# **Interferon-stimulated gene 15 (ISG15) deficiency in dendritic cells protects against contact hypersensitivity (CHS) inflammation in mouse.**

**Short Title:** *ISG15 during contact hypersensitivity reaction.*

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# **Acknowledgements**

**General:** Authors thank Dr. Miguel Vicente for critical reading and English editing of the manuscript. We thank Cell cytometry, Microscopy and Animal Facility Units from CNIC.

**Funding:** This study was supported by grant PD1-2020-120412RB-100 and PDC2021-121797-100 from the Spanish Ministry of Economy and Competitiveness (MINECO), grant S2017/BMD-3671-INFLAMUNE-CM from the Comunidad de Madrid, a grant from the Ramón Areces Foundation “Ciencias de la Vida y la Salud” (XIX Concurso-2018), a grant from Comunidad de Madrid (CAM) (S2017/BMD‐3671‐INFLAMUNE‐CM), “la Caixa” Banking Foundation (HR17-00016), BIOIMID (PIE13/041) from Instituto de Salud Carlos III, CIBER Cardiovascular (CB16/11/00272), and Fondo de Investigación Sanitaria del Instituto de Salud Carlos III and co-funding by Fondo Europeo de Desarrollo Regional FEDER). The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the Ministerio de Ciencia e Innovación (MCIN) and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (CEX2020-001041-S). Microscopy experiments were performed at the Microscopy and Dynamic Imaging Unit, CNIC, ICTS-ReDib, co-funded by MCIN/AEI /10.13039/501100011033 and ERDF "A way to do Europe" (#ICTS-2018-04-CNIC-16). I.F.-D. has been supported by a Fellowship from the Spanish Ministry of Science, Innovation, and Universities (FPU15/02539). R.C.-G. is supported by Ayudas Margarita Salas para la Formación de Jóvenes Doctores - Universidad Autónoma de Madrid (CA1/RSUE/2021–00577) from the Spanish Ministry of Universities. N.F.-G has been supported by Formación de Profesorado Universitario (FPU) Program (FPU16/03953) from the Spanish Ministry of Universities and by Investigo Program (09-PIN1-00015.6/2022) from the Comunidad de Madrid funded by Next Generation EU program through Spanish Recovery, Transformation and Resilience Plan. Funding agencies did not intervene in the design of the studies, with no copyright over the study. **Competing interests:** The authors declare that they have no competing interests.

**Availability of data and material**: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

**Authors’ contributions**: I.F.-D. and R.C.-G. designed most experimentation and analysed results; R.I.-S., M.R.-H, O.M.-G., A.R.-G. and N.F.-G. helped with the collection of data and experimental design. D.C, H.dlF. and S.G helped with project design. I.F.-D. made the figures and wrote the manuscript with input from the rest of the authors. F.S.-M. supervised and revised all the work.

**Ethics approval**. Mice were housed under specific pathogen-free conditions at Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), and experiments were approved by the CNIC Ethical Committee for Animal Welfare and by the Spanish Ministry of Agriculture, Food, and the Environment. Animal care and animal procedures license were reviewed and approved by the local Ethics Committee for Basic research at the CNIC Ethical Committee for Animal Welfare and the Órgano Encargado del Bienestar Animal (OEBA) del Gabinete Veterinario de la Universidad Autónoma de Madrid (UAM). This committee approved the document with an associated identification number PROEX 210.6-20. The human study was approved by the Hospital Universitario de La Princesa ethics committee, and all participants provided written informed consent.

# **Abstract:**

The role of ISG15 (Interferon-Stimulated Gene 15) is becoming increasingly acknowledged in cancer, pathogen infection and inflammatory immune diseases. ISG15 expression increases in human psoriatic skin, and genetic mutations of ISG15 cause dermatological alterations. Here, we show that ISG15 deficiency leads to reduced inflammation and swelling in a murine model of allergic contact dermatitis (ACD). Bone marrow transplantation and adoptive transfer approaches demonstrate that ISG15 alters dendritic cell (DC) responses in the ACD mouse model. Consistently, *Isg15*-deficient DCs secrete reduced amounts of pro-inflammatory cytokines, including IL-1β and IL-12. This study opens new avenues to potentiate immunotherapies to treat immune-related dermatological disorders.

Word count: **101**

**Keywords:** Contact hypersensitivity, ISG15, Dendritic cells, Inflammation

# **Introduction**

The skin is an essential defensive barrier against external aggression. Diverse subtypes of immune cells reside in the skin together with non-immune cells, e.g. keratinocytes (KC) or fibroblasts. The onset of protective and regenerative skin responses to combat pathogens or repair damage may cause inflammation. A common inflammatory skin disease is allergic contact dermatitis (ACD), which is a type of manifestation of contact dermatitis (CD) triggered by the formation of self-complexes with haptens (small molecules with molecular weights below 500 Da) in the epidermis (1). ACD is a type IV delayed hypersensitivity reaction divided into two temporally dissociated phases. The first (afferent) phase involves initial contact with the hapten and sensitization. This phase is characterized by the recognition and capture of hapten complexes by dermal dendritic cells (dDCs) and Langerhans cells (LCs) (2,3). During this phase, KCs, mast cells and infiltrating macrophages promote an inflammatory response. DCs migrate towards the draining lymph node (dLN), where they prime T cells through antigen (Ag)-presentation, promoting the emergence of T cell memory populations. The response is mainly mediated by Th1 and cytotoxic CD8+ T lymphocyte (CTL) responses (4). A second contact with the hapten leads to the so-called elicitation or efferent phase, which happens rapidly (first 24h) after hapten exposure. It includes dermal oedema, secretion of pro-inflammatory cytokines and recruitment of immune cells such as neutrophils (5). Th1 and CTL Ag specific T cells are recruited into the skin, which will be later attenuated by regulatory T cells (Tregs) (1,4).

