific knockout of IL-38 (K14Cre/+;IL-38f/f) in mice, they show that IL-38 deficiency suppresses the development and progression of DMBA/TPA induced skin tumors, with decreased inflammatory cell infiltrations and inflammatory cytokines. The authors also show that IL-38 directly promotes proliferation and migration of skin cancer cells, through regulation of cell cycle proteins, by using an in vitro approach based on overexpression (Oe-IL-38) or silencing (LV-shIL-38) of IL-38 in A431 cSCC cell line. With the same system, they show that IL-38 function on cell migration and proliferation is mediated through its binding to the IL-1Rrp2 receptor to activate downstream JNK/AP1.
In general, the data presented were for the most part clear and convincing with proper controls. The experiments performed are presented in a logical order, they are appropriate and well-executed, and based on several complementary approaches (in vivo model, analysis of publicly-available databases, cell culture for mechanistic/molecular/biochemical analysis).

Note that a previous study from the Dinarello lab already indicated that IL-1Rrp2 is involved in IL-38 signaling (PMID: 22315422). My specific comments are provided below.

Major comments
• There are several typos and sentences that are cropped or whose syntax needs to be improved as their meaning is unclear. This is particularly (but not solely) the case in the second part of the discussion.
• The anti-IL-38 staining in Fig1 is not that clear, possibly due to the resolution of the image or because IL-38 appears to be a (soluble) cytoplasmic cytokine. Can the authors detect IL-38 in the tissue of their keratinocyte-specific IL-38 knockout mice (K14Cre/+;IL-38f/f), to validate their staining?
• Based on their findings from Fig.5, the authors conclude that IL-38 binds to IL-1Rrp2, but not "to the other ... receptors" that

were tested. However, there are consistently weak but detectable bands for IL-38 (both for the 1-152aa and 20-152aa variant) to bind IL1RAcp-Fc in Fig5A and B.
• Along these lines, a previous study reported that IL-38 binds IL1RAPL1 on skin gd T cells (PMID: 30995480) to modulate the function of these cells. Yet, Fig.5 does not provide evidence for IL-38 to bind IL-1RAPL1. The authors should at least comment on these apparently different results in the discussion.
• In the discussion, the authors comments on the counter-intuitive findings that IL-38 levels are reduced in tumor tissues, while IL-38 promotes keratinocyte proliferation to support skin hyperplasia and tumorigenesis in their model. Yet they do not show (or at least discuss) on which cells IL-1Rrp2 is otherwise expressed in the tumor environment. Do immune cells potentially also express it and respond to IL-38 (see also my previous remark)? How much of a contribution to tumor development do the IL-38-dependent inflammatory cues produced by the malignant keratinocytes make? These aspects may possibly explain the apparently contradictory phenotypes the author observe, and should be at least reflected in the discussion.

Minor Comments:
• The authors should use the correct nomenclature while mentioning genes / transcripts versus proteins, in dependence on the species they refer to.
• It should be more clearly indicated how the histology was quantified (apparently automatically)? Was this done on whole images or per field of view?
• Similarly, it is not clear how AP1 activation was quantified (likely via a luciferase reporter assay, yet this is not clear).
• Page 1, 2nd paragraph, Introduction: 'The *IL-1* family of cytokines...'
• Fig 1, legend: labels (F) and (G) are not correct
• Fig 2: Missing figure legend on E
• The results in Fig 4 rather indicate that IL-38 promotes epidermal cancer cell proliferation (than hyperplasia).
• The first paragraph of the result section entitled "IL-38 forms a complex with IL-1Rrp2 and activates JNK/AP-1 signal transduction pathway in an IL-1Rrp2-dependent manner" seems to be more suited for the introduction part.
• As a minor side note: the lead contact is provided in the paragraph on data availability.
• I guess all study participants had *given* written informed consent, and no therapy had been given *before sample collection* (i.e. samples were taken at diagnosis). Please precise or improve accordingly.

Dear Editor,

Thank you very much for your kind letter about our paper Manuscript #EMBOR-2021-53791V1 entitled "Interleukin-38 promotes skin tumorigenesis in an IL-1Rrp2-dependent manner". In this revised manuscript, we have carefully addressed all the comments from the reviewers and revised the manuscript accordingly. Our responses are given in a point-by-point manner below. All the changes of the manuscript are highlighted in yellow. Your consideration for this manuscript is highly appreciated. Based on these new data, we believe that the manuscript is significantly improved and suitable for publication in *EMBO reports*. We hope the revised version is now suitable for publication, and we look forward to hearing from you in due course. Thank you very much for your kindness.

Yours sincerely,

Jiong Li, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, Sichuan 610041, China. Email: lijionghh@scu.edu.cn

Responses to Reviewer #1:

Thank you for your review of our paper. We have answered each of your points below.

Question: Figure 1: IL-38 showed different level between normal skin and cSCC. But if there is lower expression of IL-38 in both human and mouse skin tumor, how does it act to promote skin cancer development? It is hard to understand even-though they describe in discussion. Please see below comment about Figure 6. In addition, this work suggests that blocking IL-38 could have preventive effect but not a therapeutic impact as the cytokine is lost in tumors - this point needs to be discussed in the paper.

Response: Thank you very much for your comments. We indeed observed the lower expression of IL-38 in both human and mouse skin tumors, the potential role of IL-38 should be addressed in skin tumor mouse model. Consistent with our results, decreased IL-38 levels were also observed in other cancers, such as non-small cell lung cancer and colorectal cancer (Chen et al, 2020; Wang et al, 2018). Note that another two IL-1 family members, IL-33 and IL-37, also showed apparent discrepancies between their expression level and function in mouse model of disease (Akimoto et al, 2016; Volpe et al, 1997). IL-33 expression was inversely correlated with the stages of human lung cancers, but IL-33 enhances lung cancer progression by selecting for more malignant cells in the tumor microenvironment (Akimoto et al, 2016). The expression of IL-37 increased in oral squamous cell carcinoma (OSCC) compared with normal control (Volpe et al, 1997). However, recombinant IL-37b treated cells showed decreased production of LPS-stimulated IL-6, TNF-α, and IL-1β, which have been reported to promote malignant transformation and tumor aggression in oral cancer (Akimoto et al, 2016; Volpe et al, 1997; Wang et al, 2010; Yoshida et al, 2012). Moreover, many studies have determined that IL-38 is increased in multiple diseases, while increased IL-38 inhibited the progression of inflammatory bowel disease, myocardial infarction, and type 2 diabetes, and blockade of IL-38 promoted the progression of acute respiratory syndrome, and fatal sepsis (Chai et al, 2020; Liu et al, 2020; Wei et al,

2020; Xie et al, 2020; Xu et al, 2018). Thus, the differential expression data give us the association between these modulators and diseases, while mouse model and cell model help to clarify their potential functions and mechanisms during diseases pathological processes.

In our results, IL-38 promoted skin tumorigenesis via regulation of pro-tumorigenic microenvironment and epidermal cell hyperplasia in DMBA/TPA induced skin tumor model (Fig 3C and D and Fig4A and B). Additionally, the promoting effect of IL-38 on expression of cancer-related inflammatory cytokines and proliferation and migration of skin tumor cells reaffirmed a tumor-promoting function for IL-38 in skin tumor (Fig 3E and F and Fig 4C-J). These results discovered the potential underlying mechanism by which IL-38 regulates skin cancer.

In addition, our results showed there was no difference between *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$*-IL-38*$^{f/f}$ mice, but IL-38 keratinocyte-specific deletion suppresses the development of tumors. Moreover, overexpression or knockdown of IL-38 in tumor cells indicated that IL-38 promote the expression of cancer-related inflammatory cytokines and proliferation and migration of tumor cells. These results indicated that IL-38 could promote skin tumorigenesis, which suggest that blocking IL-38 may have a preventive and therapeutic impact on skin cancer.

In the revised manuscript, we have added new data (Fig EV3A-F) and the following sentence to the Discussion section: “In our study, for the first time, we investigated the expression pattern of IL-38 protein in the skin tissues of cSCC patients and DMBA/TPA-induced mouse tumors. We found that the expression of IL-38 in skin tumor tissues was lower than that in normal tissues, which is consistent with the results in non-small cell lung cancer and colorectal cancer (Chen et al, 2020; Wang et al, 2018). A decrease in IL-38 suggests that it might play an inhibitory role in skin tumors. However, we found that IL-38 promoted the pathogenesis of skin tumors *in vivo* and *in vitro*. Considering that IL-38 can potentially be expressed by cells other than keratinocytes, we performed experiments to detect the expression of IL-38 in DMBA/TPA-treated skin and DMBA/TPA-induced tumors of *Il-38*$^{f/f}$ and *K14*$^{Cre/+}$*-Il-38*$^{f/f}$ mice. We observed complete loss of IL-38 from skin

lysates in DMBA/TPA-treated skin and DMBA/TPA-induced tumors in *K14$^{Cre/+}$-Il-38$^{f/f}$* mice (Fig EV3A and B). To detect if this contradiction was caused by IL-1Rrp2 expression, we performed experiments to detect IL-1Rrp2 expression in skin versus tumor. Our results showed that IL-1Rrp2 expression did not differ significantly between normal skin and tumors (Fig EV3C–E). Similar apparent contradictions were observed for IL-33 and IL-37, the other two IL-1 family members. IL-33 expression was also inversely correlated with the stages of human lung cancers, but IL-33 enhances lung cancer progression by selecting more malignant cells in the tumor microenvironment (Akimoto et al, 2016). The expression of IL-37 is higher in oral squamous cell carcinoma (OSCC) than in normal controls (Volpe et al, 1997). However, recombinant IL-37b-treated cells showed decreased production of LPS-stimulated IL-6, TNF-α, and IL-1β, which have been reported to promote malignant transformation and tumor aggression in oral cancer (Akimoto et al, 2016; Volpe et al, 1997; Wang et al, 2010; Yoshida et al, 2012). Moreover, many studies have determined that IL-38 is increased in multiple diseases, while increased IL-38 inhibited the progression of inflammatory bowel disease, myocardial infarction, and type 2 diabetes, and blockade of IL-38 promoted the progression of acute respiratory syndrome, and fatal sepsis (Chai, Lin et al., 2020, Liu, Chen et al., 2020, Wei, Lan et al., 2020, Xie, Yan et al., 2020, Xu, Lin et al., 2018). Our data and those of previous studies indicate that the differential expression of a biomarker observed in clinical data may not conclusively demonstrate the real role of the biomarker in pathological processes without corresponding experiments in animal models.", corresponding description have been added in Materials and Methods sections.

