Article

The severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression

PALOMO, Jennifer, *et al*.

Abstract

The IL-1 cytokine family includes eleven members, among which Il-36α, β and γ, IL-36Ra and IL-38. The IL-36 cytokines are involved in the pathogenesis of psoriasis. IL-38 is also expressed in the skin and was previously proposed to act as an IL-36 antagonist. In this study, we thus examined expression and function of Il-38 in a mouse model of imiquimod (IMQ)-induced skin inflammation. Il-38 mRNA was detected in the epidermis and in primary mouse keratinocytes, but not in dermal fibroblasts. At the peak of IMQ-induced inflammation, skin Il-38 mRNA levels were reduced, whereas Il-36ra mRNA expression increased. The severity of IMQ-induced skin inflammation, as assessed by recording ear thickness and histological changes, was similar in Il-38 KO and WT littermate control mice, while, in contrast, Il-36ra-deficient mice displayed more severe skin pathology than their WT littermates. Il-38-deficiency had no impact on IMQ-induced expression of proinflammatory mediators in the skin in vivo, on the basal expression of various cytokines or chemokines by cultured primary keratinocytes and dermal fibroblasts in vitro, or on the [...]

Reference

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The severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression

--Manuscript Draft--

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Full Title:	The severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression
Short Title:	IL-38 in imiquimod-induced skin inflammation
Corresponding Author:	Gaby Palmer-Lourenco, PhD University of Geneva, Faculty of Medicine Geneva 4, SWITZERLAND
Keywords:	Cytokines; inflammation; Psoriasis; skin; Interleukin; keratinocyte; Mouse
Abstract:	The IL-1 cytokine family includes eleven members, among which Il-36α, β and γ, IL-36Ra and IL-38. The IL-36 cytokines are involved in the pathogenesis of psoriasis. IL-38 is also expressed in the skin and was previously proposed to act as an IL-36 antagonist. In this study, we thus examined expression and function of Il-38 in a mouse model of imiquimod (IMQ)-induced skin inflammation. Il-38 mRNA was detected in the epidermis and in primary mouse keratinocytes, but not in dermal fibroblasts. At the peak of IMQ-induced inflammation, skin Il-38 mRNA levels were reduced, whereas Il-36ra mRNA expression increased. The severity of IMQ-induced skin inflammation, as assessed by recording ear thickness and histological changes, was similar in Il-38 KO and WT littermate control mice, while, in contrast, Il-36ra-deficient mice displayed more severe skin pathology than their WT littermates. Il-38-deficiency had no impact on IMQ-induced expression of proinflammatory mediators in the skin in vivo, on the basal expression of various cytokines or chemokines by cultured primary keratinocytes and dermal fibroblasts in vitro, or on the response of these cells to Il-36β. Finally, after cessation of topical IMQ application, the resolution of skin inflammation was also not altered in Il-38 KO mice. In conclusion, Il-38-deficiency did not impact the development or resolution of IMQ-induced skin inflammation. Our observations further suggest that endogenous Il-38 does not exert Il-36 inhibitory activity in this model, or in cultured skin cells. A potential anti-inflammatory function of Il-38 in mouse skin thus still remains to be demonstrated.
Order of Authors:	Jennifer Palomo
	Sabina Troccaz
	Dominique Talabot-Ayer
	Emiliana Rodriguez
	Gaby Palmer-Lourenco, PhD
Opposed Reviewers:	
Response to Reviewers:	To the Editor: Even though, IL38 is generally considered an anti-inflammatory cytokine, your work indicated that In IL-38 deficiency in mice did not impact the development or resolution of imiquimod-induced skin inflammation. The editor and reviewers agree that these negative results are of interest for the field. However, there are some deficiencies in your work including the characterization of inflammatory infiltrate in the ears of wt and IL38 KO mice after imiquimod treatment. According to the reviewers' suggestions, and as detailed below, we have further characterized the inflammatory infiltrate in the ears of WT and Il-38KO mice. To Reviewer #1: This manuscript describes studies analyzing IL-38 knockout mice in an imiquimod-induced psoriasis model. This appears to be the first study examining IL-38 knockout

mice in any skin model. The data suggest that IL-38 does not have a significant impact on psoriasis-like characteristics or pro-inflammatory cytokine expression. While the data are mostly negative, this reviewer feels that the data will be important for other investigators in the field. A few concerns are noted below.
1) The authors confirm a lack of IL-38 mRNA expression in their knockout mice. It would be more convincing if they also showed a lack of IL-38 protein in the skin of cells from IL-38 knockout mice.

We totally agree with this remark. Unfortunately, so far, we have not been able to achieve specific detection of the mouse (m)Il-38 protein in skin or skin lysates using standard approaches. We are thus unable to satisfactorily document mIl-38 protein expression in WT skin.
Indeed, by Western blot, using 3 different anti-mIl-38 antibodies obtained through collaboration with Dr. Donzé (Adipogen International Inc.), or a commercial antibody from RnD Systems (BAF2427, anti-human IL-38), we easily detect recombinant mIl-38, as well as a band of the expected size in lysates of 293T cell overexpressing mIl-38 (Figure I in 'response to reviewers' file, and data not shown). However, using different extraction buffers and methods, we have so far not been able to detect mIl-38 either in total skin or in isolated epidermis (Figure I in the 'response to reviewers' file, and data no shown).

We further tried to quantify mIl-38 by ELISA. Using lysates of WT skin, we obtained positive ELISA signals. However, we also observed important non-specific background signals in Il-38KO skin lysates (Figure II, in the 'response to reviewers' file, and data not shown), indicating a lack of specificity of the assay in this type of samples. Thus, unfortunately, although there seems to be some more signal overall in WT, as compared to Il-38KO skin, these ELISA data remain inconclusive and do not allow for proper quantification of mIl-38 protein.

Finally, by immunohistochemistry, using all the different antibodies mentioned above, as well as one additional commercial antibody (clone H127C, eBioscience; see Takenaka et al, 2015, Biochem. Biophys. Rep.), we have so far been unable to detect specific staining (i.e. absent in Il-38KO skin) on paraffin or frozen skin sections using various protocols for fixation and epitope unmasking (data not shown).

2) The authors report "cell infiltrate" in several figures, but this is not well described. Do the authors assume these are inflammatory cells? If so, a more specific method (i.e., IHC) would be helpful in identifying specific inflammatory cell populations.
We further characterized the cell infiltrate in the ears of IMQ-treated WT and Il-38KO mice by IHC, using anti-Ly6G antibodies to stain neutrophils, anti-CD3 antibodies to stain T cells, and anti-B220 antibodies to stain B cells. Indeed, infiltrated cells are mostly inflammatory cells, and, in particular, neutrophils, consistent with previous observations in this model (van der Fits et al., 2009, J. Immunol; Walter et al., 2013, Nat. Commun.). Ly6G+ cells are observed predominantly in the dermis, as well as in characteristic neutrophil-filled abscess-like structures located just beneath the stratum corneum, as previously described in IMQ-treated mouse skin (Walter et al., 2013, Nat. Commun.). CD3+ cells are found both in dermis and epidermis. Some infrequent B220+ cells are also detected, in the dermis exclusively. This information is now included in the text of the results section (p.12) and representative photomicrographs of the different IHC stainings are shown in the new Figure S4. The corresponding methods have been implemented in the ‘methods’ section on p.8.
We did not observe any qualitative differences in infiltrate composition between WT (n=3) and Il-38 KO (n=3) mice. This is now illustrated in the new Figure S4C.

To Reviewer #2:
This report entitled “ the severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression” authored by Dr. Palomo et al demonstrated IL-38 is not an antagonist for IL-36 by comparing with the established IL-36 antagonist, IL-36Ra,
These authors employed a common IMQ-induced mouse inflammation model to evaluate inhibition of IL-36 on skin inflammation development. IL-36 is a critical cytokine in regulating the development of skin inflammations, thus inhibition of IL-36 would be a potential approach to control skin inflammations, such as psoriatic inflammation development.

	The sequence of IL-38 has homology with IL-1Ra and IL-36Ra, two established IL-36 antagonists, and thus IL-38 was proposed to be a potential antagonist of IL-36. Based on this study, they demonstrated that IL-38 is not the antagonist of IL-36. These authors developed two mouse clones, IL-38-/- and IL-36ra-/-, to evaluate the role of IL-38 on IMQ-induced skin inflammation. This study is well designed and conclusion is based on convincing evidence. However, a few concerns need to be addressed before it can be accepted for a publication in the journal. Specifically, Major concerns: 1. To evaluate the inflammation by histopathological assessment, proper references need to be cited. For example, how to quantify the cell infiltration and how to assess scabs We have added further details and appropriate references to the 'Material and Methods' section (p. 8), as follows: Infiltration of inflammatory cells was evaluated using a modification of the semi-quantitative analysis described previously [9], in which we evaluated the proportion of ear tissue containing infiltrated neutrophils, instead of using scores to reflect differential cell counts. Neutrophils infiltrating the dermis were identified morphologically on HE-stained sections. Areas containing infiltrated neutrophils were then delineated manually and the sum of all neutrophil-containing areas was normalized to the total ear surface. Consistent with previous reports [9, 35], we also observed neutrophil-filled abscess-like structures beneath the stratum corneum in IMQ-treated mice, which were identified morphologically on HE-stained sections and counted manually along the whole ear. Please note that we now refer to these structures, termed 'scabs' in the previous version of the manuscript, using the more complete histological designation 'neutrophil-filled abscess-like structures' as published by Walter et al. (2013, Nat. Commun.) for the sake of consistency with this previous publication. 2. When mention skin, it is a sum of keratinocytes, fibroblasts and variety of immune cells, etc. Thus it needs more precise in the 1st paragraph of discussion section, " ….consistent with previous observations in human skin (17, 19), but not in dermal fibroblasts". We agree and modified the sentence accordingly. It now reads: We detected Il-38 mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of IL-38 in keratinocytes in human skin [17, 19], but not in dermal fibroblasts. 3. Regarding the discussion above, what are these cytokine expression profile in human skin should be discussed thoroughly to make this study more translational to human. As mentioned above, previous studies described IL-38 expression in human keratinocytes, which is consistent with our results in mice. We also previously reported decreased expression of IL-38 mRNA in total skin samples obtained from patients with moderate to severe plaque psoriasis, as compared to normal control biopsies obtained from surgical samples of healthy skin (Boutet et al., 2016, Clin. Exp. Immunol.). These observations indeed suggest similar regulation of Il-38 expression in human and mouse in the context of skin inflammation. This is now more explicitly stated in the discussion (p.15), as follows: We detected Il-38 mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of IL-38 in keratinocytes in human skin [17, 19], but not in dermal fibroblasts. In agreement with our recent observations in psoriatic human skin [17], Il-38 mRNA levels were decreased in mouse during skin inflammation in vivo. Minor concerns: 1. a few typos were found, manuscript should be carefully proof read before submit to the journal The manuscript was proofread.
Additional Information:	

Question	Response
Financial Disclosure Please describe all sources of funding that have supported your work. **This information is required for submission and will be published with your article, should it be accepted.** A complete funding statement should do the following: Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding. **Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If the funders had **no role** in any of the above, include this sentence at the end of your statement: "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*" However, if the study was **unfunded**, please provide a statement that clearly indicates this, for example: "*The author(s) received no specific funding for this work.*" * typeset	This work was supported by the Rheumasearch Foundation (http://www.rheumasearch.ch) and the Institute of Arthritis Research (https://www.iar-suisse.ch/home.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Competing Interests You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)? **If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:*	The authors have declared that no competing interests exist.

If no authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*" * typeset	
Ethics Statement You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should **also be included in the Methods section** of your manuscript. Please write "N/A" if your study does not require an ethics statement. **Human Subject Research (involved human participants and/or tissue)** All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript. Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research. **Animal Research (involved vertebrate animals, embryos or tissues)** All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare	Animal studies were approved by the Animal Ethics Committee of the University of Geneva and the Geneva Veterinarian Office (authorizations GE-43-15 and GE-115-17) and complied with the requirements defined by the Swiss regulation (federal Animal protection ordinances and law). Mice were euthanized by exposure to gradually increasing concentrations of carbon dioxide (CO2) in a dedicated euthanasia chamber or by exsanguination (cardiac puncture) under deep terminal anesthesia, followed by cervical dislocation, as indicated in the Methods section of the manuscript.

and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
Data Availability PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found. Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. **Please note that simply stating 'data available on request from the author' is not acceptable.** ***If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.*** Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	Yes - all data are fully available without restriction

Please describe where your data may be found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details. If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.” If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example: “Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.” “Data are from the XXX study whose authors may be contacted at XXX.” * typeset	All relevant data are within the paper and its Supporting Information files.
Additional data availability information:	

Sirs:

Here enclosed please find a revised version of manuscript PONE-D-17-41406 « The severity of imiquimod-induced skin inflammation is independent of endogenous IL-38 expression » by J. Palomo et al.

We would like to thank the editor and the reviewers for their comments, which helped us improve the quality of the manuscript. You will find attached a rebuttal letter responding to each point raised by the editor and the two reviewers.

We hope that our manuscript will meet now your expectations for acceptance in PLOS ONE.

Sincerely,

Gaby Palmer, PhD

The severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression

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Abbreviations used in this article: IMQ, imiquimod

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Disclosures: The authors have no financial conflicts of interest.