Post-translational modifications (PTMs) are the most important and functionally diverse regulatory systems of protein location, stability and function. PTMs include a wide group of modifications, e.g. phosphorylation, acetylation, etc. A crucially important PTM is elicited by the binding of small proteins. Ubiquitin (UB) and Ubiquitin-like-modifiers (UBLs) are included in this group and characterized by a β-grasp fold and the ability to covalently modify proteins owing to a conjugation enzyme machinery (6,7). Interferon-Stimulated Gene 15 (ISG15) is an inducible modifier expressed mainly in response to type I IFN and bacterial lipopolysaccharide (LPS), viral double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α), retinoic acid or certain genotoxic stressors (8). ISG15 can act as a PTM or as a free molecule. It interacts with or modifies a wide variety of protein targets. Integrin LFA-1 is a putative receptor for free extracellular ISG15 (9). On the other hand, its conjugation to proteins is controlled by the sequential action of three enzymes: activating E1 (Ube1L), conjugating E2 (UbCM8) and E3 ligase (HERC6). ISG15 can modulate the function of multiple proteins in diverse ways in processes such as viral infection, exosome secretion, immune modulation, autophagy or tumorigenesis (10).

Several studies revealed that ISG15 mRNA levels are upregulated in psoriatic lesional skin samples (11–14), but not in atopic dermatitis (15,16). Moreover, mutations in ISG15 in several human patients triggered diverse dermatological manifestations, including severe IFNopathies and susceptibility to mycobacterial diseases (17,18). ISG15 mutant carriers display hyperinflammation, alterations in connective tissue and compromised epidermis integrity (19), suggesting that ISG15 is an important player in skin homeostasis and skin disorders with an inflammatory component.

In this study, we have observed that the levels of ISG15 in lesional skin AD human samples is increased compared to its non-lesional counterpart. Furthermore, we show that the deficiency of ISG15in a contact hypersensitivity (CHS) mouse model of ACD reduces inflammation. Transplantation and adoptive transfer experimentation allows us to rule out a major role for T lymphocytes and reveals that ISG15 on DCs is essential for the development of skin inflammation in the mouse model of CHS. The absence of ISG15 in DCs decreased secretion of pro-inflammatory cytokines such as IL-1β and IL-12. Free extracellular ISG15 in combination with IL-12 restores IL-1β secretion to normal levels. These observations reveal a central role of ISG15 in the development of skin inflammatory diseases, which may be important for the development of new therapies.

# **Materials and Methods**

**Antibodies (Abs) and other reagents.** Abs and reagents used are listed in Supplementary Table 1.

**Generation of bone marrow-derived dendritic cells (BMDCs).** Bone marrow (BM) cell suspensions were extracted from medullary cavity of femur and tibia from mice. ACK lysis buffer (Lonza, 10-548E) was used to lysate erythrocytes for 30 sec at room temperature (RT) and cell suspensions were filtered through a 70-μm cell sieve (Fisher Scientific, 10788201). BM cell suspension was cultured on non-treated 150-mm Petri dishes at a concentration of 0.5·106 cells/mL in complete RPMI-1640 supplemented with 20 ng/mL of recombinant mouse Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF, PeproTech). Adhered cells were removed from culture at day 3. Cells (floating + attached) were maintained and passed every two days by detachment with PBS 1x – 5 % BSA – 5 mM EDTA (PBE). GM-CSF BMDCs were collected at day 9 for experiment and characterized as CD11c+MHCII+Gr-1- cells by flow cytometry.

**Mice.** *Isg15-*/- mice, also referred to as KO or ISG15KO, were generated at the laboratory of Klaus-Peter Knobeloch by an insertion of pgk-neo cassette, which results in exon 2 deletion in ES cells. The injection was performed into C57BL/6 blastocysts to generate germ line chimeras (20). For bone-marrow transplant and adoptive transfer experiments, B6SJL CD45.1 (*Ptprca*) and Rag1-/- mice were used, respectively. *In vivo* experiments were conducted with sex- and age-matched mice (8-12 weeks) kept on a regular 12 h light/dark cycle (7a.m. - 7 p.m. light period), with food and water available *ad libitum*.

**Contact hypersensitivity mouse model.** This is a two-step procedure, sensitization and elicitation. Sensitization was performed by applying 200 μL of 3% oxazolone (OXA)-ethanol solution to shaved abdomen of mice. After 4 days, 40 μL of 1% OXA-ethanol was topically administered to one ear of mice on both sides, leaving the other as control (only ethanol as vehicle). Ear thickness was measured every day since its administration, which informs on inflammation status due to swelling and oedema. Inflammation decreases with time and information is collected at several time points. For mice groups, males between 6-12 weeks were selected.

