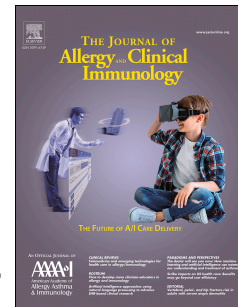


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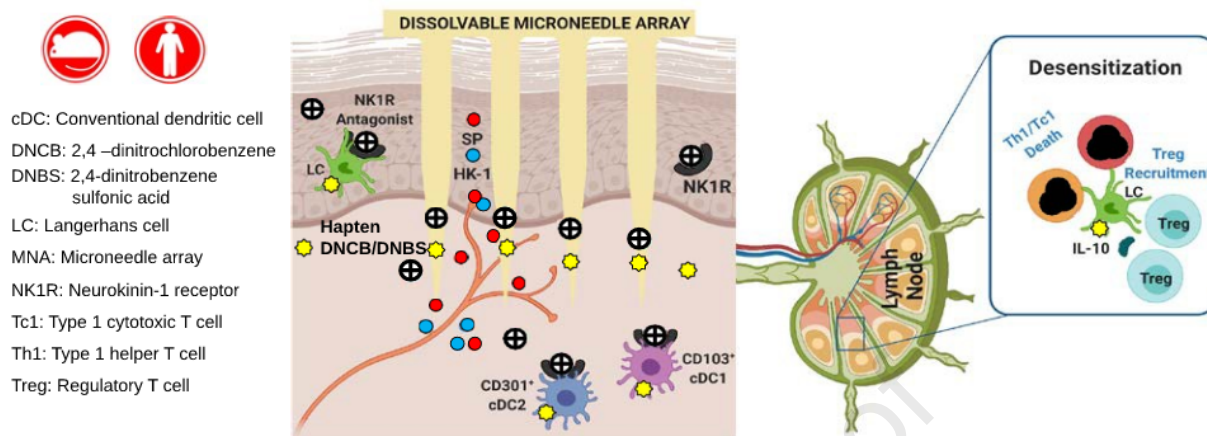
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MNA delivery of NK1R antagonists and haptens in the skin desensitizes allergic contact dermatitis



Skin co-delivery of contact sensitizers and neurokinin-1 receptor antagonists integrated in microneedle arrays suppresses allergic contact dermatitis

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ABSTRACT

Background: Allergic contact dermatitis (CD) is a chronic inflammatory skin disease caused by type-1 biased adaptive immunity for which there is an unmet need for antigen (Ag)-specific immunotherapies. Exposure to skin sensitizers stimulates secretion of the proinflammatory neuropeptides substance P (SP) and hemokinin 1 (HK1), which signal via the neurokinin-1 receptor (NK1R) to promote the innate and adaptive immune responses of CD. Accordingly, mice lacking the NK1R develop impaired CD.

Nonetheless, the role and therapeutic opportunities of targeting the NK1R in CD remain to be elucidated.

Objective: To develop an Ag-specific immunosuppressive approach to treat CD by skin co-delivery of hapten and NK1R antagonists integrated in dissolvable microneedle arrays (MNA).

Methods: In vivo mouse models of contact hypersensitivity and ex-vivo models of human skin were used to delineate the effects and mechanisms of NK1R-signaling and the immunosuppressive effects of the contact sensitizer-NK1R-antagonists-MNA in CD.

Results: We demonstrate in mice that CD requires NK1R signaling by SP and HK1. Specific deletion of the NK1R in keratinocytes and dendritic cells, but not in mast cells prevented CD. Skin co-delivery of hapten- or Ag-NK1R-antagonist-MNA inhibited neuropeptide-mediated skin inflammation in mouse and human skin, promoted deletion of Ag-specific effector T cells and increased regulatory T cells, which prevented CD onset and relapses locally and systemically in an Ag-specific manner.

Conclusions: Our findings demonstrate that immune-regulation by engineering localized skin neuroimmune networks can be used to treat cutaneous diseases that like CD, are caused by type-1 immunity.

KEY MESSAGES

- Skin exposure to haptens stimulates secretion of the neuropeptides substance P (SP) and hemokinin-1 (HK1), which signal via the neurokinin-1 receptor (NK1R) to initiate the inflammatory response that promotes the T-cell stimulatory function of skin-resident dendritic cells (DC).
- Simultaneous skin delivery of hapten and NK1R antagonists incorporated in dissolvable microneedle arrays prevents hapten-induced skin inflammation, reduces the migration of conventional dermal DC without affecting the migration of epidermal Langerhans cells (LC), and inhibits the adaptive immune response of contact dermatitis.