Short title: IL-38 in imiquimod-induced skin inflammation

Abstract

The IL-1 cytokine family includes eleven members, among which Il-36α, β and γ, IL-36Ra and IL-38. The IL-36 cytokines are involved in the pathogenesis of psoriasis. IL-38 is also expressed in the skin and was previously proposed to act as an IL-36 antagonist. In this study, we thus examined expression and function of Il-38 in a mouse model of imiquimod (IMQ)-induced skin inflammation.

Il-38 mRNA was detected in the epidermis and in primary mouse keratinocytes, but not in dermal fibroblasts. At the peak of IMQ-induced inflammation, skin *Il-38* mRNA levels were reduced, whereas *Il-36ra* mRNA expression increased. The severity of IMQ-induced skin inflammation, as assessed by recording ear thickness and histological changes, was similar in Il-38 KO and WT littermate control mice, while, in contrast, Il-36ra-deficient mice displayed more severe skin pathology than their WT littermates. Il-38-deficiency had no impact on IMQ-induced expression of proinflammatory mediators in the skin *in vivo*, on the basal expression of various cytokines or chemokines by cultured primary keratinocytes and dermal fibroblasts *in vitro*, or on the response of these cells to Il-36β. Finally, after cessation of topical IMQ application, the resolution of skin inflammation was also not altered in Il-38 KO mice.

In conclusion, Il-38-deficiency did not impact the development or resolution of IMQ-induced skin inflammation. Our observations further suggest that endogenous Il-38 does not exert Il-36 inhibitory activity in this model, or in cultured skin cells. A potential anti-inflammatory function of Il-38 in mouse skin thus still remains to be demonstrated.

Introduction

Psoriasis is a chronic disease characterized by localized or generalized skin lesions including erythematous plaques and lamellar scales [1], [2]. In a significant proportion of patients, the skin disease is accompanied by arthritis [3]. To date, many aspects of psoriasis pathogenesis remain unclear, but a dysregulated crosstalk between immune and skin cells is believed to underlie epidermal hyper-proliferation and hyperplasia, leukocyte infiltration and vascular proliferation in the papillary dermis [4, 5]. Historically, studies first focused on immune cells, but recently, non-immune cells, in particular keratinocytes and fibroblasts, were shown to play important roles in the disease process [6]. Various environmental triggers can induce or exacerbate psoriasis in humans, among which imiquimod (IMQ), a Toll-Like Receptor (TLR)7 agonist that activates the innate immune response [7, 8]. Similarly, topical application of the IMQ-containing Aldara cream on mouse skin causes cutaneous inflammation with leukocyte influx and epidermal hyperplasia, resembling human psoriatic lesions [9-11].

The IL-1 family of cytokines includes seven agonists, IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ, and four established or hypothetical antagonists, IL-1Ra, IL-36Ra, IL-37 and IL-38 [12, 13]. The three IL-36 agonists signal through the same receptor, composed of the specific alpha chain IL-36R (IL-1Rrp2) and the common beta chain IL-1 receptor accessory protein [14]. IL-36 cytokines and IL-36R are mainly expressed by keratinocytes, but also by dendritic cells and Th0 cells [15, 16]. IL-36 agonists are involved in the pathogenesis of skin inflammation both in mouse models and in human psoriasis [17]. In particular, in mice, IL-36 plays a crucial role in the development of IMQ-induced skin inflammation, which is exacerbated in absence of its antagonist IL-36Ra [15]. In humans, IL-36Ra deficiency results in a severe form of pustular psoriasis [18].

Conversely, little is known about IL-38, which was initially proposed to act as an antagonist based on its sequence homology with IL-1Ra and IL-36Ra [19]. Consistently, anti-

inflammatory effects were subsequently reported for IL-38 in cultured cells [20-23] and in mice [23-26]. In humans, polymorphisms in the *IL1F10* locus are associated with rheumatic diseases [27-30] and IL-38 expression or serum levels have been recorded in some autoimmune pathologies [21, 24, 31-33], but overall few data are available concerning the role of IL-38 in inflammatory diseases. The identity of the IL-38 receptor(s) also remains elusive, although IL-1 receptor type I [19], IL-36R [20] and interleukin-1 receptor accessory protein-like 1 (IL1RAPL1, also named TIGIRR-2) [22] have successively been proposed as candidates. Recently, we observed reduced *IL-38* transcript levels in human psoriatic skin, whereas *IL-38* expression was increased in colonic biopsies of Crohn's disease patients and in synovial tissues of patients suffering from rheumatoid arthritis [17].

Given the importance of the IL-36 system in the skin and since IL-38 was previously proposed to act as an IL-36R antagonist [20], in the present study we examined the expression and function of endogenous Il-38 in the context of IMQ-induced skin inflammation. We confirmed that *Il-38* was mainly expressed by keratinocytes in the mouse, as previously reported for human skin [19], but not by dermal fibroblasts. Furthermore, our data obtained using Il-38 KO mice indicate that, in contrast to Il-36ra-deficiency, lack of endogenous Il-38 does not impact the development or resolution of IMQ-induced skin inflammation.

Materials and methods

Mice

Il-38 (*Il-1f10*)–deficient mice (*Il-38*$^{-/-}$; Balb.129/Sv-Il1f10) and *Il-36ra* (*Il-1f5*)–deficient mice (*Il-36ra*$^{-/-}$; Balb.B6.129S5/SvEv-Il36rn) [34] were created by Amgen Inc. (Seattle, WA, USA). *Il-38*$^{-/-}$ mice were generated by targeting of the *Il1f10* gene in 129Sv ES GS1 cells, resulting in the deletion of all coding exons and leading to a complete loss of *Il-38* mRNA expression (S1 Fig). Genotyping of *Il-38*$^{-/-}$ mice was performed using a 3-primer PCR combining a forward primer specific for the wild-type (WT) (5'-TGG CCC AGC TGA GCC CCA GCA GCC AGT-3') or the KO (5'-CAG CTT CTG TTC CAC ATA CAC TTC-3') allele with a common reverse primer (5'-TGC TGA GCA AGA AGA TCT CAG ACT-3') (S1 Fig). Genotyping of *Il-36ra*$^{-/-}$ mice was performed using a 3-primer PCR combining a forward primer specific for the WT (5'-GAA AAG AGA GAG TGA ATG GGA G-3') or the KO (5'-GAT TGC ACG CAG GTT CTC-3') allele with a common reverse primer (5'-GAG CTC CAT GAT GTT CAC TGG-3'). *Il-38* and *Il-36ra*-deficient mice were backcrossed onto the BALB/cJ background using a marker-assisted selection protocol (MASP). The purity of the BALB/cJ background, as assessed by genome-wide single nucleotide polymorphism (SNP) scanning using a 384 SNP panel with SNPs spread across the genome at 7 Mbp intervals (Charles River Laboratories, Wilmington, MA) was > 97% and > 99% for *Il-38*$^{-/-}$ and *Il-36ra*$^{-/-}$ mice respectively. For both mouse lines, heterozygous breedings were then set up to obtain *Il-38*$^{-/-}$ or *Il-36ra*$^{-/-}$ mice and their respective WT co-housed littermates for experiments. All mice were bred and maintained in the conventional area of the animal facility at the Geneva University School of Medicine and housed in open cages, enriched with Nordic aspen bedding (Tapvei, Harjumaa, Estonia), nestlets, and a mouse house, in groups of 2-6 individuals on a 12h light/dark cycle. The temperature in the room was maintained between 20-24° Celsius and hygrometry was 30-70%. Extruded food and tap water were

provided *ad libitum*. Mice were monitored daily for signs of distress (signs of dehydration, unresponsiveness to extraneous stimuli, hunched posture, or labored breathing) and would have been euthanized should these signs have appeared. Animal studies were approved by the Animal Ethics Committee of the University of Geneva and the Geneva Veterinarian Office (authorizations GE-43-15 and GE-115-17) and complied with the requirements defined by the Swiss regulation (federal animal protection ordinances and law). Experiments were performed according to the appropriate codes of practice and all efforts were made to minimize suffering.

Isolation of skin, epidermis and primary culture of keratinocytes and dermal fibroblasts

To harvest untreated skin from tails and ears for RNA extraction and for the isolation and culture of primary cells, naïve mice were euthanized by exposure to gradually increasing concentrations of carbon dioxide (CO_2) in a dedicated euthanasia chamber. For the comparison of cytokine expression in naïve total skin and in the epidermis, a fragment of shaved abdominal skin was removed, rinsed in PBS / 100 U/ml penicillin / 100 µg/ml streptomycin and incubated in Keratinocyte-Serum Free Medium (K-SFM) (Life Technologies, Carlsbad, Ca, USA) / 10mg/ml Dispase II (Sigma-Aldrich, Saint-Louis, Mi, USA) overnight at 4°C. The piece of skin from each mouse was cut into halves. One part was immediately frozen in liquid N_2, while, for the second part, the epidermis was detached from the dermis, collected and frozen. For keratinocyte culture, mouse tails were removed, rinsed in PBS / 100 U/ml penicillin / 100 µg/ml streptomycin and incubated in K-SFM / 10mg/ml Dispase II overnight at 4°C. Epidermis was then detached from the dermis and gently mixed 3 times for 1 minute with 0.05% Trypsin / 0.02% EDTA. Isolated cells were cultured in

collagen type IV coated plates, in K-SFM complemented with 53.4 µg/ml Bovine Pituitary Extract (BPE) and 6.6 ng/ml human recombinant EGF (Life Technologies). The cells were used when they reached 80% confluence. For dermal fibroblast culture, ears were removed, minced and incubated for 2 h in HBSS / Ca^{2+} / Mg^{2+} / 2 mg/ml collagenase (Sigma-Aldrich) at 37°C. The tissue was then digested for 30 min in 0.05% Trypsin / 0.02% EDTA at 37°C and the cells and tissue pieces were cultured in Petri dishes in DMEM / 10% FBS / 1 x non-essential amino acids / 100 U/ml penicillin / 100 µg/ml streptomycin to recover fibroblasts, which were used after the third passage. Purity of the isolated epidermal fraction and of keratinocyte and fibroblast cultures was verified by analyzing mRNA expression of keratinocyte-specific *Keratin 14* and fibroblast-specific *Collagen 1a* and *Vimentin* markers (S2 Fig).

IMQ-induced skin inflammation

Psoriasis-like skin inflammation was induced in adult, age-matched, 8 to 12-week-old female *Il-38*$^{-/-}$ or *Il-36ra*$^{-/-}$ mice and their respective WT littermates by daily application of a topical dose of 12.5mg of Aldara™ cream (Meda Pharma GmbH, Frankfurt, Germany), containing 5% (0.625mg) of imiquimod (IMQ), on one ear during 7-8 days. Body weight was recorded and ear thickness was measured daily using a pocket thickness gage (Mitutoyo Europe GmbH, Dusseldorf, Germany). At the end of the experiment, mice were euthanized under deep terminal anesthesia by exsanguination (cardiac puncture) followed by cervical dislocation. Ears were collected for histological analysis and RNA extraction.

Histopathological evaluation and immunohistochemistry

Ears were fixed in 4% buffered formaldehyde and embedded in paraffin. Ear sections (4µm) were deparaffinized and stained with hematoxylin and eosin (HE; Diapath S.p.A., Milano,

Italy). Ly6G, CD3 and B220 expression was examined by immunohistochemistry on paraffin sections using the following antibodies: rat anti-mouse Ly6G (clone 1A8, BD Bioscience, 1/1000), rat anti-human CD3 (clone CD3-12, AbD Serotec, Kidlington, UK, 1/200), or rat anti-mouse B220 (clone RA3-6B2, BD Bioscience, 1/200). Tissue sections were deparaffinized and antigens retrieved by pressure-cooking in 10 mM citrate buffer, pH 6 (anti-Ly6G or anti-B220 staining) or in 10 mM Tris, 1 mM EDTA buffer, pH 9 (anti-CD3 staining). Slides were blocked for endogenous peroxidase activity and incubated with anti-Ly6G, CD3 or B220 antibodies in antibody diluent (S2022, Dako AG, Baar, Switzerland) overnight at 4°C. Subsequently, slides were incubated with appropriate HRP-conjugated secondary antibodies in antibody diluent and developed with diaminobenzidine (Dako). Slides were scanned on a Mirax Midi slide scanner (Carl Zeiss Microscopy, Feldbach, Switzerland). The ZEN blue software (Carl Zeiss Microscopy) was used for image acquisition and measurements. Total ear area was determined on HE-stained sections using the Definiens Developer XD2 software (Definiens, Munich, Germany) and different histopathological parameters were determined in a blinded manner. The average epidermal thickness was estimated by taking 20 measures along the ear. Infiltration of inflammatory cells was evaluated using a modification of the semi-quantitative analysis described previously [9], in which we evaluated the proportion of ear tissue containing infiltrated neutrophils, instead of using scores to reflect differential cell counts. Neutrophils infiltrating the dermis were identified morphologically on HE-stained sections. Areas containing infiltrated neutrophils were then delineated manually and the sum of all neutrophil-containing areas was normalized to the total ear surface. Consistent with previous reports [9, 35], we also observed neutrophil-filled abscess-like structures beneath the stratum corneum in IMQ-treated mice, which were identified morphologically on HE-stained sections and counted manually along the whole ear.