**Skin painting.** 10 μL of FITC 1% dissolved in acetone:dibutyl-phtalate (1:1) was administered in mice ears for sensitization. FITC 1% dissolved in acetone was used as vehicle control. Mice were sacrificed 48 h after administration and auricular LN (aLN) were processed and analysed for DC migration by flow cytometry.

**Bone-marrow transplant.** B6SJL CD45.1+ mice were irradiated with 6.5 Gy twice (with 3 h gap in between doses). The day after irradiation mice were intravenously injected with 107 cells of Bone Marrow (BM) from WT and ISG15-deficient (CD45.2+ haplotype) donor mice. After two months, CD45.1 (receptor) vs CD45.2 (BM-donor) expression was monitored in blood by flow cytometry to determine the percentage of chimerism and BM-transplant success.

**Adoptive transfer experiments.** *Rag1*-/- mice (deficient in B and T cells) were intravenously injected with LN-cells isolated from a specific genotype of OXA-treated mice. In that matter, cell suspension from axillar, brachial, and inguinal LNs from *Isg15+/+* or *Isg15-/-* mice, after 4 days of OXA application, were used as donors. To study the contribution of T cells, *Isg15+/+* or *Isg15-/-* mice were intravenously injected with 40 ·106 CD3+ T cells or PBS and treated or not with 1% OXA in the ear. CD3+ T cells were isolated from sensitized (3% OXA) C57BL/6 mice using a cocktail of biotinylated antibodies (IgM, B220, CD19, MHCII, CD11c, CD11b, DX5, F4/80, GR-1, Ter119 and TCRγδ) and the EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit (STEMCELL Technologies; 19860).

**Mice tissue sample preparation for flow cytometry (FC).** Before collecting samples, mice were perfused with PBS 1x or 0.9% NaCl solution for 4-5 min with continuous flow. This procedure removes any circulating cells remaining in the tissues (dorsal and ear skin). Skin samples were collected in complete RPMI-1640. Samples were treated with an enzyme digestion cocktail solution - 0.083 mg/mL LiberaseTM (Roche), 0.5 mg/mL Collagenase IV (Sigma-Aldrich) and 100 µg/mL DNase (Sigma-Aldrich) in RPMI medium - for 35 min at 37ºC. Then, reaction was blocked with a solution containing 1% FBS in PBS 1x and 5 mM EDTA (PFE). Digested suspensions were filtered through 70-μm cell strainer (Fisher Scientific, 10788201). Remaining tissue was torn away by mechanical action of Stainless-Steel Beads (7 mm, Qiagen) in TissueLyser LT (Qiagen) for 20 oscillations 3 min at RT and filtered again through the same strainer. Spleen and LN cell suspensions were obtained from mice by grinding organs through a 70-μm cell strainer (Fisher Scientific, 10788201). ACK lysis buffer (Lonza, 10-548E) was used to lysate erythrocytes for 5 min at RT only in spleen suspensions. If cells of interest include myeloid compartment, both LNs and spleen were treated with an enzyme digestion cocktail solution (250 μg/mL Liberase TL and 100 μg/mL DNAse I) for 20 min at 37ºC. Then, reaction was blocked with PFE. All tissue samples were suspended in a TrucountTM (BD, 340334) solution for determining absolute counts of cell populations.

**Flow cytometry (FC).** Cell suspensions were transferred to a 96-V-well plate (BRAND™) and incubated in LIVE/DEAD® Fixable Yellow Dead Cell Stain (Invitrogen), FcBlock (Tonbo), 5 mM EDTA and PBS 1x for 20 min at 4ºC. Solution was washed out with PBE. Cells were incubated with surface primary Ab cocktail (for dilution see Supplementary Table 1) for 1 h at 4ºC. Solution was washed out with PBE. For fixation, 1% Paraformaldehyde (PFA) in 5 mM EDTA - PBS 1x was used for 15 min at 4ºC. When intracellular antigens (Ags) were targeted, after washing out surface primary Ab cocktail, cells were incubated with Cytofix/Cytoperm™ (BD) for 20 min at 4ºC. Solution was washed out with Perm/Wash™ Buffer (BD). Then, cells were incubated with intracellular primary Ab cocktail in Perm/Wash™ Buffer for 1-2 h at 4ºC. Alternatively, for intranuclear staining, after washing out surface primary Ab cocktail, cells were incubated with FoxP3 Fixation/Permeabilization staining Buffer (eBioscience, 00-5523) for 20 min at 4ºC. Solution was washed out with FoxP3 Permeabilization staining Buffer (eBioscience, 00-8333). Then, cells were incubated with intranuclear or intracellular primary Ab cocktail in Permeabilization Buffer for 1 h at 4ºC. Cells were analysed in FACS CantoTM II, LSRFortessaTM or FACS SymphonyTM. As single positive controls UltraComp eBeads (Invitrogen, 01-2222-41) or cells were used.

**RNA extraction for mice tissues.** Back skin, ear, LNs and spleen were collected in cryotubes and introduced in liquid nitrogen at the moment of extraction. RNA was isolated with TRI Reagent solution (Ambion, TR118). Previous to following manufacturer protocol, tissue sample in TRI reagent solution was torn away by mechanical action of Stainless-Steel Beads (7 mm, Qiagen) previously cleaned and sterilized by heat. In the case of skin samples, the procedure consisted in 3 cycles of 50 oscillations for 5 min in cold TissueLyser LT (Qiagen). For smooth tissues, such as lymph nodes or spleen, only one cycle is needed. Then, beads and remaining tissue was removed by centrifugation and proceeded to continue with manufacturer instructions of TRI Reagent solution. DNA contamination was removed with TURBO DNA-free™ Kit (Thermo Fisher Scientific, AM1907). RNA purity and concentration were analysed in a Nanodrop-1000 Spectrophotometer (Thermo Fisher Scientific).