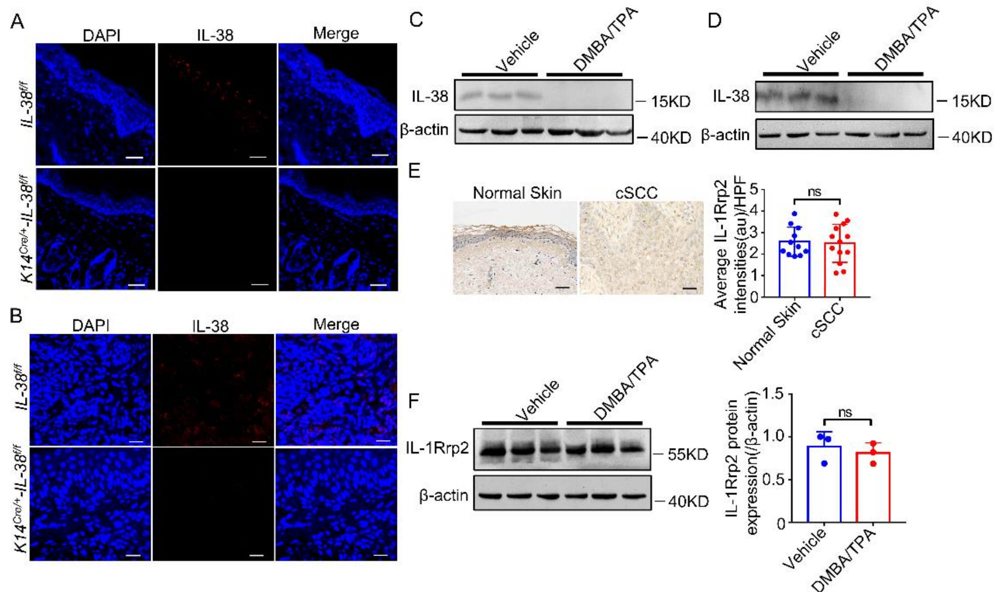


Figure EV3. The expression of IL-38 and IL-1Rrp2 in tissues.

(A and C) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 3 weeks to induce the skin inflammation. (A) Representative immunofluorescent staining micrographs of IL-38 in the skin of $Il\text{-}38^{f/f}$ and $K14^{Cre/+}\text{-}Il\text{-}38^{f/f}$ mice. Scale bars represent 100 µm. (C) Relative expression of IL-38 in the skin of $Il\text{-}38^{f/f}$ (n = 3) and $K14^{Cre/+}\text{-}Il\text{-}38^{f/f}$ (n = 3) mice were detected by western blot.

(B and D) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 32 weeks to induce the skin tumors. (B) Representative immunofluorescent staining micrographs of IL-38 in the tumors of $Il\text{-}38^{f/f}$ and $K14^{Cre/+}\text{-}Il\text{-}38^{f/f}$ mice. Scale bars represent 100 µm. (D) Relative expression of IL-38 in the tumors of $Il\text{-}38^{f/f}$ (n = 3) and $K14^{Cre/+}\text{-}Il\text{-}38^{f/f}$ (n = 3) mice were detected by western blot.

(E) Representative immunohistochemical staining micrographs of IL-1Rrp2 from normal patients (n=11) and tumors of cSCC patients (n=13). Scale bars represent 100 µm. The graph shows average intensities of IL-1Rrp2 per high-powered field in tissues. Mean ± SD.

(F) Relative expression of IL-1Rrp2 in mouse normal skin (n=3) and DMBA/TPA induced tumors (n=3). The graph shows the quantification of mean IL-1Rrp2 expression in tissues. Mean ± SD.

Question: Is IL-38 the only cytokine from IL-1 family that shows differential expression in skin cancer compared to normal skin in mouse and human? The known roles of other IL-1-like cytokine, like IL-18 and IL-33, in skin cancer needs to be discussed. Also, can authors determine the levels of these other cytokines in their human and mouse skin/tumor samples shown in Figure 1?

Response: We truly appreciate your kind suggestions. IL-38 is not the only cytokine from IL-1 family that shows differential expression in skin cancer compared with normal skin in mouse and human. Based on the reviewer's request, we have added corresponding data in revised manuscript. We searched NCBI GEO database to identify the expression of IL-1 family members in skin cancer compared with normal skin. Our analysis of the GEO database revealed that *IL-1α, IL-1β, IL-18* and *IL-36γ* expression was lower in human skin tumor cells compared with normal human keratinocytes, and *IL-18, IL-33, IL-36α, IL-36β, IL-36γ*, and *IL-37* showed no significant difference in gene expression (Fig EV1B). Moreover, *IL-18* was lower in DMBA/TPA induced tumors compared with normal mouse skin consistent with the above result, while *IL-1β* and *IL-33* expression increased in tumors, and *IL-1α, IL-36α, IL-36β*, and *IL-36γ* showed no significant difference in gene expression (Fig EV1C). Furthermore, we have performed experiments to detect IL-18 expression in human and mouse skin tumors. Our results showed that the expression of IL-18 decreased in human cutaneous squamous cell carcinomas (cSCC) and DMBA/TPA induced mouse tumors compared with normal skin tissues (Fig EV1D-F). These results indicated that other IL-1 family members have similar properties to IL-38.

In addition, IL-18 mRNA expression was found to be significantly lower in melanoma tissues than normal tissues (Gil & Kim, 2019), and IL-18 was reported to promote the growth of B16F10 melanoma cells (Cho et al, 2000) and enhance the ability of melanoma cells to migrate via the generation of ROI and the MAPK pathway(Jung et al, 2006), which consistent with our findings. The high expression of IL-33 was found to associate with better overall survival in melanoma patients (Wagner et al,

2020). The role of IL-33 in melanoma has been investigated in mouse models extensively and researchers observe both its pro- and anti-cancer effects, which may be due to the timing and dosage of IL-33 administration, and the specificity of IL-33 (Gao et al, 2013; Gao et al, 2015; Jevtovic et al, 2020; Long et al, 2018; Schuijs et al, 2020). These results indicate that IL-1 family members play an important role in progression of skin cancer.

In the revised manuscript, we have added new data (Fig EV1B-F) and the following sentence to the Results and Discussion section: "Moreover, we searched the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) and identified multiple IL-1 family members exhibiting differential expression in skin cancer compared with normal skin searching (Fig EV1B and C). Further, experiments to detect IL-18 expression in normal human and mouse skin and tumors demonstrated that the expression of IL-18 decreased in human cutaneous squamous cell carcinoma (cSCC) and DMBA/TPA-induced mouse tumors compared with its expression in normal skin tissues, which is consistent with the findings in the GEO database (Fig EV1D–F). Collectively, these results suggest a possible role of IL-38 in skin tumorigenesis." and "The role of other IL-1 family members, such as IL-18 and IL-33, in melanoma has been investigated in mouse models extensively. IL-18 mRNA expression was found to be significantly lower in melanoma tissues than normal tissues (Gil & Kim, 2019), and IL-18 was reported to promote the growth of B16F10 melanoma cells (Cho et al, 2000) and enhance the ability of melanoma cells to migrate via the generation of ROI and the MAPK pathway(Jung et al, 2006), which consistent with our findings. The high expression of IL-33 was found to associate with better overall survival in melanoma patients (Wagner et al, 2020). The role of IL-33 in melanoma has been observed both its pro- and anti-cancer effects, which may be due to the timing and dosage of IL-33 administration, and the specificity of IL-33 (Gao et al, 2013; Gao et al, 2015; Jevtovic et al, 2020; Long et al, 2018; Schuijs et al, 2020). Our data and those of previous studies indicate that IL-1 family members play an

important role in progression of skin cancer.", corresponding description have been added in Discussion and Materials and Methods sections.

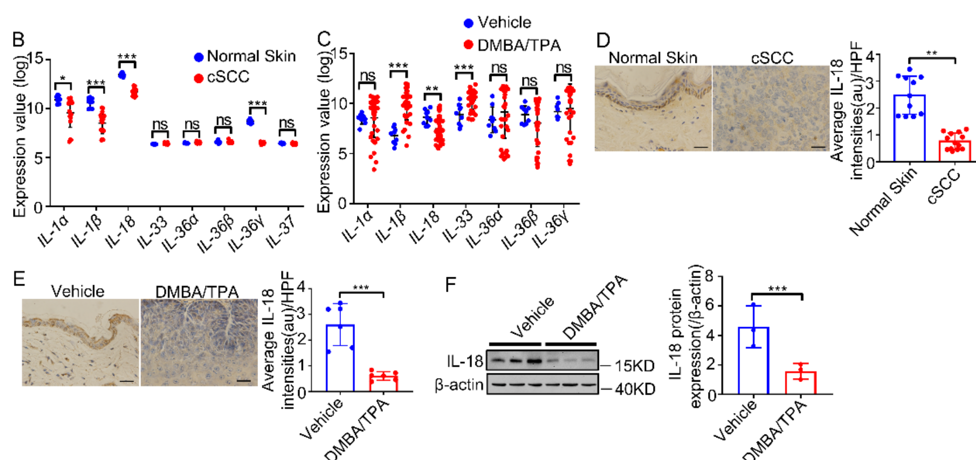


Figure EV1. The expression of IL-1 family members in cSCC and DMBA/TPA induced mouse tumors.