RNA extraction and RT qPCR

Total RNA was extracted using TRIzol® reagent (Life Technologies) and treated with RNAse free DNAse set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (100-500ng) was then reverse transcribed using SuperScript II Reverse transcriptase (Invitrogen, Waltham, USA). The mRNA expression levels were determined by quantitative PCR using the SYBR® Green PCR Master Mix (Applied Biosystem, Waltham, USA) according to the manufacturer's protocol. The primer sequences (Eurofins, Ebersberg, Germany) are detailed in Table 1. Relative levels of mRNA expression were normalized to ribosomal protein L32 (*Rpl32*) mRNA levels using a comparative method ($2^{-\Delta Ct}$). Non-reverse-transcribed RNA samples and Buffer were included as negative controls.

Table 1. Primers used for qPCR.

Gene	Accession number	Primer sequence	Amplicon (pb)
Col1a1	NM_007742.4	Fwd 5'-GGCTCCTGCTCCTCTTAG-3' Rev 5'-ACAGTCCAGTTCTTCATTGC-3'	194
Cxcl1	NM_008176.3	Fwd 5'-ACTCAAGAATGGTCGCGAGG-3' Rev 5'-GTGCCATCAGAGCAGTCTGT-3'	123
K14 Var1 Var2	NM_016958.2 NM_001313956.1	Fwd 5'-ATCGAGGACCTGAAGAGCAA-3' Rev 5'-GGCTCTCAATCTGCATCTCC-3'	220
Il-1a	NM_010554.4	Fwd 5'-GGGAAGATTCTGAAGAAGAG-3' Rev 5'-GAGTAACAGGATATTTAGAGTCG-3'	319
Il-1b	NM_008361.4	Fwd 5'-TGTGAAATGCCACCTTTTGA-3' Rev 5'-GTGCTCATGTCCTCATCCTG-3'	248
Il-6	NM_031168.2	Fwd 5'-TGAACAACGATGATGCACTTGCAGA-3' Rev 5'-TCTGTATCTCTCTGAAGGACTCTGGCT-3'	211
Il-18	NM_008360.1	Fwd 5'-CAGGCCTGACATCTTCTG-3' Rev 5'-CTGACATGGCAGCCATT-3'	104
Il-36a	NM_019450.3	Fwd 5'-TAGTGGGTGTAGTTCTGTAGTGTGC-3' Rev 5'-GTTCGTTCAAGAGTGTCCAGATAT-3'	268
Il-36b	NM_027163.4	Fwd 5'-ACAAAAAGCCTTTCTGTTCTATCAT-3' Rev 5'-CCATGTTGGATTTACTTCTCAGACT-3'	186
Il-36g	NM_153511.3	Fwd 5'-AGAGTAACCCCAGTCAGCGTG-3' Rev 5'-AGGGTGGTGGTACAAATCCAA-3'	186
Il-36r	NM_133193.3	Fwd 5'-AAACACCTAGCAAAAGCCCAG-3' Rev 5'-AGACTGCCCGATTTTCCTATG-3'	262
Il-36ra	NM_019451.2	Fwd 5'-TGGAGCTCATGATGGTTCTG-3' Rev 5'-TAATGACCTTCTCTGCGTGC-3'	123
Il-38	NM_153077.2	Fwd 5'-CCTGGCGTGTGTAAAGACAA-3' Rev 5'-CAGATCCCAAGCTTCTCTGG-3'	125
Rpl32	NM_172086.2	Fwd 5'-CACCAGTCAGACCGATATGTGAAAA-3' Rev 5'-TGTTGTCAATGCCTCTGGGTTT-3'	64
S100a9 Var1 Var2	NM_001281852.1 NM_009114.3	Fwd 5'-CACCCTGAGCAAGAAGGAAT-3' Rev 5'-TGTCATTTATGAGGGCTTCATTT-3'	95

Tnfa	NM_013693.3	Fwd 5'-AGTTCTATGGCCCAGACCCT-3' Rev 5'-GTCTTTGAGATCCATGCCGT-3’	159
Vim	NM_011701.4	Fwd 5’-CGGCTGCGAGAGAAATTGC -3’ Rev 5’-CCACTTTCCGTTCAAGGTCAAG-3’	124

Statistical analysis

Data were analyzed using Prism version 6 (Graphpad Software, La Jolla, USA). Unpaired Mann-Whitney comparison tests, two-way ANOVA followed by a Holm–Sidak’s comparison test, or paired two-way ANOVA followed by a Sidak post-test were used, as indicated. Values are expressed as mean ± SEM. Statistical significance was defined at a p-value < 0.05.

Results

Expression of Il-38 and of Il-36 family cytokine mRNA in naïve mouse skin and in primary mouse skin cells

Keratinocytes express various pattern recognition receptors and act as early detectors of microbial or endogenous danger signals. After activation, they secrete chemokines, cytokines and anti-microbial peptides. In human skin, keratinocytes were suggested to be the main source of IL-38 [19]. In mouse skin, we previously observed *Il38* mRNA expression [17], but its cellular source had not been described. We first examined the mRNA expression of *Il-38* and of the different *Il-36* agonists and antagonist in total skin and isolated epidermis of naïve BALB/c mice. We detected similar levels of *Il-38*, *Il-36ra*, *Il-36a*, *Il-36b* and *Il-36g* mRNA in total skin and in epidermis (Fig 1A). We further investigated the expression of *Il-38* and of the *Il-36* agonists and antagonist in cultured primary keratinocytes and dermal fibroblasts isolated from naïve BALB/c mouse skin. We observed *Il-38* mRNA expression in keratinocytes, but not in dermal fibroblasts. Similarly, transcripts for *Il-36* agonists and *Il-36ra* were detected in keratinocytes only (Fig 1B). In contrast, Il-36 receptor (*Il-36r*) mRNA expression was observed in both skin cell types (S2B Fig).

Il-38 deficiency has no impact on the development of IMQ-induced psoriasis

Consistent with our previous observations during the development of IMQ-induced skin inflammation in C57BL/6 mice [17], we confirmed decreased *Il-38* mRNA expression at the peak of IMQ-induced inflammation in the skin of BALB/c mice, whereas the mRNA levels of *Il-36ra* and of the *Il-36* agonists were increased after IMQ treatment (S3 Fig).

We then went on to investigate the involvement of endogenous Il-38 in the pathogenesis of IMQ-induced skin inflammation, using Il-38-deficient mice (S1 Fig). Homozygous *Il-38*$^{-/-}$ mice are healthy, fertile, and show weight gain similar to that of their WT littermates from birth to adult age. They do not display any spontaneous phenotype in our conventional animal facility. *Il-38*-deficiency had no effect on the severity of IMQ-induced skin inflammation (Fig 2). Ear thickness increased similarly after IMQ application in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 2A), and both groups of mice displayed comparable histopathological alterations on day 7 of IMQ-treatment (Fig 2B). Immunohistochemical analyses confirmed infiltration of IMQ-treated ears by inflammatory cells, in particular neutrophils, as reported previously [9, 35]. Abundant infiltration of Ly6G^{+} cells was observed predominantly in the dermis (S4A Fig). In addition, characteristic neutrophil-filled abscess-like structures were found just beneath the stratum corneum [35] (S4B Fig). CD3^{+} T cells were detected both in the dermis and the epidermis, while some infrequent B220^{+} B lymphocytes were detected in the dermis exclusively (S4A Fig). We did not observe any qualitative differences in infiltrate composition between WT and Il-38 KO mice, as illustrated by anti-Ly6G and anti-CD3 staining of ear sections after 7 days of IMQ-treatment (S4C Fig). Furthermore, histopathological scoring indicated that the extent of neutrophil infiltration, the epidermal thickness and the numbers of neutrophil-filled abscess-like structures were similar in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 2C).

We compared these observations with the response of mice deficient in Il-36Ra, a well-known inhibitor of IL-36-dependent IMQ-induced skin inflammation [15]. We confirmed that *Il-36ra*-deficiency resulted in an aggravation of skin pathology. Indeed, *Il-36ra*$^{-/-}$ mice developed a more severe disease, as shown by an increased ear thickening, as compared to their WT littermates (Fig 2D). This was associated with more severe histopathological changes (Fig 2E and F).

IL-38 deficiency has no impact on IMQ-induced expression of proinflammatory mediators *in situ* or in cultured skin cells

We examined whether the lack of endogenous *Il-38* could nevertheless influence the local expression of proinflammatory mediators in the skin. Thus, we analyzed mRNA expression of various cytokines and chemokines in the ear after 7 days of IMQ application. We did not find any significant differences in *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Cxc-l1*, *Il-6*, *Il1-α*, *IL1-β*, *Il-18* or *Tnfα* mRNA expression between *Il-38*$^{-/-}$ and WT mice, while *Il-38* was obviously not expressed in *Il-38*$^{-/-}$ mice (Fig 3A and S5 Fig).

We further investigated the expression of proinflammatory mediators by cultured primary keratinocytes and dermal fibroblasts isolated from *Il-38*-deficient and WT mice, at baseline and upon stimulation with rec. mouse Il-36β. Although both cell types express *Il36r* (S2B Fig), keratinocytes and fibroblasts displayed differential responses to Il-36β. Indeed, in keratinocytes, Il-36β enhanced mRNA expression of *Il-36α, Il-36γ* and *Il-38,* and of the anti-microbial peptide *S100a9* (Fig 3B), while stimulation of dermal fibroblast with Il-36β strongly induced expression of *Il-6* and *Cxcl-1*. Basal expression levels of the various transcripts examined did not differ significantly in cells isolated from *Il-38*$^{-/-}$ or from WT mice, except for the expression of *Il-38* itself. Il-38 deficiency also lacked any major impact on the response of cultured keratinocytes or fibroblasts to Il-36β (Fig 3B).

IL-38 deficiency does not alter IMQ-induced psoriasis resolution

Although Il-38 was not required for the development of IMQ-induced skin inflammation, we wondered whether it might still be involved in the resolution of the pathology. To answer this question, after 7 days of IMQ topical application, *Il-38*$^{-/-}$ mice and their WT littermates were kept untreated for 5 days. As in Fig 2, the severity of peak skin inflammation was again

279 similar in *Il-38*$^{-/-}$ and WT mice. Afterwards, the gradual decrease of ear thickness was also
280 similar in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 4A). Those results were further
281 confirmed by histological analysis. Indeed, neutrophil infiltration, epidermal thickness, and
282 the number of neutrophil-filled abscess-like structures were comparable on day 11 in the
283 presence or in the absence of Il-38 (Fig 4B and C).
284

Discussion

IL-36 cytokines are involved in the pathogenesis of psoriasis [36], as well as in the development of IMQ-induced skin inflammation in the mouse [15, 34]. Since IL-38 is also expressed in the skin [17] and was previously proposed to act as an IL-36 antagonist [20], we studied the expression and function of Il-38 in the IMQ model. We detected *Il-38* mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of *IL-38* in keratinocytes in human skin [17, 19], but not in dermal fibroblasts. In agreement with our recent observations in psoriatic human skin [17], *Il-38* mRNA levels were decreased in mouse during skin inflammation *in vivo*. However, lack of endogenous *Il-38* did not impact the development or resolution of IMQ-induced psoriasis. In our hands, Il-38-deficiency did also not change expression of proinflammatory mediators in inflamed skin *in situ* or in cultured skin cells, nor modify the response of primary keratinocytes and dermal fibroblasts to IL-36 stimulation.

Our *in vivo* data indicate that, in contrast to Il-36ra-deficiency, the absence of Il-38 does not impact the course of IMQ-induced skin inflammation, implying that endogenous Il-38 does not act as an Il-36 antagonist in this context. As it has been suggested that the anti-inflammatory properties of IL-38 are inferior as compared to IL-36Ra [20], it is conceivable that Il-38 deficiency is counterbalanced by the presence of Il-36Ra, whose role was confirmed in this study. Alternatively, the biological function of IL-38 might be unrelated to IL-36 inhibition. Several recent studies indeed demonstrated broader anti-inflammatory properties of IL-38 and/or suggested different mechanisms of action [20-26]. In contrast to several of these studies based on overexpression or injection of recombinant exogenous Il-38 [21, 23-26], we were not able to detect any anti-inflammatory, or other, activity of the endogenous protein in our model.

We further showed that, similarly to what was observed in human keratinocytes [17], murine keratinocytes, but not dermal fibroblasts, express IL-36 agonists, as well as IL-36Ra and IL-38. However, both keratinocytes and dermal fibroblasts expressed the Il36r, although, interestingly, the two cell types responded in a different way to IL-36. Indeed, keratinocytes rather amplified the IL-36 signaling by upregulating Il-36α expression, while fibroblasts produced pro-inflammatory mediators, such as Il-6 and the neutrophil-attracting chemokine Cxcl1. This is consistent with the role of keratinocytes as skin sentinels, which can detect early skin damage and release danger signals and pro-inflammatory mediators. This primary response can then be strongly amplified by dermal fibroblasts, which produce signals to recruit and activate immune cells. Since IL-38 was previously described to antagonize the effects of IL-36 stimulation [20], we also investigated the effects of Il-36 stimulation on primary skin cells isolated from WT and *Il-38*$^{-/-}$ mice. However, Il-38-deficiency did not influence the response of primary keratinocytes or dermal fibroblasts to Il-36. Consistent with our *in vivo* observations, these *in vitro* results thus again failed to provide any evidence for an Il-36 inhibitory function of endogenous Il-38.

In conclusion, while this study does not exclude an inhibitory role of IL-38 in other contexts, our results indicate that Il-38-deficiency does not impact the development or resolution of IMQ-induced skin inflammation. Our observations further suggest that endogenous Il-38 does not exert Il-36 inhibitory activity in this model, or in cultured skin cells. An anti-inflammatory function, or any other role, of Il-38 in mouse skin thus still remain to be demonstrated.