**Reverse transcription and real-time quantitative PCR (qPCR).** Total RNA (0.5 to 2 μg) was reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, 4374966). Then, qPCR was performed using GoTaq® qPCR Master Mix (SYBR Green, Promega, A6001) in an AB7900-384 thermocycler (Applied Biosystems). Dilutions were performed to cDNA when needed, ranging from 15 to 100 ng per well. PCR reactions were performed by triplicate in 384-well plates. Expression levels of target genes were normalized to housekeeping genes *β-Actin* and *Ywhaz*. Gene-specific primers used are listed in Supplementary Table 2.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Cytokine production was analysed in the supernatant of GM-CSF BMDCs treated with different Toll-like receptor (TLR) agonist for a 12-24 h at 37ºC. Dilutions were performed to maintain measures in standard curve range. Detection was based on colorimetric quantification of absorbance at 450nm, corrected by subtraction at 570nm in a microplate reader (Bio-Rad 550) using MaxiSorp® flat-bottom 96 well plates (Nunc). Cytokines analysed are described in Supplementary Table 3.

**Western blot (WB).** Cells were lysed in WB lysis buffer supplemented with protease inhibitors (Complete, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Proteins were separated by SDS-PAGE in non-reducing or reducing conditions on 8-12% acrylamide/bisacrylamide (29:1, 30%, BioRad) gels and transferred to a nitrocellulose or PVDF membrane (400 Ma for 120 min at 4ºC). Membranes were blocked with 5% BSA-TBS for 1 h, incubated with primary Abs overnight at 4ºC (for dilution see Supplementary Table 1) and peroxidase-conjugated secondary Abs (1:5000) for 40 min at RT. Proteins were visualized with Immobilon (Millipore, WBLUF0500) using ImageQuant LAS-4000 Mini (GE Healthcare) or iBright 1500 (Invitrogen).

**Histology and Hematoxylin/Eosin stain (H&E).** Back skin, ear, LN and spleen were collected in tissue embedding cassettes with pads and introduced in 10% Formalin pH 7.2 - 7.4 solution (BIO OPTICA, 05-K01022) for 24 h at RT. Tissue cassettes were then transferred to 70% ethanol solution for tissue processor and prepare samples for paraffin embedment. Blocks were grinded 200 μm between levels and stained with hematoxylin/eosin. Microscope slides were digitalized and analysed with NDP.view2 software.

**Immunofluorescence of tissues**. Skin punch biopsy specimens (4 mm) were obtained from patients with allergic contact dermatitis (lesional and non-lesional skin) and healthy volunteers, and prepared in OCT blocks. Patients had a PGA score (Physician Global Assessment) of 3. Samples were kept under -80ºC conditions. Slides were dried for 20 min at RT. Tissue samples were fixed with 4% PFA for 20 min. After that, several washes were performed before permeabilization with PBS 1x - 0.2 % Triton X-100 for 15 min. Samples were blocked with a solution of serum obtained from secondary Abs species together with 100 μg/mL of human γ-globulins for 1 h. Biopsies were incubated with the corresponding primary or biotinylated primary Abs (for dilution see Supplementary Table 1) in PBS 1x for 1 h. After washing twice with PBS 1x, slides were incubated with secondary Abs or streptavidin in PBS 1x for 45 min (for dilution see Supplementary Table 1). DAPI solution was used for staining nucleus (Supplementary Table 1). Images were obtained with Leica SP8 navigator confocal microscope (Leica) with 63x 1.4 OIL objective and processed with ImageJ (same settings and processing were applied to all images).

**Statistical analysis.** Data were analysed with GraphPad Prism software (La Jolla, CA). Normality was studied with D'Agostino-Pearson omnibus or Shapiro-Wilk normality test. When data passed the normality test (α = 0.05), a parametric test was applied, Student’s *t*-test for two groups or one-way analysis of variance (ANOVA) test with Tukey’s post-test for more than two groups. When working with dependent samples, a paired *t* test was conducted. For nonparametric data, Mann-Whitney *U* test or Kruskal-Wallis with Dunn’s post-test were applied for two or more groups, respectively. Every experiment was analysed through at least three biological replicates. Graphs show the distribution of each sample and mean ± standard deviation (SD). Tukey-style Box and whiskers plot represents median, lower quartile, upper quartile and two independent whiskers allowing outliers. Statistically significant differences were considered when *P* ≤ 0.05 (depicted as \*), *P* ≤ 0.01 (\*\*), *P* ≤ 0.001 (\*\*\*) and *P* ≤ 0.0001 (\*\*\*\*). Not significant differences were represented as ns (*P* > 0.05). When signal was not detected was represented as n.d.

# **Results**

## **Contact hypersensitivity (CHS) inflammatory response is reduced in *Isg15-*deficient mice**

Several studies have reported differences in ISG15 mRNA expression in human psoriatic vs. healthy skin samples (11–14). Conversely, its expression during ACD has yet to be addressed. We then explored by immunofluoresece whether *Isg15* levels were altered in lesional skin of patients suffering from this condition. ISG15 protein levels were elevated in ACD patients in the eczemas skin areas but not in non-lesional sections or in skin from healthy donors **(Fig. 1)**.