(B) Relative expression of IL-1 family members in human normal tissues (n = 9) and cSCC (n = 18) were analyzed using Geo Datasets (GSE98767). Mean ± SD.

(C) Relative expression of IL-1 family members in normal tissues (n = 9) and cSCC (n = 38) of mice analyzed using Geo Datasets (GSE63967). Mean ± SD.

(D) Representative micrographs of human skin sections stained with anti-IL-18 antibody from normal patients (n = 11) and tumors of cSCC patients (n = 13). Scale bars represent 100 μm. The graph shows the quantification of mean IL-18 expression per high-powered field in tissues. Mean ± SD.

(E) The dorsal hair of normal C57/BL6 mice was shaved and treated with DMBA/TPA twice a week for 32 weeks to induce skin tumors. Representative micrographs of mouse normal skin (n = 5) and tumor (n = 5) sections stained with anti-IL-18 antibody. Scale bars represent 100 μm. The graph shows the quantification of mean IL-38 expression in tissues. Mean ± SD.

(F) Representative western blot bands indicating IL-18 in mouse normal skin (n = 3) and DMBA/TPA-induced tumors (n = 3). The graph shows the quantification of mean IL-18 expression in tissues. Mean ± SD.

Question: Figure 2: Authors have demonstrated that IL-38 is decreased in DMBA/TPA model. Considering that IL-38 can potentially be expressed by cells other than keratinocytes, what is IL-38 expression level in the skin/tumor of IL-38 f/f versus K14-Cre, IL-38 f/f after DMBA/TPA treatment? Do authors see complete loss of IL-38 from skin lysate by deleting the cytokine expression in the keratinocytes? How about from DMBA/TPA-induced tumors comparing of IL-38 f/f to K14-Cre, IL-38 f/f? It is important to know if levels in K14-Cre, IL-38 f/f tumors are even lower than IL-38 f/f to support some degree of role for IL-38 in tumor progression? This especially critical as authors argue K14-Cre, IL-38 f/f have more invasive tumors but based on Figure 1 data, it is assumed that IL-38 is already lost in early tumors of WT mice.

Response: We thank the Reviewer for raising this point. In our results, we showed that IL-38 is mainly expressed in keratinocytes of epidermis. Moreover, we have performed experiments to detect the expression of IL-38 in DMBA/TPA treated skin and DMBA/TPA-induced tumors of *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$-*IL-38*$^{f/f}$ mice. We found that IL-38 was completely lost or under detectable in DMBA/TPA treated skin and DMBA/TPA-induced tumors of *K14*$^{Cre/+}$-*IL-38*$^{f/f}$ mice (Fig EV3A–D). We indeed observed the lower expression of IL-38 in both human and mouse skin tumors, the potential role of IL-38 should be addressed in skin tumor mouse model. As mentioned in Question 1, decreased IL-38 levels were also observed in other cancers (Chen et al, 2020; Wang et al, 2018), and another two IL-1 family members, IL-33 and IL-37, also showed the same apparent discrepancies (Akimoto et al, 2016; Volpe et al, 1997). Moreover, IL-38 also showed the same apparent discrepancies in multiple diseases as mentioned in Question 1 (Chai et al, 2020; Liu et al, 2020; Wei et al, 2020; Xie et al, 2020; Xu et al, 2018). Thus, the differential expression data give us the association between these modulators and diseases, while mouse models and cell models help to clarify their potential functions and mechanisms during diseases pathological processes. In our results, IL-38 promoted skin tumorigenesis via regulation of pro-tumorigenic microenvironment and epidermal cell

hyperplasia in DMBA/TPA induced skin tumor model (Fig 3C and D and Fig4A and B). Additionally, the promoting effect of IL-38 on expression of cancer-related inflammatory cytokines and proliferation and migration of skin tumor cells reaffirmed a tumor-promoting function for IL-38 in skin tumor (Fig 3E and F and Fig 4C-J). These results discovered the potential underlying mechanism by which IL-38 regulates skin cancer.

In the revised manuscript, we have added we have added new data (Fig EV3A-D) and the following sentence to the Results and Discussion sections: "IL-38 was completely lost or under detectable in DMBA/TPA treated skin and DMBA/TPA-induced tumors of *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice (Fig EV3A–D)." and "Considering that IL-38 can potentially be expressed by cells other than keratinocytes, we performed experiments to detect the expression of IL-38 in DMBA/TPA-treated skin and DMBA/TPA-induced tumors of *Il-38*$^{f/f}$ and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice. We observed complete loss of IL-38 from skin lysates in DMBA/TPA-treated skin and DMBA/TPA-induced tumors in *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice (Fig EV3A–D).", corresponding description have been added in Materials and Methods sections.

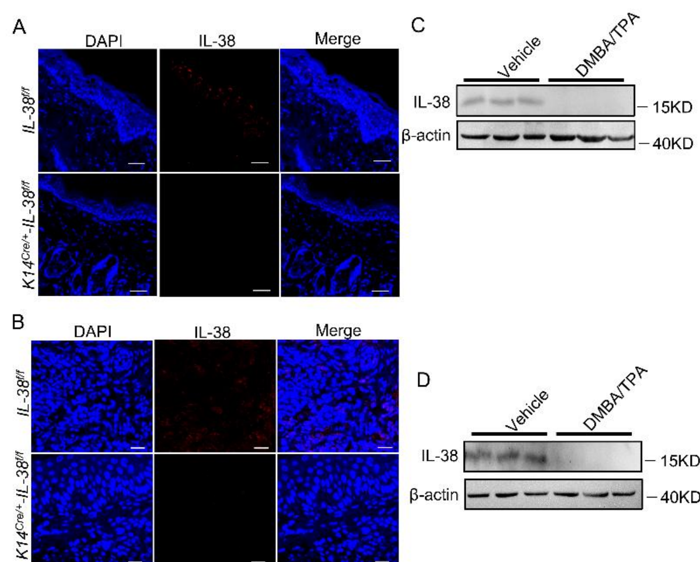


Figure EV3. (A and C) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 3 weeks to induce the skin inflammation. (A) Representative immunofluorescent staining micrographs of IL-38 in the skin of *Il-38*$^{f/f}$ and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice. Scale bars represent 100 μm. (C) Relative expression of IL-38 in the skin of

Il-38$^{f/f}$ (n = 3) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ (n = 3) mice were detected by western blot.

(B and D) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 32 weeks to induce the skin tumors. (B) Representative immunofluorescent staining micrographs of IL-38 in the tumors of *Il-38*$^{f/f}$ and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice. Scale bars represent 100 μm. (D) Relative expression of IL-38 in the tumors of *Il-38*$^{f/f}$ (n = 3) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ (n = 3) mice were detected by western blot.

Question: Figure 3: what is IL-38 expression level in normal keratinocyte cell lines compared to SCC cell lines, not only A431 cells? Is there a similar pattern as seen In vivo? If SCC cell lines have lower IL-38 expression, how does knockdown of IL-38 reveal cytokine expression changes? Or SCC cell lines highly express IL-38??

Response: We thank the Reviewer for raising this point. Based on the reviewer's comments, we have added new data in the revised manuscript, which showed that IL-38 expression was lower in squamous cell carcinomas (SCC) cell lines compared with normal keratinocyte cell lines consistent to the results in vivo (Fig EV4A and B).

In the revised manuscript, we have added new data (Fig EV4A and B) and the following sentence to the Results section: "IL-38 expression was lower in SCC cell lines than in normal keratinocyte cell lines, consistent with the *in vivo* results (Fig EV4A and B).", corresponding description have been added in Materials and Methods sections.

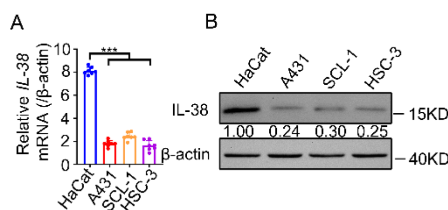


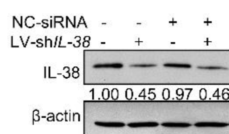
Figure EV4. (A) Relative expression of *IL-38* in normal skin cells (n=7) and SCC cells (n=7) determined using qPCR. Mean ± SD.

(B) Relative expression levels of IL-38 in normal skin and SCC cells were detected using western blot.

Question: For example, there are dramatic changes in the level of Cxcl1 and Tnf-alpha with/without knockdown of IL-38 in figure 3. But there are only smaller changes of Cxcl1 and Tnf-alpha in figure 6 and 7. Does this reflect the heterogenicity in the efficiency of IL-38 knockdown in different experiment?? The data suggest that the SCC cell line used has high IL-38 expression unlike the findings in vivo

Response: Thank you very much for your comments. Based on the reviewer's request, we have added the corresponding data in revised manuscript. The result showed that the expression of IL-38 has no significant difference in IL-38 knockdown cells transfected with NC-siRNA or not as shown below. The cells in figure 6 and 7 were transfected with NC-siRNA before IL-38 knockdown, which may result in differential sensitivity between the cells in figure 3 and figure 6 and 7. Moreover, we found that IL-38 expression was lower in squamous cell carcinomas (SCC) cell lines compared with normal keratinocyte cell lines consistent to the results in vivo (Fig EV4A and B).