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Figure legends

Fig 1. Expression of *Il-38* and *Il-36* family cytokines in total skin, epidermis, cultured primary keratinocytes and dermal fibroblasts. Basal *Il-38*, *Il-36ra*, *Il-36a*, *Il-36b* and *Il-36g* mRNA expression was quantified by real-time RT-qPCR in total skin (n=5) and epidermis (n=5) of naïve BALB/c WT mice (A); and in cultured primary keratinocytes (Kera, n=4 independent cultures) and dermal fibroblasts (Fibro, n=5 independent cultures) isolated from naïve BALB/c WT mice (B). Data were expressed relative to *L32* levels. Results are shown as individual values and mean ± SEM.

Statistical analysis was performed using an unpaired Mann-Whitney comparison test. A *p*-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

Fig 2. Il-38-deficiency does not influence the severity of IMQ-induced skin inflammation. *Il-38*$^{-/-}$ mice (n=5) and WT littermates (n=5) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days. Ear thickness was followed daily (A) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears on day 7. Scale bar = 100µm. (B); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-filled abscess-like structures were evaluated (C). Results are from one experiment representative of two and are expressed as mean ± SEM of individual mice (n = 5 mice per group). *Il-36ra*$^{-/-}$ mice (n=6) and WT littermates (n=4) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 8 days. Ear thickness was followed daily (D) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears on day 8. Scale bar = 100µm. (E); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-filled abscess-like structures were evaluated (F). Results are expressed as mean ± SEM of individual mice (n = 4-6 mice per group). Statistical

analysis was performed using a paired two-way ANOVA followed by a Sidak post-test for A and and D, and an unpaired Mann-Whitney comparison test in C and F. A p-value < 0.05 was considered significant. ** p<0.01, * p<0.05.

Fig 3. Expression of proinflammatory mediators in IMQ-treated skin and in cultured skin cells of Il-38 deficient mice. *Il-38*$^{-/-}$ mice and WT littermates were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days. Skin mRNA levels for *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Il-38*, *Cxcl-1 and Il-6* were quantified by real-time RT-qPCR on day 7 in the IMQ-treated ears (A). Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 5 mice per group. Statistical analysis was performed by unpaired Mann-Whitney comparison test. A p-value < 0.05 was considered significant. ** p<0.01. Cultured primary keratinocytes (Kera) and dermal fibroblasts (Fibro) isolated from naïve *Il-38*$^{-/-}$ (dark symbols) or WT mice (white symbols), were stimulated with rec. mouse Il-36β at 100ng/ml for 6 h, or left unstimulated (Med). *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Il-38*, *Cxcl-1, Il-6* and *S100a9* mRNA levels were quantified by real-time RT-qPCR (B). Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 6–9 biological replicates per group. Statistical analysis was performed by two-way ANOVA followed by a Holm–Sidak's comparison test. A p-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

Fig 4. Il-38-deficiency does not affect resolution of IMQ-induced inflammation. *Il-38*$^{-/-}$ mice (n=5) and WT littermates (n=5) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days, then left untreated until day 11. Ear thickness was followed daily (A) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears at day 11. Scale bar =

502 100 µm. (B); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-
503 filled abscess-like structures were evaluated (C). Results are representative of 2 independent
504 experiments and expressed as mean ± SEM of individual mice (n = 5 mice per group).
505 Statistical analysis was performed using a paired two-way ANOVA followed by a Sidak's
506 post-test for A and an unpaired Mann-Whitney comparison test in C. A p-value < 0.05 was
507 considered significant.
508

Supporting information

S1 Figure. Generation of mice deficient for IL-38. Schematic representation of *Il1f10* gene invalidation: in the targeted allele, a neomycin selection cassette was inserted to replace all coding exons of the *Il1f10* gene (A). Mouse genotyping was performed on total DNA extracted from ear biopsies. PCR products for the WT (150 bp) and KO (250 bp) alleles are shown in *Il-38*$^{-/-}$, *Il-38*$^{+/-}$ and *Il-38*$^{+/+}$ (WT) DNA samples (B). *Il-38* mRNA levels were quantified by real-time RT-qPCR on skin samples from naïve *Il-38*$^{-/-}$ and WT mice. Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 3 per group (C).

S2 Figure. Expression of keratinocyte and fibroblast specific markers in total skin, epidermis and primary skin cells. Basal mRNA expression of keratinocyte-specific *Keratin 14*, as well as of fibroblast-specific *Collagen 1a* and *Vimentin* was quantified by real-time RT-qPCR in total skin (n=5) and epidermis (n=5) of naïve BALB/c WT mice (A). *Keratin 14*, *Collagen 1a, Vimentin*, and *Il-36r* mRNA levels were quantified by real-time RT-qPCR in cultured primary skin keratinocytes (Kera, n=4 independent cultures) and dermal fibroblasts (Fibro, n=5 independent cultures) isolated from the skin of naïve WT BALB/c mice (B). Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed using an unpaired Mann-Whitney comparison test. A *p*-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

S3 Figure. Expression of IL-38 and IL-36 family members in IMQ-treated skin of WT BALB/c mice. WT BALB/c mice were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ) for 8 days (n=7). Skin mRNA levels for *Il-38*, *Il-36ra*, *Il-36α*, *Il-36β* and *Il-36γ* were quantified by real-time RT-qPCR in the non-treated ear (Ctr) and

in the IMQ-treated ear on day 8. Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed by unpaired Mann-Whitney comparison test. A p-value < 0.05 was considered significant. ** p<0.01, *** p<0.001.

S4 Figure. Characterization of the inflammatory infiltrate in IMQ-treated ears. Representative HE (upper left panel), anti-Ly6G (brown staining, upper right panel), anti-CD3 (brown staining, lower left panel), and anti-B220 (brown staining, arrows, lower right panel) stained sections are shown for IMQ-treated WT ears at the peak of inflammation on day 7 (A). Representative **a**nti-Ly6G (brown staining, left panel) and HE (right panel) stained sections including neutrophil-filled abscess-like structures located just beneath the stratum corneum (arrows) are shown for IMQ-treated WT ears at the peak of inflammation on day 7 (B). Representative anti-Ly6G (brown staining, left panels) and anti-CD3 (brown staining, right panels) stained sections are shown for IMQ-treated ears of WT (upper panels) or *Il-38*$^{-/-}$ (lower panels) littermate mice at the peak of inflammation on day 7 (C). Scale bar = 100 µM.

S5 Figure. IMQ-induced expression of proinflammatory mediators in the skin of *Il-38* deficient mice. *Il-38*$^{-/-}$ mice and WT littermates were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days (n=5). Skin mRNA levels for *Il-1α*, *Il-1β*, *Il-18* and *Tnfa* were quantified by real-time RT-qPCR on day 7. Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed by unpaired Mann-Whitney comparison test. No significant differences were observed between the groups.

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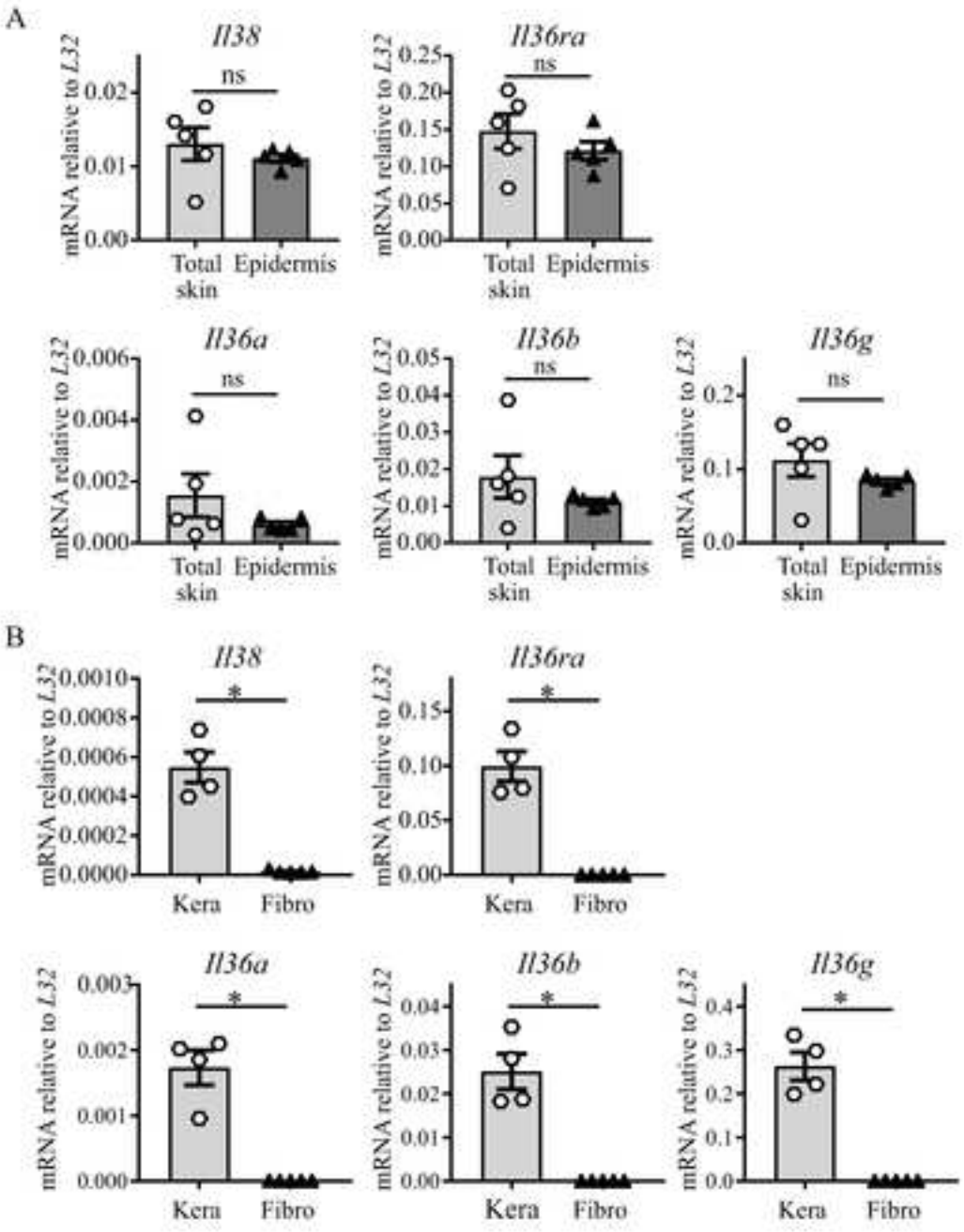
Figure 1



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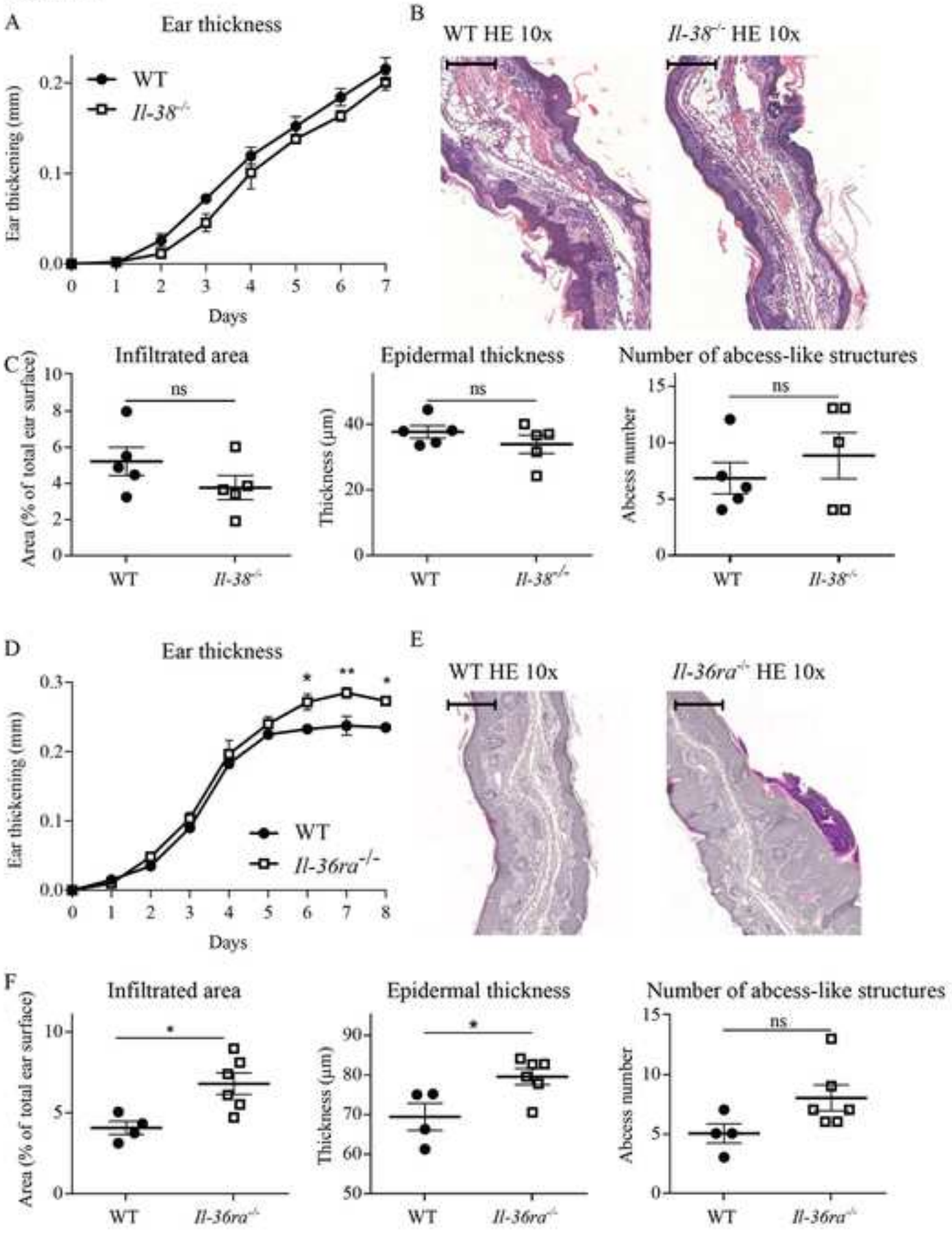