ACD is a type IV hypersensitivity reaction mediated by Th1 and CD8+ T cells. These T cells are responders in virus and pathogen infections, in which IFN type I responses are highly represented. In addition, a recent report highlighted that *Isg15* mutations lead to necrotizing skin lesions in an IFN-I-dependent manner (17), suggesting that *Isg15* levels and/or mutational state may play a general role in inflammatory responses. To address this in the context of ACD, we developed a murine model triggered by oxazolone (OXA). We triggered sensitization by applying OXA in the ventral area of the mouse. After four days, we elicited local inflammation and swelling by direct application of a lower dose of OXA in the ear **(Fig. 2A)**.

By measuring ear thickness with a digital calliper, we found that *Isg15-*deficient mice elicited less inflammation in the ear after the second challenge compared to wild-type mice **(Fig. 2B)**. After four days of challenge, we collected tissue sections of the ear, which revealed marked histological differences between vehicle and OXA-treated ears **(Fig. 2C)**. Epidermal thickness quantification revealed histological differences in ISG15-deficient mice, but no significant differences were observed in the dermis **(Fig. 2D)**.

We next assessed whether *Isg15* expression increased during sensitization, elicitation or both CHS phases. We analysed dLNs after 24, 48 and 72h post-sensitization with OXA. We detected elevated expression of *Isg15* mRNA in wild type-sensitized animals, but not in *Isg15*-deficient mice at any time point **(Fig. 2E)**. ISG15 was also highly expressed during elicitation after 24 h, decreasing 4 days post-challenge in auricular LNs (aLNs) **(Fig. 2F)**. Moreover, ISG15 expression was induced in the ear skin 24h after OXA challenge **(Fig. 2G).** Therefore, *Isg15* expression is induced during elicitation of early response both in dLNs and skin, and its absence reduces the extent of the inflammatory response.

## **ISG15 controls immune cells recruitment to the skin during CHS response**

To characterize the immunological alterations of *Isg15-*deficient mice leading to ACD, we first assessed the different immune cell populations during the development of reaction. During sensitization, we observed reduced expression of *Cxcl2, Cd86* and *Ccr7* after 48 h post-challenge in the absence of ISG15 **(Fig. Sup. 1A)**. CD86 and CCR7 are involved in DC maturation and migration towards dLNs, and CXCL2 is secreted by macrophages for neutrophil recruitment. Consistenly, reduced levels of these proteins correlate with a reduction in total cell numbers of macrophages and migratory and resident cDC2 **(Fig. Sup. 1B)**.

Re-exposure to the hapten also triggered reduced recruitment of neutrophils after 24h and 7 days post-challenge in the skin of *Isg15-*deficient mice **(Fig. 3A)**. Likewise, numbers of macrophages and monocytes were reduced after 7 days of OXA treatment **(Fig. 3A)**. In fact, we observed a global reduction of CD45+ leukocytes, including CD4+ and CD8+ T cells, in the ear skin of *Isg15-*deficient mice at day 7 during elicitation **(Fig. 3B)**. Regarding dLN, CD45+ cells were also reduced at day 7, but no significant differences were observed in the numbers of CD4+ and CD8+ T cell populations **(Fig. 3C).**

## **The hematopoietic compartment is responsible for limiting inflammation in *Isg15*-deficient mice**

We next explored the mechanism underlying the decreased recruitment of leukocytes to the challenged region of the skin in the absence of ISG15. KCs, albeit non-immune, play a crucial role in the initiation of the immune response through secretion of inflammatory factors upon insult, including lipid mediators, such as IL-1α/β and TNFα (4,5). Decreased levels of these initiators would translate into reduced recruitment of myeloid and lymphoid cells to the tissue. To address this possibility, we performed bone marrow transplant (BMT) experiments (21). CD45.1+ receptor mice were subjected to lethal irradiation and reconstituted with BM cells from CD45.2+ *Isg15*-WT and *Isg15*-KO donor mice **(Fig. 4A).** BM reconstitution after 2 months exceeded 90% of donor cells in most cases **(Fig. 4B).** Full analysis of ear thickness during the elicitation phase showed reduced values in *Isg15*-deficient BM-transplanted mice **(Fig. 4C)**. Importantly, dDCs but not epidermal LCs are replenished from BM-derived monocytes after γ-irradiation (22). Hence, it seems that ISG15 depletion in the hematopoietic compartment accounts for the observed reduction in skin inflammation, but the role of specific T cells and/or myeloid cells is not clarified yet.

We next performed additional adoptive transfer experiments. We first transferred OXA-challenged LN-cells from a WT or KO donor mice to a Rag1-/- recipient mice **(Fig. 4D)**. Rag1-deficient mice do not have B or T cells as *Rag1* deletion impairs V(D)J recombination (23). However, due to their intrinsic immunodeficiency, these mice are not good responders to OXA challenge. During 7 days post OXA-challenge, ear thickness was similar between WT and KO LN-cell transferred mice, and both of them responded better than mice receiving PBS alone **(Fig. 4E)**. These results suggest that lymphocyte populations are not responsible for the difference in inflammation between *Isg15*-WT and -deficient mice. To better substantiate these observations, we isolated sensitized CD3+ T cells from WT mice and intravenously injected them into *Isg15*-WT or KO mice during the elicitation phase. Differences in inflammation were still observed in the ear of *Isg15*-deficient mice **(Fig. 4F)**, hence, antigen-specific T cells are unlikely to be the main drivers of these differences.