In the revised manuscript, we have added new data (Fig EV4A and B) and the following sentence to the Results section: "IL-38 expression was lower in SCC cell lines than in normal keratinocyte cell lines, consistent with the *in vivo* results (Fig EV4A and B).", corresponding description have been added in Materials and Methods sections.



Relative expression of IL-38 in IL-38 knockdown cells transfected with NC-siRNA or not were detected by western blot.

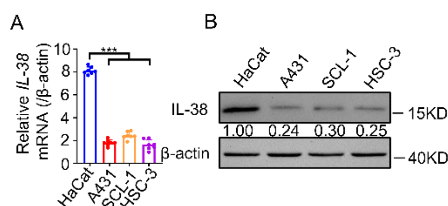


Figure EV4. (A) Relative expression of *IL-38* in normal skin cells (n=7) and SCC cells (n=7) determined using qPCR. Mean ± SD.

(B) Relative expression levels of IL-38 in normal skin and SCC cells were detected using western blot.

Question: IL-38 also induces anti-inflammatory genes (PMID: 22315422 IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist), how about the expression of this set of genes in the cell line upon IL-38 induction versus knockdown?

Response: We thank the Reviewer for raising this point. Indeed, previous report showed that IL-6 production induced by LPS was significantly higher in the presence of IL-38 (van de Veerdonk et al, 2012). We have now performed qPCR to detect IL-6 expression in A431 upon IL-38 overexpression or knockdown, we found that overexpression or knockdown of IL-38 in A431 showed no significant difference compared with control cells (Fig EV4I and J).

In the revised manuscript, we have added new data (Fig EV4I and J) and the following sentence to the Results section: "A previous study reported that LPS-induced IL-6 production was significantly higher in the presence of IL-38 (van de Veerdonk et al, 2012). The findings also demonstrated that neither the overexpression nor the knockdown of IL-38 exerted no significant difference in the expression of *IL-6* compared with its expression in control cells (Fig EV4I and J).", corresponding description have been added in Materials and Methods sections.

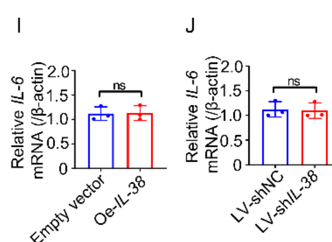


Figure EV4. (I) Relative expression of *IL-6* in *IL-38*-overexpressed A431 cells (n=3) was determined using qPCR. Mean ± SD.

(J) Relative expression of *IL-6* in *IL-38*-knockdown A431 cells (n=3) was determined using qPCR. Mean ± SD.

(K) The flow cytometry gating strategy for immune cell detection in DMBA/TPA-treated skin.

Question: Figure 6: IL-38 promotes skin tumorigenesis even though IL-38 is lost in cSCC - what is IL-1Rrp2 receptor expression in skin versus tumor? If this receptor expression is also changed, it may help explain the complex of role of IL-38 in skin tumorigenesis. IL-38 can also bind to IL-36 receptor, how about IL-36 receptor expression as a control in skin and tumor.

Response: We thank the Reviewer for raising this point. In the revised manuscript, we have added corresponding data in revised manuscript. IL-1Rrp2 is the aliases for IL-36 receptor. We have performed experiments to detect IL-1Rrp2 expression in skin versus tumor. Our results showed that IL-1Rrp2 expression had no significant difference between normal skin and tumors (Fig EV3E-F).

In the revised manuscript, we have added new data (Fig EV3E-F) and the following sentence to the Results and Discussion sections: “IL-1Rrp2 expression showed no significant difference between normal skin and tumors (Fig EV3E-F).” and “To detect if the contradiction caused by IL-1Rrp2 expression, we performed experiments to detect IL-1Rrp2 expression in skin versus tumor. Our results showed that IL-1Rrp2 expression showed no significant difference between normal skin and tumors (Fig EV3E-F).”, corresponding description have been added in Materials and Methods sections.

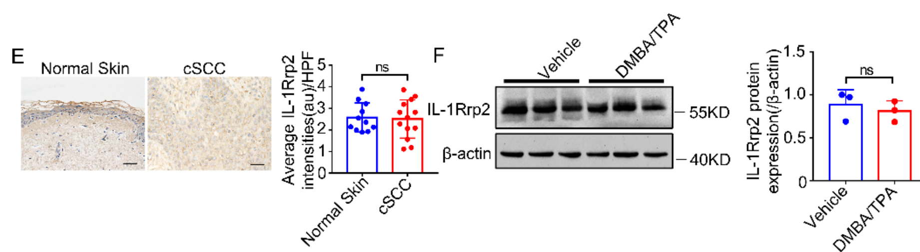


Figure EV3. (E) Representative immunohistochemical staining micrographs of IL-1Rrp2 from normal patients (n=11) and tumors of cSCC patients (n=13). Scale bars represent 100 μm. The graph shows average intensities of IL-1Rrp2 per high-powered field in tissues. Mean ± SD.

(F) Relative expression of IL-1Rrp2 in mouse normal skin (n=3) and DMBA/TPA induced tumors (n=3). The graph shows the quantification of mean IL-1Rrp2 expression in tissues. Mean ± SD.

Question: Revise picture to show a clearer IL-38 positive staining in figure 1C

Response: We thank the Reviewer for raising this point. We have now performed immunofluorescence to determine the IL-38 levels in skin tumors. We found that expression of IL-38 protein is significantly lower in DMBA/TPA induced tumors compared with normal mouse skin in line with findings in human patients (Fig 1C). We are so sorry to have made a print error in figure legend, and we have corrected the error in figure legend 1 in the revised manuscript.

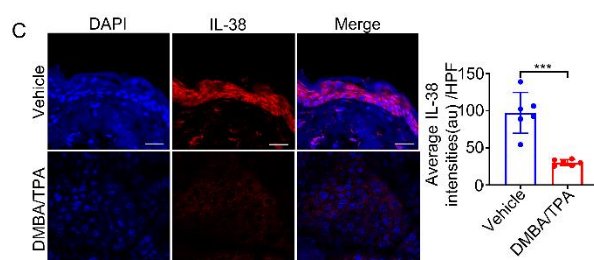


Figure 1. (C) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 32 weeks to induce skin tumors. Representative micrographs of mouse normal skin (n = 6) and tumor (n = 6) sections stained with anti-IL-38 antibody. The graph shows the quantification of mean IL-38 expression per high-powered field in tissues. Scale bars represent 100 µm.

Question: The labels in Figure 1 are off (no G label in the figure itself...)

Response: We truly appreciate your kind comments. We are so sorry to have made a print error in figure legend, and we have corrected the error in figure legend 1 in the revised manuscript.

Question: Add IL-38 expression level in Figure 3C and D.

Response: We thank the reviewer for this important comment. Based on the reviewer's request, we have added IL-38 expression data in Figure 3E and F (Figure 3C and D before) in the revised manuscript.

Question: Hard to see Ki67 staining in IHC, could change to immunofluorescent-stained image with Ki67.

Response: We thank the Reviewer for raising this point. Based on the reviewer's comments, we have changed Ki67 staining in IHC to immunofluorescent-stained image with Ki67 (Fig 4B).

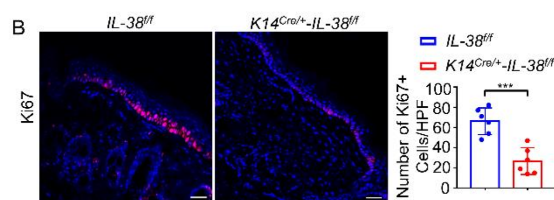


Figure 4. (B) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 3 weeks to induce the skin inflammation. Representative micrograph sections stained with anti-Ki67 antibody from the skin of *Il-38$^{f/f}$* (n = 6) and *K14$^{Cre/+}$-Il-38$^{f/f}$* mice (n = 6) Scale bars represent 100 µm. The graph shows average numbers of Ki67^{+} positive cells per high-powered field. Mean ± SD.

Question: Add IL-38 western blot data in Figure 4I and J.

Response: Thank you very much for your comments. Based on the reviewer's request, we have added IL-38 western blot data in Figure 4I and J in the revised manuscript.

Question: Revise description of Figure 5G in manuscript.

Response: We truly appreciate your kind comments. We have revised the description of Figure 5G in the revised manuscript, "Whereas overexpression of full-length IL-1Rrp2 significantly activated JNK upon IL-38 stimulation. Mutant IL-1Rrp2 completely lost the ability to activate JNK at 15 min (Fig 5G)." is revised to "Whereas overexpression of full-length IL-1Rrp2 significantly activated JNK upon IL-38 stimulation, mutant IL-1Rrp2 inhibited IL-38-induced JNK activation (Fig 5G)."

Responses to Reviewer #2:

We truly appreciate your kind comments. Our answers to your points are
as follows.

Question: The manuscript needs to be edited to a major extent, as many sentences are incomprehensible. The analyses of the in vivo studies are difficult to interpret, as figure legends often do not correspond with what is shown in graphs. Many claims are not sufficiently backed up by the data.

Response: We truly appreciate your kind comments. The manuscript has been edited by two native English speakers, and the language and grammar have been carefully reviewed in our revised manuscript. We are so sorry to have made a print error in figure legends, and we have corrected the error in the revised manuscript. Based on your comments, we have added corresponding data in revised manuscript to back up our claims.