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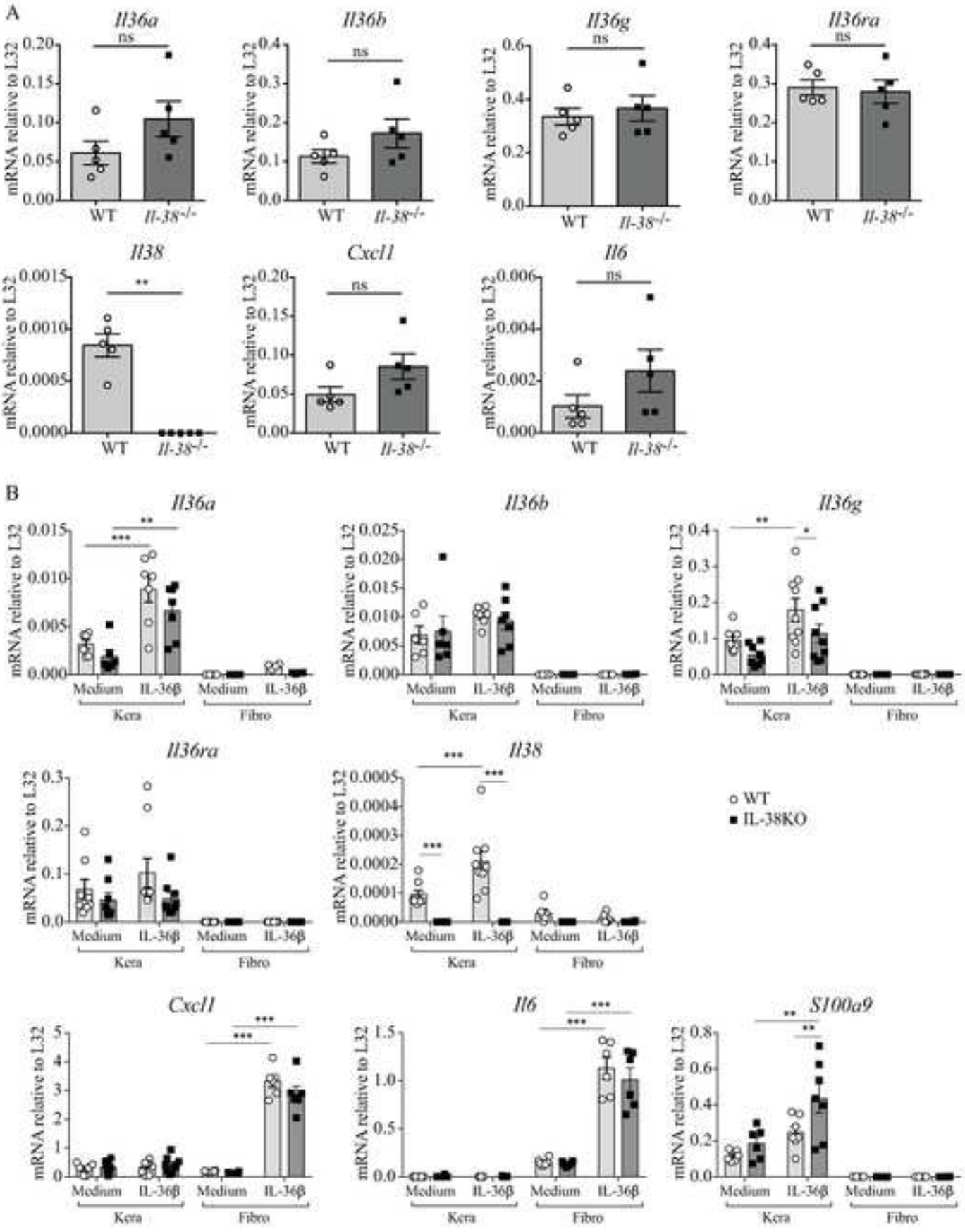

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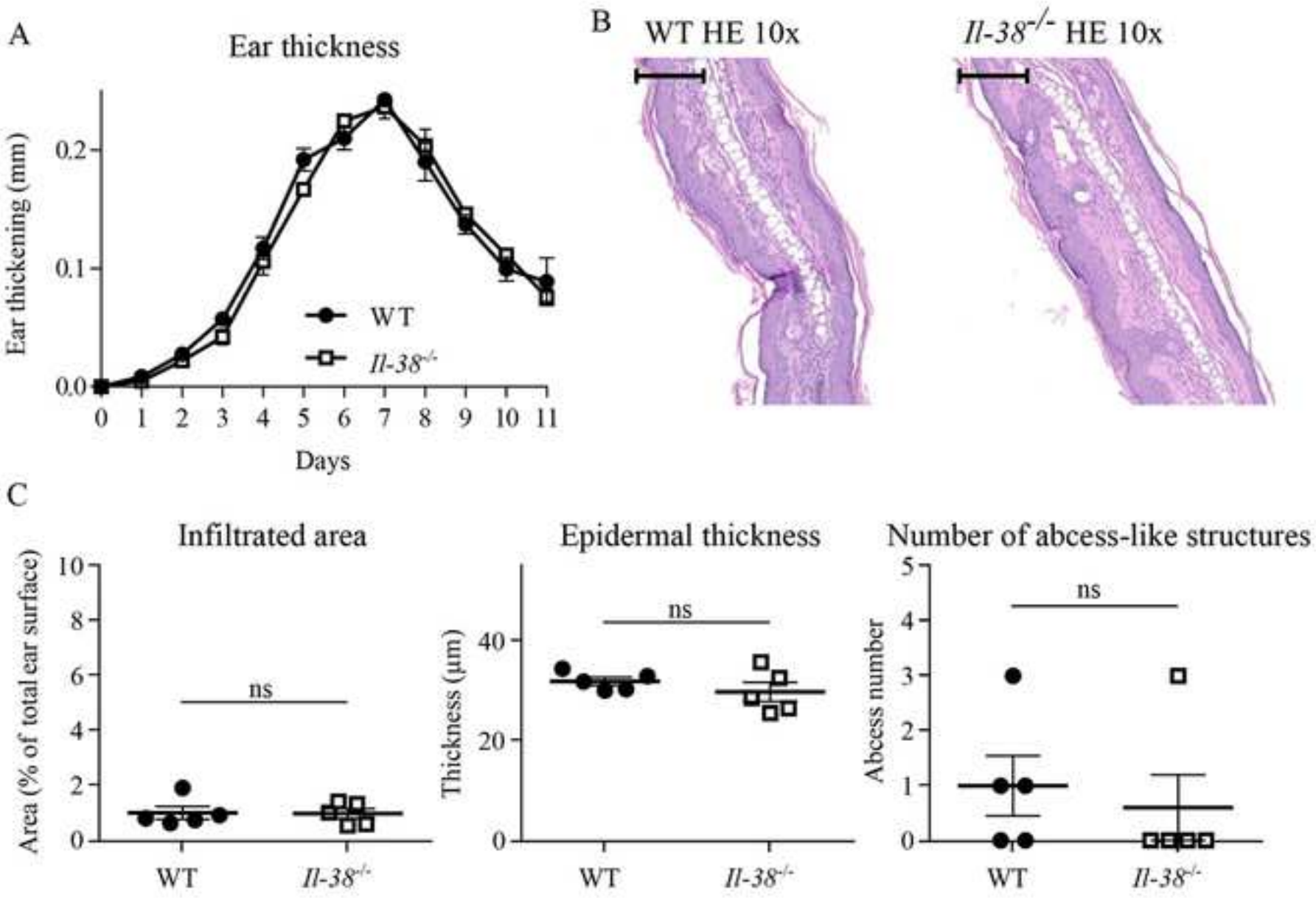

Supplementary Figure 1

Supplementary Figure 2

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Supplementary Figure 5

The severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression

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Short title: IL-38 in imiquimod-induced skin inflammation

Abstract

The IL-1 cytokine family includes eleven members, among which Il-36α, β and γ, IL-36Ra and IL-38. The IL-36 cytokines are involved in the pathogenesis of psoriasis. IL-38 is also expressed in the skin and was previously proposed to act as an IL-36 antagonist. In this study, we thus examined expression and function of Il-38 in a mouse model of imiquimod (IMQ)-induced skin inflammation.

Il-38 mRNA was detected in the epidermis and in primary mouse keratinocytes, but not in dermal fibroblasts. At the peak of IMQ-induced inflammation, skin *Il-38* mRNA levels were reduced, whereas *Il-36ra* mRNA expression increased. The severity of IMQ-induced skin inflammation, as assessed by recording ear thickness and histological changes, was similar in Il-38 KO and WT littermate control mice, while, in contrast, Il-36ra-deficient mice displayed more severe skin pathology than their WT littermates. Il-38-deficiency had no impact on IMQ-induced expression of proinflammatory mediators in the skin *in vivo*, on the basal expression of various cytokines or chemokines by cultured primary keratinocytes and dermal fibroblasts *in vitro*, or on the response of these cells to Il-36β. Finally, after cessation of topical IMQ application, the resolution of skin inflammation was also not altered in Il-38 KO mice.

In conclusion, Il-38-deficiency did not impact the development or resolution of IMQ-induced skin inflammation. Our observations further suggest that endogenous Il-38 does not exert Il-36 inhibitory activity in this model, or in cultured skin cells. A potential anti-inflammatory function of Il-38 in mouse skin thus still remains to be demonstrated.

Introduction

Psoriasis is a chronic disease characterized by localized or generalized skin lesions including erythematous plaques and lamellar scales [1], [2]. In a significant proportion of patients, the skin disease is accompanied by arthritis [3]. To date, many aspects of psoriasis pathogenesis remain unclear, but a dysregulated crosstalk between immune and skin cells is believed to underlie epidermal hyper-proliferation and hyperplasia, leukocyte infiltration and vascular proliferation in the papillary dermis [4, 5]. Historically, studies first focused on immune cells, but recently, non-immune cells, in particular keratinocytes and fibroblasts, were shown to play important roles in the disease process [6]. Various environmental triggers can induce or exacerbate psoriasis in humans, among which imiquimod (IMQ), a Toll-Like Receptor (TLR)7 agonist that activates the innate immune response [7, 8]. Similarly, topical application of the IMQ-containing Aldara cream on mouse skin causes cutaneous inflammation with leukocyte influx and epidermal hyperplasia, resembling human psoriatic lesions [9-11].

The IL-1 family of cytokines includes seven agonists, IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ, and four established or hypothetical antagonists, IL-1Ra, IL-36Ra, IL-37 and IL-38 [12, 13]. The three IL-36 agonists signal through the same receptor, composed of the specific alpha chain IL-36R (IL-1Rrp2) and the common beta chain IL-1 receptor accessory protein [14]. IL-36 cytokines and IL-36R are mainly expressed by keratinocytes, but also by dendritic cells and Th0 cells [15, 16]. IL-36 agonists are involved in the pathogenesis of skin inflammation both in mouse models and in human psoriasis [17]. In particular, in mice, IL-36 plays a crucial role in the development of IMQ-induced skin inflammation, which is exacerbated in absence of its antagonist IL-36Ra [15]. In humans, IL-36Ra deficiency results in a severe form of pustular psoriasis [18].

Conversely, little is known about IL-38, which was initially proposed to act as an antagonist based on its sequence homology with IL-1Ra and IL-36Ra [19]. Consistently, anti-

inflammatory effects were subsequently reported for IL-38 in cultured cells [20-23] and in mice [23-26]. In humans, polymorphisms in the *IL1F10* locus are associated with rheumatic diseases [27-30] and IL-38 expression or serum levels have been recorded in some autoimmune pathologies [21, 24, 31-33], but overall few data are available concerning the role of IL-38 in inflammatory diseases. The identity of the IL-38 receptor(s) also remains elusive, although IL-1 receptor type I [19], IL-36R [20] and interleukin-1 receptor accessory protein-like 1 (IL1RAPL1, also named TIGIRR-2) [22] have successively been proposed as candidates. Recently, we observed reduced *IL-38* transcript levels in human psoriatic skin, whereas *IL-38* expression was increased in colonic biopsies of Crohn's disease patients and in synovial tissues of patients suffering from rheumatoid arthritis [17].

Given the importance of the IL-36 system in the skin and since IL-38 was previously proposed to act as an IL-36R antagonist [20], in the present study we examined the expression and function of endogenous Il-38 in the context of IMQ-induced skin inflammation. We confirmed that *Il-38* was mainly expressed by keratinocytes in the mouse, as previously reported for human skin [19], but not by dermal fibroblasts. Furthermore, our data obtained using Il-38 KO mice indicate that, in contrast to Il-36ra-deficiency, lack of endogenous Il-38 does not impact the development or resolution of IMQ-induced skin inflammation.