On the other hand, during CHS, DCs represent a key component that triggers the immune response against the hapten by capturing and presenting antigen to T cells (2). Remarkably, our data show that OXA-instructed *Isg15*-deficient GM-CSF BMDCs transference to wild-type mice decreased inflammation upon OXA challenge **(Fig. 4G).**

## **DC activation and subsets in the skin of *Isg15-*deficient mice during elicitation**

The previous results indicate that DCs are major mediators of the effects of ISG15 depletion in the murine ACD model. To unveil the DC populations involved in this effect, we first characterized the DC populations present in skin, including LCs and four different types of dDCs after 24 h of 1% OXA-treatment during elicitation **(Fig. 5A)**. We did not observe any differences in total cell numbers of LCs, CD11b+ dDC **(Fig. 5B)** or conventional, type 1-like DCs **(Fig. 5C)**. Moreover, the activation status of these DCs, measured by co-stimulatory molecules such as CD40 and CD86 after OXA administration, was unaffected by ISG15 deletion **(Fig. 5D)**.

To assess the global migratory capacities of these cells *in vivo*, we performed skin-painting experiments, in which mice are challenged with an inflammatory stimulus, e.g. dibutyl phthalate, together with a tracing fluorophore, e.g. FITC **(Fig. Sup. 2A)**. Mice were challenged in the ear and the presence of FITC+ migratory DCs was analysed in aLNs by FC. We did not observe significant differences in total cell counts of *Isg15*-deleted vs. wild type migratory DCs from the ear skin into dLNs **(Fig. Sup. 2B)**, indicating that the defect observed is not caused by impaired DC migration capabilities.

## ***Isg15*-deficient BMDCs are less pro-inflammatory**

DCs have a different TLR pattern expression and responsiveness depending on their origin and function (24–26). Therefore, we next performed a detailed study of ISG15 inducible expression on BMDCs by different TLR agonists, from extracellular ligands such as LPS (TLR4), Pam3CSK4 (TLR2/1), Pam2CSK4 (TLR2/6); to intracellular ones such as pI:C (TLR3), IMQ (TLR7/8) and CpG (TLR9). Consistent with the reported function of ISG15 in antiviral responses, we observed higher *Isg15* mRNA expression when cells were pulsed with TLR stimuli based on DNA/RNA (but also LPS) after 12 and 24 h **(Fig. 6A).** This also translated into a higher levels of proteinISGylation upon TLR-agonist treatment **(Fig. 6B)**.

Next, we assessed the activation and maturation status of *Isg15-*KO DC cells in response to various PAMPs by monitoring the cellular levels of activation markers MHCII, CD40 and CD86. Our results showed that there were no differences in the percentage of DC expressing the typical DC markers CD11c and MHCII **(Fig. Sup. 3A**). Even a reduction was observed in MHCII surface levels upon LPS stimulation **(Fig. Sup. 3A)**, no significant differences were displayed in CD40 and CD86, neither in cell percentage (data not shown) nor in geometric mean fluorescence intensity (MFI) **(Fig. Sup. 3B).**

DCs are key producers of some inflammatory cytokines that will determine the fate of the immune response (27,28). ISG15-KO BMDCs express lower levels of *Il-1b* mRNA expression in response to various PAMPs, particularly 12 h after treatment **(Fig. Sup. 3C).** Furthermore, these differences were more pronounced when we measured protein secretion levels after 12h and 24 h of treatment **(Fig. 6C)**. We also measured the levels of IL-12, which is important for Th1 differentiation, CD8+ cytotoxicity and NK cell activation (27,29) **(Fig. 6C, Fig. Sup. 3C).** *P35* mRNA expression, which is a common subunit to IL-12 and IL-35, was not significant in most of the treatments **(Fig. Sup. 3C**). However, IL-12 (p70) secretion was reduced upon TLR agonist treatment and sustained after 24 h **(Fig. 6C)**. Similar to IL-12, *Tnfα* mRNA expression levels were similar between genotypes, but we observed a reduction in protein secretion after 12 h post-treatment **(Fig. 6C and Sup. 3C)**. Furthermore, administration of free extracellular recombinant ISG15 in combination with IL-12 increased the levels of IL-1β secretion in *Isg15*-depleted cells to wild type levels upon LPS treatment **(Fig. 6D)**.

# **Discussion**

Due to its ubiquitous nature and importance in the landscape of PTM, ISG15 has been investigated in the context of pathogenic infection and cancer. However, very few of these studies addressed its role in skin-related pathologies. At steady state, *Isg15-*deficient (KO) mice are apparently normal, which means that ISG15 depletion does not affect the composition of the main cellular compartments of the immune system, even after pI:C challenge (20). Here, we prove the functional involvement of ISG15 in the inflammatory immune response in a mouse model of ACD. By analysing the elicitation and sensitization phases of the disease together with transplantation and adoptive transfer experimental approaches, we have ruled out an effect for ISG15 in T cells and have shown that ISG15-depleted DCs do not activate properly in response to OXA challenge, and express lower levels of inflammatory cytokines. Importantly, exogenous application of extracellular ISG15 to DCs *in vitro,* corrected this defect.