Question: Quantification of IL-38 staining in Figure 1 is of poor quality. This Reviewer does not see any IL-38 staining in mouse tissues. These stainings should be performed by immunofluorescence (with the addition of appropriate controls).

Panels are not properly indicated in figure legend 1: panel f shows immunoblotting of IL-38; there is no panel G.

Response: Thank you very much for your kind reminder. In the revised manuscript, we have now performed immunofluorescence with the addition of appropriate controls to determine the IL-38 levels in skin tumors. We found that expression of IL-38 protein is significantly lower in DMBA/TPA induced tumors compared with normal mouse skin in line with findings in human patients (Fig 1C). We are so sorry to have made a print error in figure legend, and we have corrected the error in figure legend 1 in the revised manuscript.

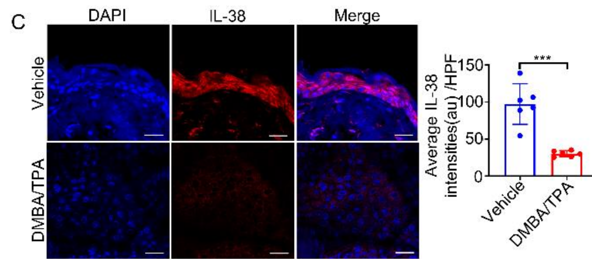


Figure 1. (C) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 32 weeks to induce skin tumors. Representative micrographs of mouse normal skin (n = 6) and tumor (n = 6) sections stained with anti-IL-38 antibody. The graph shows the quantification of mean IL-38 expression per high-powered field in tissues. Scale bars represent 100 μm.

Question: If the authors want to claim that there is no significant effect on epidermal barrier as is now stated on page 3, they need to perform epidermal barrier functionality assays, such as assessment of transepidermal water loss, dye permeability assays,...

Response: We thank the Reviewer for raising this point. Based on your comments, we have added corresponding data in revised manuscript. We have now performed dye permeability and transepidermal water loss assays (TEWL) to investigate epidermal barrier function in *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$*-IL-38*$^{f/f}$ mice. *K14*$^{Cre/+}$*-IL-38*$^{f/f}$ neonates exhibited the same patches of blue staining compared with *IL-38*$^{f/f}$ (Fig EV2J). In addition, no significant differences were found in TEWL level between *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$*-IL-38*$^{f/f}$ neonates (Fig EV2K). These results further demonstrated that there is no significant effect on epidermal barrier between *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$*-IL-38*$^{f/f}$ mice.

In the revised manuscript, these new data are shown in Supplementary Figure EV2J and K, and we have added the following sentence to the Results section: "Our results showed that keratinocyte-specific IL-38 deficiency had no significant effect on the epidermal barrier (Fig EV2G–M).", corresponding description have been added in Materials and Methods sections.

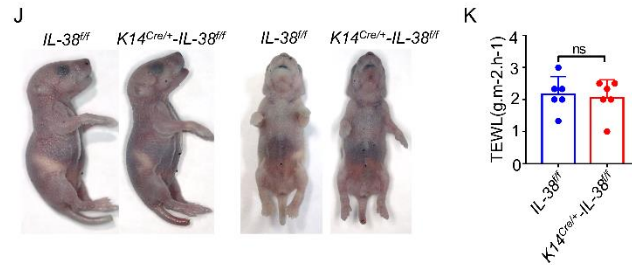


Figure EV2. (J) Skin barrier-dependent dye exclusion assay using toluidine blue in *Il-38*$^{f/f}$ mice (n = 5) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ littermate (n = 5) at birth.

(K) TEWL assay measured on ventral surface of newborn *Il-38*$^{f/f}$ mice (n = 6) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ littermate (n = 6). Mean ± SD.

Question: The graph is labelled as percentage of tumor-free survival, what is meant by this? Did animals die during the protocol? Or do the authors show percentage of mice that are tumor-free? The percentage of mice that develop tumors should be shown for the entire length of the promotion phase. This panel is not described in figure legend.

Response: We thank the Reviewer for raising this point. There is no animal die during the protocol and Figure 2E showed the tumor bearing ratio of *IL-38*$^{f/f}$ and *K14*$^{Cre/+}$-*IL-38*$^{f/f}$ mice. As you say, it shows the percentage of mice that are tumor-free. Based on your comments, we have changed the form of the graph to show the percentage of mice that develop tumors in revised manuscript (Fig 2E).

In the revised manuscript, these new data are shown in Fig 2E, and we have added the following sentence to the Results section: “The first tumors were observed in both *Il-38*$^{f/f}$ mice as well as in the *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice 8 weeks after beginning the DMBA/TPA treatment. After 15 weeks, all *Il-38*$^{f/f}$ mice developed tumors on their back skin, while approximately 37.5% *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice remained tumor-free (Fig 2E).”, corresponding description have been added in Materials and Methods sections.

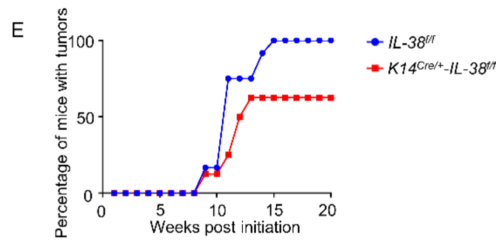


Figure 2. (E) Tumor bearing ratio of *Il-38*$^{f/f}$ (n = 12) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice (n = 8) treated with DMBA/TPA.

Question: The authors claim that 12.94% of tumours in control mice develop into SCCs. This is extremely high for mice on C57Bl6 background. How did the authors investigate conversion? The local micro-invasive foci that the authors show in Fig2f are not clear and do not convincingly point to malignancy. The authors should perform Keratin-8 staining to back up this claim.

Response: We thank the Reviewer for this good suggestion. Based on Reviewer's comments, we have made the corresponding corrections in the revised manuscript. We have now performed Keratin-8 staining in tumors to investigate malignant conversion. The result showed that multiple tumors in *IL-38*$^{f/f}$ mice were positive for the presence of K8 consistent with the finding in Fig 2G (Fig 2F before).

In the revised manuscript, these new data are shown in Fig 2G, and we have added the following sentence to the Results section: "To further investigate malignant conversion, we performed keratin8 (K8) staining in tumors. Multiple tumors in *Il-38*$^{f/f}$ mice were positive for the presence of K8 (Fig 2G).", corresponding description have been added in Materials and Methods sections.

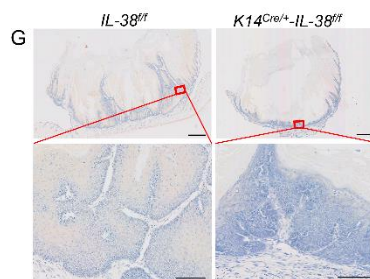


Figure 2. (G) Representative histological micrographs stained with K8 from tumors 32 weeks after initiation. Top pictures, scale bars represent 800 µm; bottom pictures, scale bars represent 100 µm.

Question: In Figure 3 the authors show flow cytometry data as percentage of total live cells. In the corresponding figure legends they claim these graphs show percentage of total immune cells. It is not clear what is shown here, hence the data are hard to interpret. Also, flow cytometry gating strategy is missing and amount of immune cells have not been standardized for the skin surface area that was digested. How long were these mice treated with DMBA/TPA? Which antibodies were used to distinguish different immune cell populations? This is essential information to assess the quality of these data and is sorely lacking from the manuscript.

Response: We thank the Reviewer for this important comment. We are so sorry to have made a print error in figure legend, and we have corrected the error in the legend of Fig 3C (Fig 3A before) in the revised manuscript, "total immune cells" is corrected to "total live cells". Based on the reviewer's request, we have added the flow cytometry gating strategy in Fig EV4K, and corresponding description have been added in Materials and Methods sections.

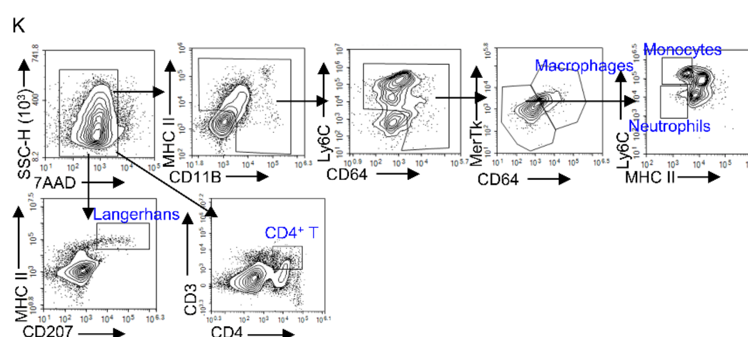


Figure EV4. (K) The flow cytometry gating strategy for immune cell detection in DMBA/TPA treated skin.

Question: The authors claim that IL-38 affects cell proliferation and migration in a SCC cancer cell line. However, with the scratch-wound assay, proliferation and migration can not be interpreted separately. If the authors want to make claims about migration potential, they have to incubate the cells with mitomycin to rule out any proliferation effects.

Response: We thank the Reviewer for raising this point. In the revised manuscript, we have made the corresponding corrections in the revised manuscript. We have now incubated the cells with mitomycin to rule out any proliferation effects in the scratch-wound assay. Cell and migration were enhanced in the cancer cell line A431 with overexpression of IL-38 (Fig 4G) and inhibited if IL-38 were silenced (Fig 4H). Further, we found that recombinant IL-38 can promote tumor cells migration and treatment with shRNA against IL-38 suppressed cells migration, but both of them had no effect on the tumor cells with IL-1Rrp2 (Fig 6F and H), or JNK knockdown (Fig 7F and H). These results indicate that IL-38 promote the migration of tumor cells depend on IL-1Rrp2/JNK signaling pathway.