Materials and methods

Mice

Il-38 (*Il-1f10*)–deficient mice (*Il-38*$^{-/-}$; Balb.129/Sv-Il1f10) and *Il-36ra* (*Il-1f5*)–deficient mice (*Il-36ra*$^{-/-}$; Balb.B6.129S5/SvEv-Il36rn) [34] were created by Amgen Inc. (Seattle, WA, USA). *Il-38*$^{-/-}$ mice were generated by targeting of the *Il1f10* gene in 129Sv ES GS1 cells, resulting in the deletion of all coding exons and leading to a complete loss of *Il-38* mRNA expression (S1 Fig). Genotyping of *Il-38*$^{-/-}$ mice was performed using a 3-primer PCR combining a forward primer specific for the wild-type (WT) (5'-TGG CCC AGC TGA GCC CCA GCA GCC AGT-3') or the KO (5'-CAG CTT CTG TTC CAC ATA CAC TTC-3') allele with a common reverse primer (5'-TGC TGA GCA AGA AGA TCT CAG ACT-3') (S1 Fig). Genotyping of *Il-36ra*$^{-/-}$ mice was performed using a 3-primer PCR combining a forward primer specific for the WT (5'-GAA AAG AGA GAG TGA ATG GGA G-3') or the KO (5'-GAT TGC ACG CAG GTT CTC-3') allele with a common reverse primer (5'-GAG CTC CAT GAT GTT CAC TGG-3'). *Il-38* and *Il-36ra*-deficient mice were backcrossed onto the BALB/cJ background using a marker-assisted selection protocol (MASP). The purity of the BALB/cJ background, as assessed by genome-wide single nucleotide polymorphism (SNP) scanning using a 384 SNP panel with SNPs spread across the genome at 7 Mbp intervals (Charles River Laboratories, Wilmington, MA) was > 97% and > 99% for *Il-38*$^{-/-}$ and *Il-36ra*$^{-/-}$ mice respectively. For both mouse lines, heterozygous breedings were then set up to obtain *Il-38*$^{-/-}$ or *Il-36ra*$^{-/-}$ mice and their respective WT co-housed littermates for experiments. All mice were bred and maintained in the conventional area of the animal facility at the Geneva University School of Medicine and housed in open cages, enriched with Nordic aspen bedding (Tapvei, Harjumaa, Estonia), nestlets, and a mouse house, in groups of 2-6 individuals on a 12h light/dark cycle. The temperature in the room was maintained between 20-24° Celsius and hygrometry was 30-70%. Extruded food and tap water were

provided *ad libitum*. Mice were monitored daily for signs of distress (signs of dehydration, unresponsiveness to extraneous stimuli, hunched posture, or labored breathing) and would have been euthanized should these signs have appeared. Animal studies were approved by the Animal Ethics Committee of the University of Geneva and the Geneva Veterinarian Office (authorizations GE-43-15 and GE-115-17) and complied with the requirements defined by the Swiss regulation (federal animal protection ordinances and law). Experiments were performed according to the appropriate codes of practice and all efforts were made to minimize suffering.

Isolation of skin, epidermis and primary culture of keratinocytes and dermal fibroblasts

To harvest untreated skin from tails and ears for RNA extraction and for the isolation and culture of primary cells, naïve mice were euthanized by exposure to gradually increasing concentrations of carbon dioxide (CO_2) in a dedicated euthanasia chamber. For the comparison of cytokine expression in naïve total skin and in the epidermis, a fragment of shaved abdominal skin was removed, rinsed in PBS / 100 U/ml penicillin / 100 µg/ml streptomycin and incubated in Keratinocyte-Serum Free Medium (K-SFM) (Life Technologies, Carlsbad, Ca, USA) / 10mg/ml Dispase II (Sigma-Aldrich, Saint-Louis, Mi, USA) overnight at 4°C. The piece of skin from each mouse was cut into halves. One part was immediately frozen in liquid N_2, while, for the second part, the epidermis was detached from the dermis, collected and frozen. For keratinocyte culture, mouse tails were removed, rinsed in PBS / 100 U/ml penicillin / 100 µg/ml streptomycin and incubated in K-SFM / 10mg/ml Dispase II overnight at 4°C. Epidermis was then detached from the dermis and gently mixed 3 times for 1 minute with 0.05% Trypsin / 0.02% EDTA. Isolated cells were cultured in

collagen type IV coated plates, in K-SFM complemented with 53.4 µg/ml Bovine Pituitary Extract (BPE) and 6.6 ng/ml human recombinant EGF (Life Technologies). The cells were used when they reached 80% confluence. For dermal fibroblast culture, ears were removed, minced and incubated for 2 h in HBSS / Ca^{2+} / Mg^{2+} / 2 mg/ml collagenase (Sigma-Aldrich) at 37°C. The tissue was then digested for 30 min in 0.05% Trypsin / 0.02% EDTA at 37°C and the cells and tissue pieces were cultured in Petri dishes in DMEM / 10% FBS / 1 x non-essential amino acids / 100 U/ml penicillin / 100 µg/ml streptomycin to recover fibroblasts, which were used after the third passage. Purity of the isolated epidermal fraction and of keratinocyte and fibroblast cultures was verified by analyzing mRNA expression of keratinocyte-specific *Keratin 14* and fibroblast-specific *Collagen 1a* and *Vimentin* markers (S2 Fig).

IMQ-induced skin inflammation

Psoriasis-like skin inflammation was induced in adult, age-matched, 8 to 12-week-old female *Il-38*$^{-/-}$ or *Il-36ra*$^{-/-}$ mice and their respective WT littermates by daily application of a topical dose of 12.5mg of Aldara™ cream (Meda Pharma GmbH, Frankfurt, Germany), containing 5% (0.625mg) of imiquimod (IMQ), on one ear during 7-8 days. Body weight was recorded and ear thickness was measured daily using a pocket thickness gage (Mitutoyo Europe GmbH, Dusseldorf, Germany). At the end of the experiment, mice were euthanized under deep terminal anesthesia by exsanguination (cardiac puncture) followed by cervical dislocation. Ears were collected for histological analysis and RNA extraction.

Histopathological evaluation and immunohistochemistry

Ears were fixed in 4% buffered formaldehyde and embedded in paraffin. Ear sections (4µm) were deparaffinized and stained with hematoxylin and eosin (HE; Diapath S.p.A., Milano,

Italy). Ly6G, CD3 and B220 expression was examined by immunohistochemistry on paraffin sections using the following antibodies: rat anti-mouse Ly6G (clone 1A8, BD Bioscience, 1/1000), rat anti-human CD3 (clone CD3-12, AbD Serotec, Kidlington, UK, 1/200), or rat anti-mouse B220 (clone RA3-6B2, BD Bioscience, 1/200). Tissue sections were deparaffinized and antigens retrieved by pressure-cooking in 10 mM citrate buffer, pH 6 (anti-Ly6G or anti-B220 staining) or in 10 mM Tris, 1 mM EDTA buffer, pH 9 (anti-CD3 staining). Slides were blocked for endogenous peroxidase activity and incubated with anti-Ly6G, CD3 or B220 antibodies in antibody diluent (S2022, Dako AG, Baar, Switzerland) overnight at 4°C. Subsequently, slides were incubated with appropriate HRP-conjugated secondary antibodies in antibody diluent and developed with diaminobenzidine (Dako). Slides were scanned on a Mirax Midi slide scanner (Carl Zeiss Microscopy, Feldbach, Switzerland). The ZEN blue software (Carl Zeiss Microscopy) was used for image acquisition and measurements. Total ear area was determined on HE-stained sections using the Definiens Developer XD2 software (Definiens, Munich, Germany) and different histopathological parameters were determined in a blinded manner. The average epidermal thickness was estimated by taking 20 measures along the ear. Infiltration of inflammatory cells was evaluated using a modification of the semi-quantitative analysis described previously [9], in which we evaluated the proportion of ear tissue containing infiltrated neutrophils, instead of using scores to reflect differential cell counts. Neutrophils infiltrating the dermis were identified morphologically on HE-stained sections. Areas containing infiltrated neutrophils were then delineated manually and the sum of all neutrophil-containing areas was normalized to the total ear surface. Consistent with previous reports [9, 35], we also observed neutrophil-filled abscess-like structures beneath the stratum corneum in IMQ-treated mice, which were identified morphologically on HE-stained sections and counted manually along the whole ear.

RNA extraction and RT qPCR

Total RNA was extracted using TRIzol® reagent (Life Technologies) and treated with RNAse free DNAse set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (100-500ng) was then reverse transcribed using SuperScript II Reverse transcriptase (Invitrogen, Waltham, USA). The mRNA expression levels were determined by quantitative PCR using the SYBR® Green PCR Master Mix (Applied Biosystem, Waltham, USA) according to the manufacturer's protocol. The primer sequences (Eurofins, Ebersberg, Germany) are detailed in Table 1. Relative levels of mRNA expression were normalized to ribosomal protein L32 (*Rpl32*) mRNA levels using a comparative method ($2^{-\Delta Ct}$). Non-reverse-transcribed RNA samples and Buffer were included as negative controls.

Table 1. Primers used for qPCR.

Gene	Accession number	Primer sequence	Amplicon (pb)
Col1a1	NM_007742.4	Fwd 5'-GGCTCCTGCTCCTCTTAG-3' Rev 5'-ACAGTCCAGTTCTTCATTGC-3'	194
Cxcl1	NM_008176.3	Fwd 5'-ACTCAAGAATGGTCGCGAGG-3' Rev 5'-GTGCCATCAGAGCAGTCTGT-3'	123
K14 Var1 Var2	NM_016958.2 NM_001313956.1	Fwd 5'-ATCGAGGACCTGAAGAGCAA-3' Rev 5'-GGCTCTCAATCTGCATCTCC-3'	220
Il-1a	NM_010554.4	Fwd 5'-GGGAAGATTCTGAAGAAGAG-3' Rev 5'-GAGTAACAGGATATTTAGAGTCG-3'	319
Il-1b	NM_008361.4	Fwd 5'-TGTGAAATGCCACCTTTTGA-3' Rev 5'-GTGCTCATGTCCTCATCCTG-3'	248
Il-6	NM_031168.2	Fwd 5'-TGAACAACGATGATGCACTTGCAGA-3' Rev 5'-TCTGTATCTCTCTGAAGGACTCTGGCT-3'	211
Il-18	NM_008360.1	Fwd 5'-CAGGCCTGACATCTTCTG-3' Rev 5'-CTGACATGGCAGCCATT-3'	104
Il-36a	NM_019450.3	Fwd 5'-TAGTGGGTGTAGTTCTGTAGTGTGC-3' Rev 5'-GTTCGTTCAAGAGTGTCCAGATAT-3'	268
Il-36b	NM_027163.4	Fwd 5'-ACAAAAAGCCTTTCTGTTCTATCAT-3' Rev 5'-CCATGTTGGATTTACTTCTCAGACT-3'	186
Il-36g	NM_153511.3	Fwd 5'-AGAGTAACCCCAGTCAGCGTG-3' Rev 5'-AGGGTGGTGGTACAAATCCAA-3'	186
Il-36r	NM_133193.3	Fwd 5'-AAACACCTAGCAAAAGCCCAG-3' Rev 5'-AGACTGCCCGATTTTCCTATG-3'	262
Il-36ra	NM_019451.2	Fwd 5'-TGGAGCTCATGATGGTTCTG-3' Rev 5'-TAATGACCTTCTCTGCGTGC-3'	123
Il-38	NM_153077.2	Fwd 5'-CCTGGCGTGTGTAAAGACAA-3' Rev 5'-CAGATCCCAAGCTTCTCTGG-3'	125
Rpl32	NM_172086.2	Fwd 5'-CACCAGTCAGACCGATATGTGAAAA-3' Rev 5'-TGTTGTCAATGCCTCTGGGTTT-3'	64
S100a9 Var1 Var2	NM_001281852.1 NM_009114.3	Fwd 5'-CACCCTGAGCAAGAAGGAAT-3' Rev 5'-TGTCATTTATGAGGGCTTCATTT-3'	95

Tnfa	NM_013693.3	Fwd 5'-AGTTCTATGGCCCAGACCCT-3' Rev 5'-GTCTTTGAGATCCATGCCGT-3’	159
Vim	NM_011701.4	Fwd 5’-CGGCTGCGAGAGAAATTGC -3’ Rev 5’-CCACTTTCCGTTCAAGGTCAAG-3’	124

Statistical analysis

Data were analyzed using Prism version 6 (Graphpad Software, La Jolla, USA). Unpaired Mann-Whitney comparison tests, two-way ANOVA followed by a Holm–Sidak’s comparison test, or paired two-way ANOVA followed by a Sidak post-test were used, as indicated. Values are expressed as mean ± SEM. Statistical significance was defined at a p-value < 0.05.