There are different types of DCs in the skin, from epidermal LCs to four subsets of dDCs. LCs are located in the epidermis and mediate tolerance to weak haptens (30), but they are also necessary to induce inflammatory responses in response to strong haptens (31,32). When the hapten dose is very high, it can permeate deeper in the skin reaching the dermis, where other dDCs populations are located, triggering the inflammatory response as well (2,33). Importantly, LCs are radioresistant (22), while dDCs are radiosensitive and repopulated from BM-derived monocytes after γ–irradiation (22). Then, dDCs but not LCs might be contributing to the differential response between genotypes. In CHS responses, ROS and hyaluronidase activity increase due to hapten challenge. These lead to hyaluronic acid fragments that act as DAMPs, activating TLR2 and TLR4 signalling in these DCs (4). These pathways stimulate the production of several inflammatory cytokines, such as IL-18 and IL-1β, which also require activation of the inflammasome pathway to be released (34). We have observed that, *in vitro*, DCs deficient in *Isg15* secrete less IL-1β, which may account for reduced severity of CHS in ISG15-deficient mice.

LFA-1 integrin is a membrane receptor for extracellular, secreted ISG15 in NK cells. ISG15-LFA-1 interaction triggers a Src-dependent signalling pathway that, in synergy with IL-12, triggers secretion of IFN-γ- and IL-10-containing vesicles(9). Conversely, LFA-1 might not be the unique ISG15 receptor, as ISG15 can directly modulate diverse cell types (including red blood cells, macrophages, DCs, NK cells, neutrophils, and T cells, some of which do not express LFA-1) and be secreted by different cell types, or detected in blood and other secreted vesicles (neutrophilic granules, microparticles, exosomes, lysosomes and apoptotic bodies) (35). Here, we show that the combination of free ISG15 + IL-12 restored IL-1β secretion to normal levels in ISG15-KO BMDCs. However, whether LFA-1 is driving this process is currently unknown. Although the rescue experiment employed free ISG15, its modification of endoplasmic reticulum (ER) and Golgi associated proteins can also contribute to the observed differences in cytokine secretion (36). In this regard, IL-12 and TNFα depend on ER and Golgi for its secretion, albeit through different mechanisms (37). In fact, IL-1β mechanism of secretion is still unclear, and could include lysosome or multivesicular bodies (MVBs) pathways (37). ISGylation of proteins, such as TSG101, are involved in restricting MVBs formation towards lysosome degradation, thus reducing exosome or virus release (38–40).

The role of IFN type I in ACD skin inflammatory processes is not yet fully understood. Prevention of IFN type I signalling can suppress CHS (41–43). Thus, the reduced CHS severity observed in *Isg15-*deficient mice is in general agreement with these previous results as ISG15 is an IFN type I-induced gene. CD8+ T cells participate in CHS (44). These cells are important to fight against virus, bacteria, and tumour cells, processes in which IFN-I and ISGs play pivotal roles. A recent study proposed that ISG15 can act as an alarmin to boost CTL responses (45). In addition, ISG15 can act as a neutrophil chemoattractant (46), impair human moDCs migration (47), regulate macrophage activation and phagocytosis (48), induce IFNγ secretion by T and NK cells (18,49), secretion of IFN-y and IL-1β by CD8α+ DCs (50), and many other immune related functions. For that reason, in the context of ACD responses, DCs might not be the only cellular actor influenced by ISG15 deficiency during the immune response. The balance between different functions of ISG15 as a free intracellular or extracellular molecule or as PTM is key for understanding its role in CHS.

The role of ISG15 in skin diseases needs additional attention, particularly if ISG15 is to be at the centre of novel therapeutic approaches. Its plasticity and versatility make ISG15 a small molecule with many partners and functions. It exerts functions as a free molecule recognized by LFA-1 or conjugated to many proteins through the ISGylation system, with a myriad of possible outcomes (35). It is important to note, that for that reason, assigning a specific function has been a difficult task. The development of new proteomic techniques and enrichment methodologies will aid to gradually fill in the gaps in order to understand the mechanisms in which ISG15 is involved and whether it can be harnessed to control inflammation.

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# **Figure legends**

**Figure 1. ISG15 is expressed in patients with allergic contact dermatitis (ACD)** Representative human skin biopsies from healthy, non lesional and lesional allergic contact dermatitis donors. Tissue sections were stained with CD45 (*green*), CD49f (*red*) and ISG15 (*gray*). Nuclei were stained with DAPI (*blue*). Scale bars = 100 μm. Images are representative of 3 patients and 3 control subjects.