In the revised manuscript, these new data are shown in Figure 4G, 4H, Figure 6F, 6H and Figure 7F, 7H, and corresponding description have been added in Materials and Methods sections.

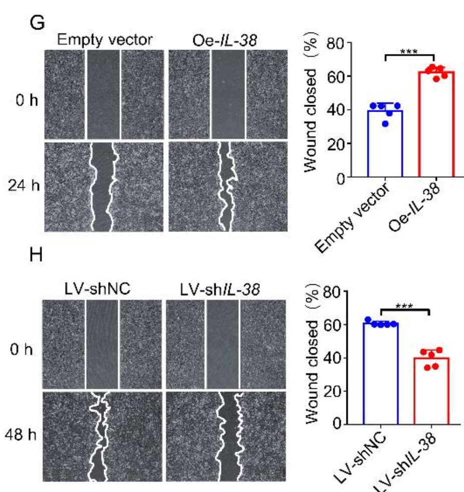


Figure 4. Representative images of the scratch assay (left) and wound closure rate (right) of A431 cells (n = 5) after overexpression (G) or knockdown (H) of *IL-38*. Mean ± SD.

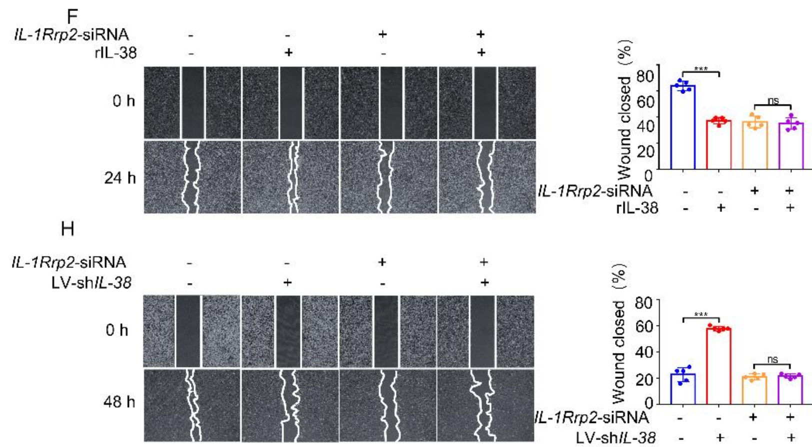


Figure 6. (F and H) Representative images of the scratch assay (left) and wound closure rate (right) of A431 cells (n = 5) treated with recombinant IL-38 (200 ng/mL) (F) or knockdown of *IL-38* (H) after *IL-1Rrp2* interference. Mean ± SD.

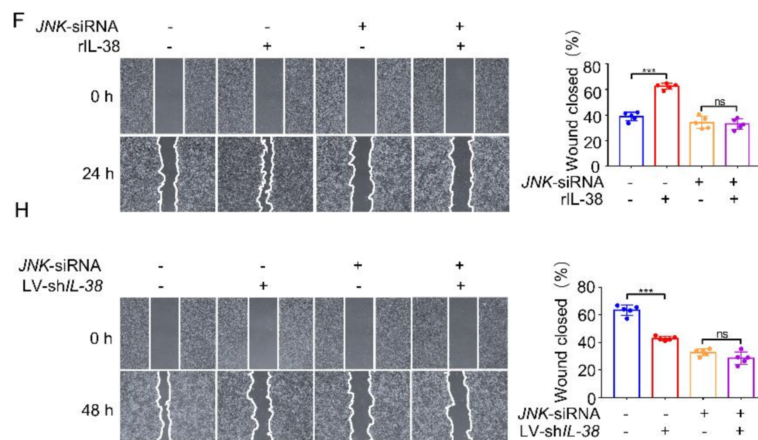


Figure 7. (F and H) Representative images of the scratch assay (left) and wound closure rate (right) of A431 cells (n = 5) treated with recombinant IL-38 (200 ng/mL) (F) or knockdown of *IL-38* (H) after *JNK* interference. Mean ± SD.

Responses to Reviewer #3:

Thank you very much for your good advice, which is very useful for improving our research. We have answered each of your points below.

Question: There are several typos and sentences that are cropped or whose syntax needs to be improved as their meaning is unclear. This is particularly (but not solely) the case in the second part of the discussion.

Response: We truly appreciate your kind comments. The manuscript has been edited by two native English speakers, and the language and grammar have been carefully reviewed in our revised manuscript.

Question: The anti-IL-38 staining in Fig1 is not that clear, possibly due to the resolution of the image or because IL-38 appears to be a (soluble) cytoplasmic cytokine. Can the authors detect IL-38 in the tissue of their keratinocyte-specific IL-38 knockout mice (K14Cre/+;IL-38f/f), to validate their staining?

Response: We thank the reviewer for this important comment. Based on the reviewer's request, we have now performed immunofluorescence to determine the IL-38 levels in skin tumors. We found that expression of IL-38 protein is significantly lower in DMBA/TPA induced tumors compared with normal mouse skin in line with findings in human patients (Fig 1C). We are so sorry to have made a print error in figure legends, and we have corrected the error in figure legend 1 in the revised manuscript.

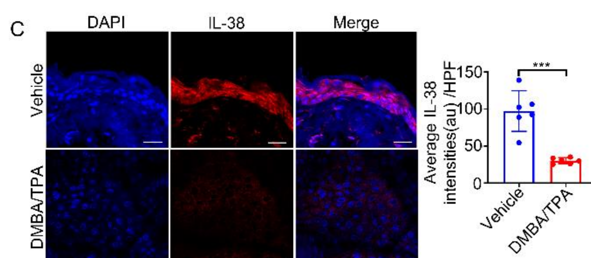


Figure 1. (C) The dorsal hair of normal C57/BL6 mice were shaved and treated with DMBA/TPA twice a week for 32 weeks to induce skin tumors. Representative micrographs of mouse normal skin (n = 6) and tumor (n = 6) sections stained with anti-IL-38 antibody. The graph shows the quantification of mean IL-38 expression per high-powered field in tissues. Scale bars represent 100 µm.

Question: Based on their findings from Fig.5, the authors conclude that IL-38 binds to IL-1Rrp2, but not "to the other ... receptors" that were

tested. However, there are consistently weak but detectable bands for IL-38 (both for the 1-152aa and 20-152aa variant) to bind IL1RAcp-Fc in Fig5A and B.

Response: We thank the Reviewer for raising this point. Indeed, as the reviver mentioned, results from Figure 5A and B, there are weak but detectable bands for IL-38 to bind IL-1RAcP-Fc. Previous studies have determined that IL-38 bound to the IL-1Rrp2-Fc but did not bind IL-1RI-Fc, IL-1RAcP-Fc, and IL-18Rα (van de Veerdonk et al, 2012), which was consistent with our results. In their receptor-binding assay, it also showed slightly increased optical density when increasing the concentration of IL-38 were added to IL-1RAcP immobilized wells (van de Veerdonk et al, 2012). The IL-1 receptor accessory protein (IL-1RAcP) is a coreceptor for the IL-1, IL-33, and IL-36 receptors (Palmer et al, 2008; Towne et al, 2004; Volpe et al, 1997). IL-1RAcP is able to associates with those receptors to form the high affinity receptor complexes which mediate IL-1, IL-33, and IL-36-dependent pathways (Ali et al, 2007; Towne et al, 2004; Wang et al, 2010). IL-38 may share IL-1RAcP as the secondary receptor with IL-1, IL-33, and IL-36, which may result in IL-38 show low affinity for IL-1RAcP.

Question: Along these lines, a previous study reported that IL-38 binds IL1RAPL1 on skin gd T cells (PMID: 30995480) to modulate the function of these cells. Yet, Fig.5 does not provide evidence for IL-38 to bind IL-1RAPL1. The authors should at least comment on these apparently different results in the discussion.

Response: We thank the Reviewer for raising this point. As previously reported by Han et al., IL-38 binds IL-1RAPL1 on skin gd T cells (Han et al, 2019). Javier Mora and their colleagues also reported that IL-38 was able to bind IL-1RAPL1, but we found that IL-1Rrp2 showed significantly higher affinity for IL-38 compared with IL-1RAPL1 in their experiment(Mora et al, 2016). Moreover, IL-1RAPL1 (Interleukin 1 Receptor Accessory Protein Like 1) is similar to IL-1RAcP (Interleukin 1 accessory proteins). IL1RAPL1 shares high amino acid sequence identities in the extracellular domain with IL-1RAcP (30.3%) (Yoshida et

al, 2012). Therefore, IL1RAPL1 more likely act as a coreceptor for receptors of IL-1 family members like IL-1RAcP.

In the revised manuscript, we have added the following sentence to the Discussion section: "As previously reported by Han et al., IL-38 binds to IL-1RAPL1 on skin γδT cells (Han et al, 2019). Mora et al. also reported that IL-38 was able to bind IL-1RAPL1, but IL-1Rrp2 showed a significantly higher affinity for IL-38 than IL-1RAPL1 (Mora et al, 2016). Moreover, IL-1RAPL1 shares high amino acid sequence identity in the extracellular domain with IL-1RAcP (30.3%) (Yoshida et al, 2012). Therefore, IL-1RAPL1 is more likely to act as a coreceptor for receptors of IL-1 family members, such as IL-1RAcP.".

Question: In the discussion, the authors comments on the counter-intuitive findings that IL-38 levels are reduced in tumor tissues, while IL-38 promotes keratinocyte proliferation to support skin hyperplasia and tumorigenesis in their model. Yet they do not show (or at least discuss) on which cells IL-1Rrp2 is otherwise expressed in the tumor environment. Do immune cells potentially also express it and respond to IL-38 (see also my previous remark)? How much of a contribution to tumor development do the IL-38-dependent inflammatory cues produced by the malignant keratinocytes make? These aspects may possibly explain the apparently contradictory phenotypes the author observe, and should be at least reflected in the discussion.