Results

Expression of Il-38 and of Il-36 family cytokine mRNA in naïve mouse skin and in primary mouse skin cells

Keratinocytes express various pattern recognition receptors and act as early detectors of microbial or endogenous danger signals. After activation, they secrete chemokines, cytokines and anti-microbial peptides. In human skin, keratinocytes were suggested to be the main source of IL-38 [19]. In mouse skin, we previously observed *Il38* mRNA expression [17], but its cellular source had not been described. We first examined the mRNA expression of *Il-38* and of the different *Il-36* agonists and antagonist in total skin and isolated epidermis of naïve BALB/c mice. We detected similar levels of *Il-38*, *Il-36ra*, *Il-36a*, *Il-36b* and *Il-36g* mRNA in total skin and in epidermis (Fig 1A). We further investigated the expression of *Il-38* and of the *Il-36* agonists and antagonist in cultured primary keratinocytes and dermal fibroblasts isolated from naïve BALB/c mouse skin. We observed *Il-38* mRNA expression in keratinocytes, but not in dermal fibroblasts. Similarly, transcripts for *Il-36* agonists and *Il-36ra* were detected in keratinocytes only (Fig 1B). In contrast, Il-36 receptor (*Il-36r*) mRNA expression was observed in both skin cell types (S2B Fig).

Il-38 deficiency has no impact on the development of IMQ-induced psoriasis

Consistent with our previous observations during the development of IMQ-induced skin inflammation in C57BL/6 mice [17], we confirmed decreased *Il-38* mRNA expression at the peak of IMQ-induced inflammation in the skin of BALB/c mice, whereas the mRNA levels of *Il-36ra* and of the *Il-36* agonists were increased after IMQ treatment (S3 Fig).

We then went on to investigate the involvement of endogenous Il-38 in the pathogenesis of IMQ-induced skin inflammation, using Il-38-deficient mice (S1 Fig). Homozygous *Il-38*$^{-/-}$ mice are healthy, fertile, and show weight gain similar to that of their WT littermates from birth to adult age. They do not display any spontaneous phenotype in our conventional animal facility. *Il-38*-deficiency had no effect on the severity of IMQ-induced skin inflammation (Fig 2). Ear thickness increased similarly after IMQ application in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 2A), and both groups of mice displayed comparable histopathological alterations on day 7 of IMQ-treatment (Fig 2B). Immunohistochemical analyses confirmed infiltration of IMQ-treated ears by inflammatory cells, in particular neutrophils, as reported previously [9, 35]. Abundant infiltration of Ly6G^{+} cells was observed predominantly in the dermis (S4A Fig). In addition, characteristic neutrophil-filled abscess-like structures were found just beneath the stratum corneum [35] (S4B Fig). CD3^{+} T cells were detected both in the dermis and the epidermis, while some infrequent B220^{+} B lymphocytes were detected in the dermis exclusively (S4A Fig). We did not observe any qualitative differences in infiltrate composition between WT and Il-38 KO mice, as illustrated by anti-Ly6G and anti-CD3 staining of ear sections after 7 days of IMQ-treatment (S4C Fig). Furthermore, histopathological scoring indicated that the extent of neutrophil infiltration, the epidermal thickness and the numbers of neutrophil-filled abscess-like structures were similar in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 2C).

We compared these observations with the response of mice deficient in Il-36Ra, a well-known inhibitor of IL-36-dependent IMQ-induced skin inflammation [15]. We confirmed that *Il-36ra*-deficiency resulted in an aggravation of skin pathology. Indeed, *Il-36ra*$^{-/-}$ mice developed a more severe disease, as shown by an increased ear thickening, as compared to their WT littermates (Fig 2D). This was associated with more severe histopathological changes (Fig 2E and F).

IL-38 deficiency has no impact on IMQ-induced expression of proinflammatory mediators *in situ* or in cultured skin cells

We examined whether the lack of endogenous *Il-38* could nevertheless influence the local expression of proinflammatory mediators in the skin. Thus, we analyzed mRNA expression of various cytokines and chemokines in the ear after 7 days of IMQ application. We did not find any significant differences in *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Cxc-l1*, *Il-6*, *Il1-α*, *IL1-β*, *Il-18* or *Tnfα* mRNA expression between *Il-38*$^{-/-}$ and WT mice, while *Il-38* was obviously not expressed in *Il-38*$^{-/-}$ mice (Fig 3A and S5 Fig).

We further investigated the expression of proinflammatory mediators by cultured primary keratinocytes and dermal fibroblasts isolated from *Il-38*-deficient and WT mice, at baseline and upon stimulation with rec. mouse Il-36β. Although both cell types express *Il36r* (S2B Fig), keratinocytes and fibroblasts displayed differential responses to Il-36β. Indeed, in keratinocytes, Il-36β enhanced mRNA expression of *Il-36α, Il-36γ* and *Il-38,* and of the anti-microbial peptide *S100a9* (Fig 3B), while stimulation of dermal fibroblast with Il-36β strongly induced expression of *Il-6* and *Cxcl-1*. Basal expression levels of the various transcripts examined did not differ significantly in cells isolated from *Il-38*$^{-/-}$ or from WT mice, except for the expression of *Il-38* itself. Il-38 deficiency also lacked any major impact on the response of cultured keratinocytes or fibroblasts to Il-36β (Fig 3B).

IL-38 deficiency does not alter IMQ-induced psoriasis resolution

Although Il-38 was not required for the development of IMQ-induced skin inflammation, we wondered whether it might still be involved in the resolution of the pathology. To answer this question, after 7 days of IMQ topical application, *Il-38*$^{-/-}$ mice and their WT littermates were kept untreated for 5 days. As in Fig 2, the severity of peak skin inflammation was again

279 similar in *Il-38*$^{-/-}$ and WT mice. Afterwards, the gradual decrease of ear thickness was also
280 similar in *Il-38*$^{-/-}$ mice and in their WT littermates (Fig 4A). Those results were further
281 confirmed by histological analysis. Indeed, neutrophil infiltration, epidermal thickness, and
282 the number of neutrophil-filled abscess-like structures were comparable on day 11 in the
283 presence or in the absence of Il-38 (Fig 4B and C).
284

Discussion

IL-36 cytokines are involved in the pathogenesis of psoriasis [36], as well as in the development of IMQ-induced skin inflammation in the mouse [15, 34]. Since IL-38 is also expressed in the skin [17] and was previously proposed to act as an IL-36 antagonist [20], we studied the expression and function of Il-38 in the IMQ model. We detected *Il-38* mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of *IL-38* in keratinocytes in human skin [17, 19], but not in dermal fibroblasts. In agreement with our recent observations in psoriatic human skin [17], *Il-38* mRNA levels were decreased in mouse during skin inflammation *in vivo*. However, lack of endogenous *Il-38* did not impact the development or resolution of IMQ-induced psoriasis. In our hands, Il-38-deficiency did also not change expression of proinflammatory mediators in inflamed skin *in situ* or in cultured skin cells, nor modify the response of primary keratinocytes and dermal fibroblasts to IL-36 stimulation.

Our *in vivo* data indicate that, in contrast to Il-36ra-deficiency, the absence of Il-38 does not impact the course of IMQ-induced skin inflammation, implying that endogenous Il-38 does not act as an Il-36 antagonist in this context. As it has been suggested that the anti-inflammatory properties of IL-38 are inferior as compared to IL-36Ra [20], it is conceivable that Il-38 deficiency is counterbalanced by the presence of Il-36Ra, whose role was confirmed in this study. Alternatively, the biological function of IL-38 might be unrelated to IL-36 inhibition. Several recent studies indeed demonstrated broader anti-inflammatory properties of IL-38 and/or suggested different mechanisms of action [20-26]. In contrast to several of these studies based on overexpression or injection of recombinant exogenous Il-38 [21, 23-26], we were not able to detect any anti-inflammatory, or other, activity of the endogenous protein in our model.

We further showed that, similarly to what was observed in human keratinocytes [17], murine keratinocytes, but not dermal fibroblasts, express IL-36 agonists, as well as IL-36Ra and IL-38. However, both keratinocytes and dermal fibroblasts expressed the Il36r, although, interestingly, the two cell types responded in a different way to IL-36. Indeed, keratinocytes rather amplified the IL-36 signaling by upregulating Il-36α expression, while fibroblasts produced pro-inflammatory mediators, such as Il-6 and the neutrophil-attracting chemokine Cxcl1. This is consistent with the role of keratinocytes as skin sentinels, which can detect early skin damage and release danger signals and pro-inflammatory mediators. This primary response can then be strongly amplified by dermal fibroblasts, which produce signals to recruit and activate immune cells. Since IL-38 was previously described to antagonize the effects of IL-36 stimulation [20], we also investigated the effects of Il-36 stimulation on primary skin cells isolated from WT and *Il-38*$^{-/-}$ mice. However, Il-38-deficiency did not influence the response of primary keratinocytes or dermal fibroblasts to Il-36. Consistent with our *in vivo* observations, these *in vitro* results thus again failed to provide any evidence for an Il-36 inhibitory function of endogenous Il-38.

In conclusion, while this study does not exclude an inhibitory role of IL-38 in other contexts, our results indicate that Il-38-deficiency does not impact the development or resolution of IMQ-induced skin inflammation. Our observations further suggest that endogenous Il-38 does not exert Il-36 inhibitory activity in this model, or in cultured skin cells. An anti-inflammatory function, or any other role, of Il-38 in mouse skin thus still remain to be demonstrated.

Acknowledgments

We would like to thank Cem Gabay for helpful discussions and Florence Coppo for maintaining the different mouse lines.

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Figure legends

Fig 1. Expression of *Il-38* and *Il-36* family cytokines in total skin, epidermis, cultured primary keratinocytes and dermal fibroblasts. Basal *Il-38*, *Il-36ra*, *Il-36a*, *Il-36b* and *Il-36g* mRNA expression was quantified by real-time RT-qPCR in total skin (n=5) and epidermis (n=5) of naïve BALB/c WT mice (A); and in cultured primary keratinocytes (Kera, n=4 independent cultures) and dermal fibroblasts (Fibro, n=5 independent cultures) isolated from naïve BALB/c WT mice (B). Data were expressed relative to *L32* levels. Results are shown as individual values and mean ± SEM.

Statistical analysis was performed using an unpaired Mann-Whitney comparison test. A *p*-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

Fig 2. Il-38-deficiency does not influence the severity of IMQ-induced skin inflammation. *Il-38*$^{-/-}$ mice (n=5) and WT littermates (n=5) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days. Ear thickness was followed daily (A) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears on day 7. Scale bar = 100µm. (B); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-filled abscess-like structures were evaluated (C). Results are from one experiment representative of two and are expressed as mean ± SEM of individual mice (n = 5 mice per group). *Il-36ra*$^{-/-}$ mice (n=6) and WT littermates (n=4) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 8 days. Ear thickness was followed daily (D) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears on day 8. Scale bar = 100µm. (E); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-filled abscess-like structures were evaluated (F). Results are expressed as mean ± SEM of individual mice (n = 4-6 mice per group). Statistical

analysis was performed using a paired two-way ANOVA followed by a Sidak post-test for A and and D, and an unpaired Mann-Whitney comparison test in C and F. A p-value < 0.05 was considered significant. ** p<0.01, * p<0.05.

Fig 3. Expression of proinflammatory mediators in IMQ-treated skin and in cultured skin cells of Il-38 deficient mice. *Il-38*$^{-/-}$ mice and WT littermates were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days. Skin mRNA levels for *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Il-38*, *Cxcl-1 and Il-6* were quantified by real-time RT-qPCR on day 7 in the IMQ-treated ears (A). Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 5 mice per group. Statistical analysis was performed by unpaired Mann-Whitney comparison test. A p-value < 0.05 was considered significant. ** p<0.01. Cultured primary keratinocytes (Kera) and dermal fibroblasts (Fibro) isolated from naïve *Il-38*$^{-/-}$ (dark symbols) or WT mice (white symbols), were stimulated with rec. mouse Il-36β at 100ng/ml for 6 h, or left unstimulated (Med). *Il-36α*, *Il-36β*, *Il-36γ*, *Il-36ra*, *Il-38*, *Cxcl-1, Il-6* and *S100a9* mRNA levels were quantified by real-time RT-qPCR (B). Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 6–9 biological replicates per group. Statistical analysis was performed by two-way ANOVA followed by a Holm–Sidak's comparison test. A p-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

Fig 4. Il-38-deficiency does not affect resolution of IMQ-induced inflammation. *Il-38*$^{-/-}$ mice (n=5) and WT littermates (n=5) were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days, then left untreated until day 11. Ear thickness was followed daily (A) and expressed as ear thickness variation vs. day 0. Microscopic histopathology was studied on HE-stained slides of IMQ-treated ears at day 11. Scale bar =

100 µm. (B); neutrophil-infiltrated areas, epidermal thickness and the number of neutrophil-filled abscess-like structures were evaluated (C). Results are representative of 2 independent experiments and expressed as mean ± SEM of individual mice (n = 5 mice per group). Statistical analysis was performed using a paired two-way ANOVA followed by a Sidak's post-test for A and an unpaired Mann-Whitney comparison test in C. A p-value < 0.05 was considered significant.

Supporting information

S1 Figure. Generation of mice deficient for IL-38. Schematic representation of *Il1f10* gene invalidation: in the targeted allele, a neomycin selection cassette was inserted to replace all coding exons of the *Il1f10* gene (A). Mouse genotyping was performed on total DNA extracted from ear biopsies. PCR products for the WT (150 bp) and KO (250 bp) alleles are shown in *Il-38*$^{-/-}$, *Il-38*$^{+/-}$ and *Il-38*$^{+/+}$ (WT) DNA samples (B). *Il-38* mRNA levels were quantified by real-time RT-qPCR on skin samples from naïve *Il-38*$^{-/-}$ and WT mice. Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM of n = 3 per group (C).