**Figure 2. *Isg15*-deficient mice develop less ear swelling and inflammation after CHS challenge.** **(A)** Scheme of CHS mouse model: (i) afferent (sensitization) phase and (ii) efferent (elicitation) phase. **(B)** Increased ear thickness of WT and KO mice for 4 days after elicitation (n = 6, one-way ANOVA test with Tukey’s post-test; \**P* < 0.05and ns, not significant). **(C)** Histological hematoxylin/eosin representative cross sections of WT and KO mice, bars size represents 100 μm. **(D)** Epidermal (*left panel*) and dermal (*right panel*) thickness quantification of histological sections (n ≥ 23, one-way ANOVA test with Tukey’s post-test; \**P* < 0.05and ns, not significant). **(E)** mRNA expression levels of *Isg15* in dLNs after 24, 48 and 72 h of sensitization (n ≥ 3, Student’s t test; \**P* < 0.05, \*\**P* < 0.01and ns, not significant). **(F)** mRNA expression of *Isg15* gene in aLN for 24 h (*left* plots) and 4 days (*right plot*) after elicitation.(n ≥ 4, one-way ANOVA test with Tukey’s post-test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001and ns, not significant). **(G)** mRNA expression levels of *Isg15* in ear skin samples after 24 h of elicitation. (n ≥ 4, one-way ANOVA test with Tukey’s post-test; \*\**P* < 0.01, \*\*\**P* < 0.001and ns, not significant). *Ywhaz* and *β-actin* were used as housekeeping genes.

**Figure 3. Reduced recruitment of immune cells in the absence of ISG15 expression during elicitation.** **(A)** Neutrophils, macrophages and monocytes total cell count in ear skin of mice after 24 h, 3- and 7-days post-elicitation. **(B)** Total cell count of hematopoietic and T cell populations in the ear of mice after 24 h, 3- and 7-days post-elicitation. All populations were gated in alive and CD45.2+ cells. **(C)** Total cell count of hematopoietic cells (CD45.2+) and T cells gated in alive cells in the aLN after 24 h, 3- and 7-days of elicitation (n ≥ 4, Student’s t test; \**P* < 0.05, \*\**P* < 0.01 and ns, not significant). P, gated population.

**Figure 4. The absence of ISG15 in hematopoietic cells, especially in DCs, plays a major role in CHS.** **(A)** Schematic representation of BMT procedure. **(B)** CD45.2+ relative frequency of cells in blood from BM-transplanted mice (n = 15, Student’s t test; ns, not significant). **(C)** Increment of ear thickness from BM-transplanted mice during the CHS elicitation phase (n = 8, Student’s t test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and ns, not significant). P, gated population**. (D)** Schematic representation of the procedure. WT and KO mice were treated with 3% OXA in the ventral area and ears. After 4 days, LN-cells were retrieved and injected intravenously in Rag1-deficient mice together with 1% OXA treatment in the ear. **(E)** Increment of ear thickness in LN-cell-transferred mice for 7 days. A control PBS intravenously injected group control was included (n = 6, Student’s t test). **(F)** *Upper panel*, WT mice were treated with 3% OXA in the ventral area and ears. After 4 days, CD3+ T cells were obtained from dLNs and purified by negative selection. Cells were injected intravenously in WT or KO mice together with 1% OXA treatment in the ear. *Lower panel*, increment of ear thickness in WT or KO mice after 24 h. A control PBS intravenously injected group control was included (n ≥ 4, Student’s t test). **(G)** *Upper panel,*WT mice were treated with 3% OXA in the ventral area. BMDCs from WT and KO mice were treated or not with 1mM OXA *in vitro* for 20 min, washed and intradermal injected in the WT mice ear after four days of sensitization. *Lower panel*, increment of ear thickness in BMDCs-transferred mice at 24 h. Non-instructed WT and KO BMDCs were injected as controls (n = 10, Student’s t test).

**Figure 5. The absence of ISG15 in DCs does not affect activation and total cell numbers in the skin. (A)** Gating strategy for DCs population in ear skin. All populations are gated on CD45.2+CD3-CD19-GR-1-MHCII+. **(B)** Total cell numbers of Langerhans cells (LCs, *left panel*) and dermal DC (dDC) CD11b+ (*right*) in WT or KO mice treated or not with 1% OXA after 24 h (n ≥ 4, Student’s t test). **(C)** Total cell numbers of cDC1 subdivided in EpCAM+ (*left*) or EpCAM- (*right*) cells in WT or KO mice treated or not with 1% OXA after 24 h (n ≥ 4, Student’s t test). **(D)** *Left,* gating strategy for activated total DCs populations gated on CD45.2+CD3-CD19-GR-1-MHCII+. *Right,* total cell numbers of activated CD40+CD86+ DCs from *Isg15*-WT or KO mice treated or not with 1% OXA after 24 h (n ≥ 4, Student’s t test).

**Figure 6. *Isg15-*deficient BMDCs secrete reduced levels of pro-inflammatory cytokines. (A)** *Isg15* mRNA expression levels by RT-qPCR analysis of GM-CSF-derived BMDCs treated for 12 h (left panel) and 24 h (right panel) with different TLR agonists (n ≥ 5, Student’s t- test; \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001; ns, not significant). **(B)** WB of BMDCs from *Isg15*-WT and KO mice treated overnight with various TLR stimuli show ISGylated proteins (upper panels). α-Tubulin (lower panels) is included as load control. **(C)** IL-1β, IL-12 p70 and TNFα protein secretion levels were analysed by ELISA in WT and KO BMDCs after various TLR stimulation at 12 h (*upper panel*) or 24 h (*lower panel*) post-treatment (n = 5, Student’s t test, \**P* < 0.05, \*\**P* < 0.01and ns, not significant). **(D)** IL-1β protein secretion levels analysed by ELISA in WT and KO BMDCs after LPS treatment in a combination with ISG15 or ISG15 + IL-12 stimulation at 24 h (n=4, Student’s t test, \**P* < 0.05, \*\**P* < 0.01and ns, not significant).