Response: We thank the Reviewer for the constructive comments. Previous report has showed that IL-1Rrp2 mRNA was most strongly expressed in keratinocytes, which was at least 10-fold more than in BMDCs, splenic $CD4^+$ T cells, bone marrow-derived macrophages, and bone marrow-derived neutrophils, whereas IL-1Rrp2 mRNA was not detected in $CD8^+$ cells and B cells (Vigne et al, 2011), which is similar to the findings reported by Foster and Baliwag et al. (Foster et al, 2014). In our results, we also found that IL-1Rrp2 is highly expressed in skin (Fig EV5A) and multiple human skin related cells, especially in skin cancer cell line (A431) (Fig EV5B). Moreover, IL-1Rrp2 knockdown in skin tumor cells inhibited IL-38-induced expression of cancer-related

inflammatory cytokines and proliferation and migration of tumor cells (Fig 6). Our data and those of previous studies reminded us that IL-38 promotes skin tumorigenesis possibly mainly via binding to IL-1Rrp2 on the surface of keratinocytes.

In the revised manuscript, we have added the following sentence to the Discussion section: "A previous report has shown that *IL-1Rrp2* mRNA was most strongly expressed in keratinocytes, which was at least ten-fold more than in bone marrow derived dendritic cells (BMDCs), splenic $CD4^+$ T cells, bone marrow-derived macrophages, and bone marrow-derived neutrophils, whereas *IL-1Rrp2* mRNA was not detected in $CD8^+$ and B cells (Vigne et al, 2011), which is similar to the findings reported by Foster and Baliwag et al. (Foster et al, 2014). In our results, we also found that IL-1Rrp2 was highly expressed in the skin (Fig EV5A) and multiple human skin-related cells, especially in the skin cancer cell line (A431) (Fig EV5B). In this regard, IL-1Rrp2 meets the requirements of a stimulatory immune checkpoint for skin cancer.".

Question: The authors should use the correct nomenclature while mentioning genes / transcripts versus proteins, in dependence on the species they refer to.

Response: We truly appreciate your kind comments. We have corrected the nomenclature of genes, transcripts and proteins in the revised manuscript.

Question: It should be more clearly indicated how the histology was quantified (apparently automatically)? Was this done on whole images or per field of view?

Response: We thank the Reviewer for raising this point. ImageJ (National Institutes of Health) was used for quantification of the fluorescence and intensities of the images. ImagePro Plus was used for to quantify the DAB intensity and the number of Ki67 and γH2AX positive cells in the image. ImageJ (National Institutes of Health) was used for further quantification of the fluorescence and intensities of the images. Quantification of histological image was analyzed by per field of view. In

the revised manuscript, corresponding description have been added in figures and Materials and Methods sections.

Question: Similarly, it is not clear how AP1 activation was quantified (likely via a luciferase reporter assay, yet this is not clear).

Response: We thank the Reviewer for raising this point. Indeed, AP1 activation was quantified via a luciferase reporter assay. In the revised manuscript, corresponding description have been added in figure legend (Fig 5H).

Question: Page 1, 2nd paragraph, Introduction: 'The *IL-1* family of cytokines...'

Response: Thank you very much for your kind reminder. We are so sorry to have made a print error in Page 1, 2nd paragraph, and we have corrected the error in the revised manuscript, "L-1" is corrected to "IL-1".

Question: Fig 1, legend: labels (F) and (G) are not correct

Response: We really appreciate your kind comments. We are so sorry to have made a print error in figure legend, and we have corrected the error in the legend of Fig 1 in the revised manuscript.

Question: Fig 2: Missing figure legend on E

Response: Thank you very much for your kind reminder. We are so sorry to have made a print error in Fig.2 legend, and we have added the legend of Fig 2E in the revised manuscript.

Question: The results in Fig 4 rather indicate that IL-38 promotes epidermal cancer cell proliferation (than hyperplasia).

Response: We thank the Reviewer for the constructive comments. We have corrected the error in the revised manuscript, "hyperplasia" is corrected to "proliferation".

Question: The first paragraph of the result section entitled "IL-38 forms a complex with IL-1Rrp2 and activates JNK/AP-1 signal transduction

pathway in an IL-1Rrp2-dependent manner" seems to be more suited for the introduction part.

Response: We truly appreciate your kind comments. In the revised manuscript, we have moved the first paragraph of the result section entitled "IL-38 forms a complex with IL-1Rrp2 and activates JNK/AP-1 signal transduction pathway in an IL-1Rrp2-dependent manner" to the introduction part.

Question: As a minor side note: the lead contact is provided in the paragraph on data availability.

Response: Thank you very much for your kind reminder. In the revised manuscript, we have deleted the lead contact in the paragraph on data availability.

Question: I guess all study participants had *given* written informed consent, and no therapy had been given *before sample collection* (i.e. samples were taken at diagnosis). Please precise or improve accordingly.

Response: Thank you very much for your kind reminder. Written informed consent was obtained from all the study participants prior to the study. All patients had not received radiotherapy, chemotherapy, cryotherapy or laser treatment before sample collection. In the revised manuscript, corresponding description have been added in Materials and Methods section accordingly.

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1st Revision - Editorial Decision 4th Feb 2022

Dear Dr. Li,

Thank you for submitting your revised manuscript. It has now been seen by two of the original referees.

My apologies for the delay in getting back to you, it took longer than anticipated to receive the referee reports given this busy time of the year.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, I need you to address the editorial points below before I can accept the manuscript.

• Please address the remaining minor concerns of referee #2.
• We note that Yuxi Zhou missing from the Author Contributions section.
• As of January 2016, new EMBO Press policy asks for all corresponding authors to link to their ORCID iDs. You can read about the change under "Authorship Guidelines" in the Guide to Authors here:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In order to link your ORCID iD to your account in our manuscript tracking system, please do the following:

1. Click the 'Modify Profile' link at the bottom of your homepage in our system.
2. On the next page you will see a box halfway down the page titled ORCID*. Below this box is red text reading 'To Register/Link to ORCID, click here'. Please follow that link: you will be taken to ORCID where you can log in to your account (or create an account if you don't have one)

3. You will then be asked to authorise Wiley to access your ORCID information. Once you have approved the linking, you will be brought back to our manuscript system.

We regret that we cannot do this linking on your behalf for security reasons.

• We note that -actin blots of EV1F and EV3F are different exposures of the same blot, which is allowed if the blots are derived from the same experiment. In which case, please state this in the figure legend.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Authors have adequately addressed my comments. Thanks!

Referee #2:

The authors have adequately responded to my questions and the additional experiments have improved the manuscript considerably.

However, there are some remaining concerns that should be addressed:
On page 3, the authors describe GEO searches for IL-1 family members and claim that differences in IL-18 expressino levels in skin tumours suggest a possible role for IL-38 in skin tumorigenesis. This is not a valid scientific argument, cytokine family members are not always co-regulated. IL-18 is secreted by pyroptotic cells, which is not the case for IL-38, so it is far-fetched that overlapping expression would implicate similar functions.

Fig 1a: add isotype controls
Fig 2E: are these differences statistically significant? Provide details on statistical test
Fig 2G: this reviewer does not see any keratin-8 expression.

Dear Editor,

Thank you very much for your kind letter about our paper Manuscript #EMBOR-2021-53791V3 entitled "Interleukin-38 promotes skin tumorigenesis in an IL-1Rrp2-dependent manner". In this revised manuscript, we have carefully addressed all the comments from the reviewers and revised the manuscript accordingly. Our responses are given in a point-by-point manner below. We return it with track changes activated. Your consideration for this manuscript is highly appreciated. Based on these new data, we believe that the manuscript is significantly improved and suitable for publication in *EMBO reports*. We hope the revised version is now suitable for publication, and we look forward to hearing from you in due course. Thank you very much for your kindness.

Yours sincerely,

Jiong Li, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, Sichuan 610041, China. Email: lijionghh@scu.edu.cn

Responses to Editor:

Thank you for your review of our paper. We have answered each of your points below.

Question: Please address the remaining minor concerns of referee #2.

Response: In this revised manuscript, we have carefully addressed all the comments from the referee #2 and revised the manuscript accordingly.

Question: We note that Yuxi Zhou missing from the Author Contributions section.

Response: We thank the Editor for raising this point. In the revised manuscript, we have added Yuxi Zhou in the Author Contributions section.

Question: As of January 2016, new EMBO Press policy asks for all corresponding authors to link to their ORCID iDs.

Response: We have added the ORCID ID of corresponding author in our manuscript system.

Question: We note that β-actin blots of EV1F and EV3F are different exposures of the same blot, which is allowed if the blots are derived from the same experiment. In which case, please state this in the figure legend.

Response: Thank you very much for your comments. Indeed, β-actin blots of EV1F and EV3F are derived from the same experiment. In the revised manuscript, we have stated this in the figure legend.

Question: During our routine analysis, we notice that there are some textual overlaps with previously published papers (please see the attached screenshots). Please rephrase the parts of the text highlighted in the screenshots in order to avoid this.

Response: We truly appreciate your kind comments. In the revised manuscript, we have rephrased the parts of the text highlighted in the screenshots.

Question: Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and

streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

Response: The 'synopsis' and 'bullet points' are as follows.