S2 Figure. Expression of keratinocyte and fibroblast specific markers in total skin, epidermis and primary skin cells. Basal mRNA expression of keratinocyte-specific *Keratin 14*, as well as of fibroblast-specific *Collagen 1a* and *Vimentin* was quantified by real-time RT-qPCR in total skin (n=5) and epidermis (n=5) of naïve BALB/c WT mice (A). *Keratin 14*, *Collagen 1a, Vimentin*, and *Il-36r* mRNA levels were quantified by real-time RT-qPCR in cultured primary skin keratinocytes (Kera, n=4 independent cultures) and dermal fibroblasts (Fibro, n=5 independent cultures) isolated from the skin of naïve WT BALB/c mice (B). Data are expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed using an unpaired Mann-Whitney comparison test. A *p*-value < 0.05 was considered significant. *** p<0.001, ** p<0.01, * p<0.05.

S3 Figure. Expression of IL-38 and IL-36 family members in IMQ-treated skin of WT BALB/c mice. WT BALB/c mice were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ) for 8 days (n=7). Skin mRNA levels for *Il-38*, *Il-36ra*, *Il-36α*, *Il-36β* and *Il-36γ* were quantified by real-time RT-qPCR in the non-treated ear (Ctr) and

in the IMQ-treated ear on day 8. Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed by unpaired Mann-Whitney comparison test. A *p*-value < 0.05 was considered significant. ** p<0.01, *** p<0.001.

S4 Figure. Characterization of the inflammatory infiltrate in IMQ-treated ears. Representative HE (upper left panel), anti-Ly6G (brown staining, upper right panel), anti-CD3 (brown staining, lower left panel), and anti-B220 (brown staining, arrows, lower right panel) stained sections are shown for IMQ-treated WT ears at the peak of inflammation on day 7 (A). Representative anti-Ly6G (brown staining, left panel) and HE (right panel) stained sections including neutrophil-filled abscess-like structures located just beneath the stratum corneum (arrows) are shown for IMQ-treated WT ears at the peak of inflammation on day 7 (B). Representative anti-Ly6G (brown staining, left panels) and anti-CD3 (brown staining, right panels) stained sections are shown for IMQ-treated ears of WT (upper panels) or *Il-38*$^{-/-}$ (lower panels) littermate mice at the peak of inflammation on day 7 (C). Scale bar = 100 µM.

S5 Figure. IMQ-induced expression of proinflammatory mediators in the skin of *Il-38* deficient mice. *Il-38*$^{-/-}$ mice and WT littermates were treated daily with a topical dose of 12.5mg of Aldara™ cream (0.625mg IMQ), for 7 days (n=5). Skin mRNA levels for *Il-1α*, *Il-1β*, *Il-18* and *Tnfa* were quantified by real-time RT-qPCR on day 7. Data were expressed relative to *L32* levels. Results represent individual values and mean ± SEM. Statistical analysis was performed by unpaired Mann-Whitney comparison test. No significant differences were observed between the groups.

To the editor:

Even though, IL38 is generally considered an anti-inflammatory cytokine, your work indicated that In IL-38 deficiency in mice did not impact the development or resolution of imiquimod-induced skin inflammation. The editor and reviewers agree that these negative results are of interest for the field.
However, there are some deficiencies in your work including the characterization of inflammatory infiltrate in the ears of wt and IL38 KO mice after imiquimod treatment.

According to the reviewers' suggestions, and as detailed below, we have further characterized the inflammatory infiltrate in the ears of WT and Il-38KO mice.

Reviewer #1:

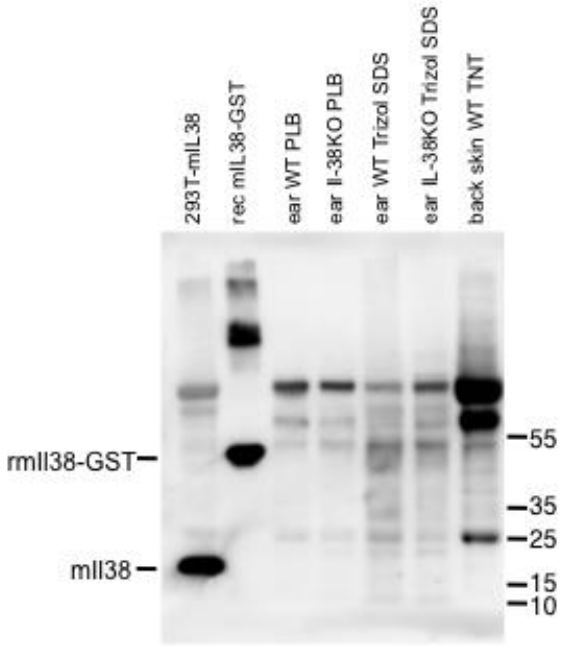
This manuscript describes studies analyzing IL-38 knockout mice in an imiquimod-induced psoriasis model. This appears to be the first study examining IL-38 knockout mice in any skin model. The data suggest that IL-38 does not have a significant impact on psoriasis-like characteristics or pro-inflammatory cytokine expression. While the data are mostly negative, this reviewer feels that the data will be important for other investigators in the field. A few concerns are noted below.

1) The authors confirm a lack of IL-38 mRNA expression in their knockout mice. It would be more convincing if they also showed a lack of IL-38 protein in the skin of cells from IL-38 knockout mice.

We totally agree with this remark. Unfortunately, so far, we have not been able to achieve specific detection of the mouse (m)Il-38 protein in skin or skin lysates using standard approaches. We are thus unable to satisfactorily document mIl-38 protein expression in WT skin.

Indeed, by Western blot, using 3 different anti-mIl-38 antibodies obtained through collaboration with Dr. Donzé (Adipogen International Inc.), or a commercial antibody from RnD Systems (BAF2427, anti-human IL-38), we easily detect recombinant mIl-38, as well as a band of the expected size in lysates of 293T cell overexpressing mIl-38 (Figure I, inserted below to the reviewers' attention, and data not shown). However, using different extraction buffers and methods, we have so far not been able to detect mIl-38 either in total skin or in isolated epidermis (Figure I and data no shown).

Figure I

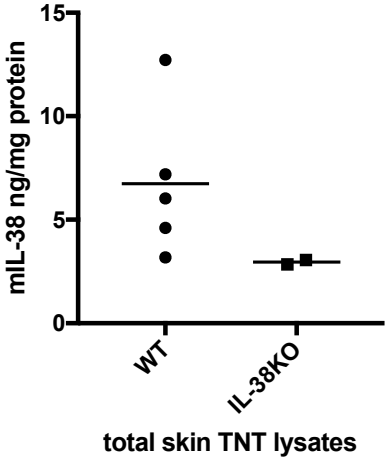


Expression of mouse Il-38 was examined in ear or back skin of WT or Il-38KO mice, as indicated. Tissues were lyzed in PLB (passive lysis buffer, Promega) or in TNT (50 mM Tris pH 7.4, 150 mM NaCl, protease inhibitors, 0.5% Triton X-100) or extracted with Trizol (Life Technologies) for purification of the protein fraction.

Proteins were separated by reducing SDS-PAGE, transferred to a PVDF membrane and Il-38 was revealed using an anti-mouse Il-38 antibody obtained from Adipogen Inc. Lysates of 293T cells transfected with an expression vector for mIL-38 and a recombinant mIl-38-GST fusion protein were included as positive controls.

We further tried to quantify mIl-38 by ELISA. Using lysates of WT skin, we obtained positive ELISA signals. However, we also observed important non-specific background signals in Il-38KO skin lysates (Figure II, inserted below to the reviewers' attention, and data not shown), indicating a lack of specificity of the assay in this type of samples. Thus, unfortunately, although there seems to be some more signal overall in WT, as compared to Il-38KO skin, these ELISA data remain inconclusive and do not allow for proper quantification of mIl-38 protein.

Figure II



Il-38 protein levels were determined in TNT total skin lysates of WT (n=5) and IL-38 KO (n=2) mice, using a DuoSet ELISA (DY2427-05, RnD Systems). Results were normalized to the total protein concentration. Data are shown as individual values and mean.

Finally, by immunohistochemistry, using all the different antibodies mentioned above, as well as one additional commercial antibody (clone H127C, eBioscience; see Takenaka et al, 2015, Biochem. Biophys. Rep.), we have so far been unable to detect specific staining (i.e. absent in Il-38KO skin) on paraffin or frozen skin sections using various protocols for fixation and epitope unmasking (data not shown).

2) The authors report "cell infiltrate" in several figures, but this is not well described. Do the authors assume these are inflammatory cells? If so, a more specific method (i.e., IHC) would be helpful in identifying specific inflammatory cell populations.

We further characterized the cell infiltrate in the ears of IMQ-treated WT and Il-38KO mice by IHC, using anti-Ly6G antibodies to stain neutrophils, anti-CD3 antibodies to stain T cells, and anti-B220 antibodies to stain B cells. Indeed, infiltrated cells are mostly inflammatory cells, and, in particular, neutrophils, consistent with previous observations in this model (van der Fits et al., 2009, J. Immunol; Walter et al., 2013, Nat. Commun.). $Ly6G^+$ cells are observed predominantly in the dermis, as well as in characteristic neutrophil-filled abscess-like structures located just beneath the stratum corneum, as previously described in IMQ-treated mouse skin (Walter et al., 2013, Nat. Commun.). $CD3^+$ cells are found both in dermis and epidermis. Some infrequent $B220^+$ cells are also detected, in the dermis exclusively. This information is now included in the text of the results section (p.12) and representative photomicrographs of the different IHC stainings are shown in the new Figure S4. The corresponding methods have been implemented in the 'methods' section on p.8.

We did not observe any qualitative differences in infiltrate composition between WT (n=3) and Il-38 KO (n=3) mice. This is now illustrated in the new Figure S4C.

Reviewer #2:

This report entitled " the severity of imiquimod-induced mouse skin inflammation is independent of endogenous IL-38 expression" authored by Dr. Palomo et al demonstrated IL-38 is not an antagonist for IL-36 by comparing with the established IL-36 antagonist, IL-36Ra,

These authors employed a common IMQ-induced mouse inflammation model to evaluate inhibition of IL-36 on skin inflammation development. IL-36 is a critical cytokine in regulating the development of skin inflammations, thus inhibition of IL-36 would be a potential approach to control skin inflammations, such as psoriatic inflammation development.

The sequence of IL-38 has homology with IL-1Ra and IL-36Ra, two established IL-36 antagonists, and thus IL-38 was proposed to be a potential antagonist of IL-36. Based on this study, they demonstrated that IL-38 is not the antagonist of IL-36.
These authors developed two mouse clones, IL-38-/- and IL-36ra-/-, to evaluate the role of IL-38 on IMQ-induced skin inflammation.
This study is well designed and conclusion is based on convincing evidence. However, a few concerns need to be addressed before it can be accepted for a publication in the journal. Specifically,

Major concerns:
1. To evaluate the inflammation by histopathological assessment, proper references need to be cited. For example, how to quantify the cell infiltration and how to assess scabs

We have added further details and appropriate references to the 'Material and Methods' section (p. 8), as follows:

Infiltration of inflammatory cells was evaluated using a modification of the semi-quantitative analysis described previously [9], in which we evaluated the proportion of ear tissue containing infiltrated neutrophils, instead of using scores to reflect differential cell counts. Neutrophils infiltrating the dermis were identified morphologically on HE-stained sections. Areas containing infiltrated neutrophils were then delineated manually and the sum of all neutrophil-containing areas was normalized to the total ear surface. Consistent with previous reports [9, 35], we also observed neutrophil-filled abscess-like structures beneath the stratum corneum in IMQ-treated mice, which were identified morphologically on HE-stained sections and counted manually along the whole ear.

Please note that we now refer to these structures, termed 'scabs' in the previous version of the manuscript, using the more complete histological designation 'neutrophil-filled abscess-like structures' as published by Walter et al. (2013, Nat. Commun.) for the sake of consistency with this previous publication.

2. When mention skin, it is a sum of keratinocytes, fibroblasts and variety of immune cells, etc. Thus it needs more precise in the 1st paragraph of discussion section, " ….consistent with previous observations in human skin (17, 19), but not in dermal fibroblasts".

We agree and modified the sentence accordingly. It now reads:

We detected *Il-38* mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of *IL-38* in keratinocytes in human skin [17, 19], but not in dermal fibroblasts.

3. Regarding the discussion above, what are these cytokine expression profile in human skin should be discussed thoroughly to make this study more translational to human.

As mentioned above, previous studies described IL-38 expression in human keratinocytes, which is consistent with our results in mice. We also previously reported decreased expression of IL-38 mRNA in total skin samples obtained from patients with moderate to severe plaque psoriasis, as compared to normal control biopsies obtained from surgical samples of healthy skin (Boutet et al., 2016, Clin. Exp. Immunol.). These observations indeed suggest similar regulation of Il-38 expression in human and mouse in the context of skin inflammation. This is now more explicitly stated in the discussion (p.15), as follows:

We detected *Il-38* mRNA expression in mouse epidermis and in primary mouse keratinocytes, consistent with expression of *IL-38* in keratinocytes in human skin [17, 19], but not in dermal fibroblasts. In agreement with our recent observations in psoriatic human skin [17], *Il-38* mRNA levels were decreased in mouse during skin inflammation *in vivo*.

Minor concerns:

1. a few typos were found, manuscript should be carefully proof read before submit to the journal

The manuscript was proofread.