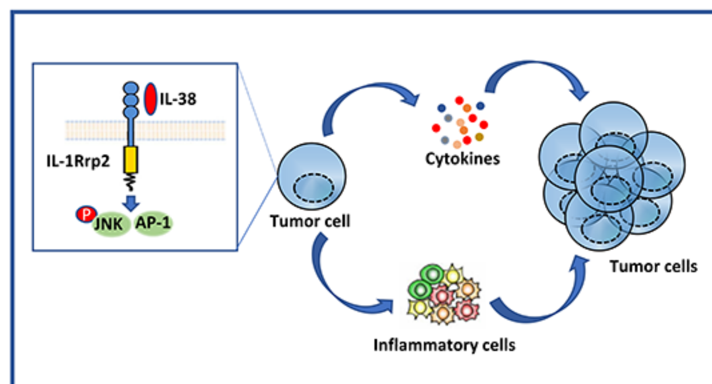
SYNOPSIS

Decreased inflammation and epidermal cell proliferation in *Il-38* cKO mice result in suppressed skin tumor formation and malignant progression. IL-1Rrp2/JNK signaling pathway is crucial for IL-38 to promote the expression of cancer-related inflammatory cytokines and proliferation and migration of tumor cells.

- Interleukin-38 (IL-38) is downregulated in human cutaneous squamous cell carcinoma and DMBA/TPA-induced mouse skin tumorigenesis.
- IL-38 keratinocyte-specific deletion dramatically ameliorates DMBA/TPA-induced skin tumors accompanied by a reduction in the number of immune cells and expression of cancer-related inflammatory cytokines.
- IL-38 forms a complex with IL-1Rrp2 and activates the JNK/AP-1 signal transduction pathway in an IL-1Rrp2-dependent manner.
- The proliferation and migration of tumor cells and expression of cancer-related inflammatory cytokines are induced by IL-38 via an IL-1Rrp2/JNK-mediated pathway.

Question: In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Response: The image for the synopsis is as follows.



Question: Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Response: Thank you very much for your comments. In the revised manuscript, we have clarified the points in the figure legends and returned it with track changes activated.

Responses to Reviewer #2:

We truly appreciate your kind comments. Our answers to your points are as follows.

Question: On page 3, the authors describe GEO searches for IL-1 family members and claim that differences in IL-18 expression levels in skin tumours suggest a possible role for IL-38 in skin tumorigenesis. This is not a valid scientific argument, cytokine family members are not always co-regulated. IL-18 is secreted by pyroptotic cells, which is not the case for IL-38, so it is far-fetched that overlapping expression would implicate similar functions.

Response: We thank the Reviewer for raising this point. We have revised the description in the revised manuscript.

In the revised manuscript, we have rephrased the parts of the text on page 3 as follows: "We analyzed IL-38 expression using the Genotype-Tissue Expression (GTEx) project (V8 dbGaP Accession phs000424.v8.p2) to determine the landscape of expression of IL-38. The analysis revealed the lowest expression of IL-38 in most tissues, while the highest expression was observed in the skin (Fig EV1A). Further, to determine IL-38 levels in skin tumors, we analyzed the expression of IL-38 in human skin cancer tissues and found that the protein was significantly lower in cSCC than in normal human skin tissues (Fig 1A and B, Fig EV1B). Analysis of IL-38 expression in normal mouse skin and DMBA/TPA-induced tumors revealed weak levels of IL-38 in tumors, consistent with findings in human patients (Fig 1C–F). Collectively, these results suggest a possible role of IL-38 in skin tumorigenesis.

At the same time, we searched the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) and identified multiple IL-1 family members exhibiting differential expression in skin cancer compared with normal skin searching (Fig EV1C and D). Further, experiments to detect IL-18 expression in normal human and mouse skin and tumors demonstrated that the expression of IL-18 decreased in human cutaneous squamous cell carcinoma (cSCC) and DMBA/TPA-induced mouse tumors compared with its expression in normal skin tissues, which is consistent with the findings in the GEO

database (Fig EV1E–G).”.

Question: Fig 1a: add isotype controls

Response: Based on the reviewer’s comments, we have added new data in the revised manuscript, which showed that positive staining with anti-IL-38 antibody in human normal skin, and staining is negative with rabbit IgG (Fig EV1B).

In the revised manuscript, we have added new data (Fig EV1B) and the following sentence to the Results section: “Further, to determine IL-38 levels in skin tumors, we analyzed the expression of IL-38 in human skin cancer tissues and found that the protein was significantly lower in cSCC than in normal human skin tissues (Fig 1A and B, Fig EV1B).”, corresponding description have been added in Materials and Methods sections.

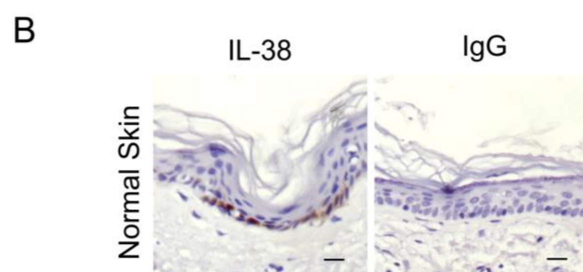


Figure EV1. (B) Representative immunohistochemical staining micrographs of IL-38 and rabbit IgG in human normal skin. Scale bars represent 200 μm.

Question: Fig 2E: are these differences statistically significant? Provide details on statistical test

Response: We thank the Reviewer for raising this point. In Fig 2E, our result showed that the first tumors were observed in both *Il-38$^{f/f}$* mice as well as in the *K14$^{Cre/+}$-Il-38$^{f/f}$* mice 8 weeks after beginning the DMBA/TPA treatment. After 15 weeks, all *Il-38$^{f/f}$* mice developed tumors on their back skin, while approximately 37.5% *K14$^{Cre/+}$-Il-38$^{f/f}$* mice remained tumor-free (Fig 2E). The percentage of mice with tumors were analyzed by Fisher’s exact test. The details on statistical test are as follows.

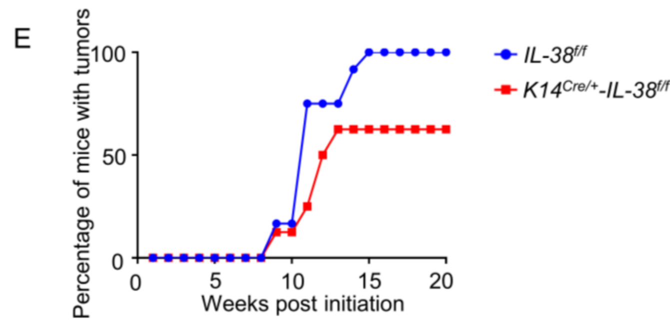


Figure 2. (E) Tumor bearing ratio of *Il-38*$^{f/f}$ (n = 12) and *K14*$^{Cre/+}$-*Il-38*$^{f/f}$ mice (n = 8) treated with DMBA/TPA.

P value and statistical significance			
Test	Fisher's exact test		
P value	0.0491		
P value summary	*		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	Yes		
Data analyzed	tumor-bear mice	tumor-free mice	Total
IL-38$^{f/f}$	12	0	12
K14$^{Cre/+}$-IL-38$^{f/f}$	5	3	8
Total	17	3	20
Percentage of row total	tumor-bear mice	tumor-free mice	
IL-38$^{f/f}$	100.00%	0.00%	
K14$^{Cre/+}$-IL-38$^{f/f}$	62.50%	37.50%	

Question: Fig 2G: this reviewer does not see any keratin-8 expression.

Response: Thank you very much for your kind reminder. In the revised manuscript, we have now performed immunofluorescence to determine Keratin-8 expression in tumors to investigate malignant conversion. The result showed that multiple tumors in *Il-38*$^{f/f}$ mice were positive for the presence of K8 consistent with the finding in Fig 2G.

In the revised manuscript, these new data are shown in Fig 2G, and we have added the following sentence to the Results section: "To further investigate malignant conversion, we performed keratin8 (K8) staining in tumors. Multiple tumors in *Il-38*$^{f/f}$ mice were positive for the presence of K8 (Fig 2G).", corresponding description have been added in Materials and Methods sections.

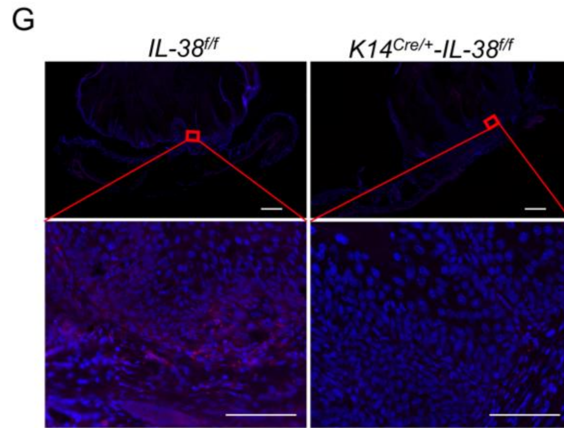


Figure 2. (G) Representative micrograph sections stained with K8 from tumors 32 weeks after initiation. Top pictures, scale bars represent 700 μm; bottom pictures, scale bars represent 100 μm.

2nd Revision - Editorial Decision 30th Mar 2022

Dear Dr. Li,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

	Please fill out these boxes (Do not worry if you cannot see all your text once you press return)
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were selected empirically from previous experimental experience with similar assays, and/or from sizes generally employed in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample sizes.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	Animals and samples from these animals were randomly selected and observed in this study.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Investigators were not blinded during data acquisition or analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators were not blinded during data acquisition or analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes.Statistical analysis was performed strictly as required.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We tested for normality using the GraphPad.
Is there an estimate of variation within each group of data?	Yes.

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have added it in the Materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have added it in the Materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have added it in the Materials and methods section.
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A "Data Availability" section was included in the manuscript.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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