CAPSULE SUMMARY

We developed an immunosuppressive approach for the prevention and treatment of allergic contact dermatitis by skin co-delivery of hapten or protein Ag and neurokinin-1 receptor antagonists integrated in dissolvable microneedle arrays.

KEYWORDS: Contact dermatitis, contact hypersensitivity, microneedle arrays, keratinocytes, neuropeptides, neurokinin-1 receptor, neurokinin-1 receptor antagonists, hapten, immunosuppressive therapies.

ABBREVIATIONS

Ag: Antigen

CD: Allergic contact dermatitis

79 cDC: Conventional dendritic cells
80 CHS: Contact hypersensitivity
81 DC: Dendritic cells
82 dLN: Draining lymph node
83 DNBS: 2,4-dinitrobenzene sulfonic acid
84 DNCB: 2,4-dinitrochlorobenzene
85 DT: Diphtheria toxin
86 HK1: Hemokinin 1
87 HPF: High power-field
88 LC: Langerhans cell
89 LN: Lymph node
90 MC: Mast cells
91 MNA: Microneedle arrays
92 Mrgpr: Mas-related G protein-coupled receptors
93 NK1R: Neurokinin-1 receptor
94 OVA: Chicken ovalbumin
95 SarSP: [Sar⁹,Met(O₂)¹¹] SP
96 SP: Substance P
97 Th1: CD4 T helper 1 cells
98 Tc1: CD8 cytotoxic T cells
99 Treg: Regulatory T cells

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INTRODUCTION

Allergic contact dermatitis (CD) is a common chronic inflammatory skin disease caused by exposure to contact sensitizers and sustained by Ag-specific T cells¹. Due to an increased exposure to skin sensitizers in domestic and working environments, the incidence of CD has escalated across age and gender differences and is currently the second highest cause of work-related diseases in the USA. Recent studies have shown that the prevalence of CD is up to 18% and affects 10.6 % of the working population^{1,2}. The management of CD includes preventing exposure to skin sensitizers and administration of corticosteroids or biologics^{1,3}. Current immunotherapies to treat severe CD are administered systemically and lack antigen (Ag)-specificity, which could result in generalized immunosuppression. Hence, there is an unmet need for Ag-specific treatments that target the physiopathology of CD.

Neuroinflammatory responses have a relevant role in the physiopathology of CD. Skin exposure to hapten or Ag initiates secretion of substance P (SP) and hemokinin 1 (HK1), which are proinflammatory neuropeptides that bind with high affinity to the neurokinin 1 receptor (NK1R), a G protein-coupled receptor that recruits $G_{\alpha q/11}$ protein subunits to activate intracellular signaling that supports innate and adaptive immunity⁴⁻⁹. As a result, NK1R signaling increases the severity of chronic inflammatory disorders that, like CD, are mediated by CD4 T helper 1 (Th1) and CD8 T cytotoxic (Tc1) cells. Accordingly, mice lacking the NK1R develop impaired CD^{4, 6, 7, 10-13}.

We hypothesized that inhibition of the proinflammatory function of SP and HK1 by NK1R antagonists, delivered simultaneously with contact sensitizers into a localized area of the skin, would enable a physiopathology-based and Ag-specific immunosuppressive approach to treat type-1 biased CD. To be feasible, this strategy requires further understanding of the role of the NK1R in skin-initiated type-1 immunity and an efficient and reliable system to co-deliver NK1R antagonists and hapten or Ag to the cutaneous microenvironment.

Recent studies have shown that besides signaling via the NK1R, SP and HK1 bind Mas-related G protein-coupled receptors (Mrgpr). Activation of mouse MrgprB2 or human MRGPRX2 by SP in sensory neurons and mast cells (MC) promotes itching, inflammatory pain and adaptive immunity^{14, 15}. These observations challenged our current understanding on the biology of the NK1R and its endogenous ligands in the innate and adaptive immunity of CD, areas of research that require in vivo models in which the NK1R can be deleted in a cell type-specific fashion.

Here we demonstrate that type-1 biased skin contact hypersensitivity (CHS) requires simultaneous NK1R signaling by SP and HK1 but does not require signaling the MrgprB2. We used cell-type specific deletion of the NK1R in mice to identify the skin cells that could be targets of NK1R agonists, including keratinocytes, conventional dendritic cells (cDC) and MC, and we described their downstream regulatory effects on the T cell response that causes CHS. Based on these data, we developed an immunosuppressive approach that utilizes dissolvable multicomponent microneedles arrays (MNA) to co-deliver NK1R antagonists and hapten or Ag to a circumscribed area of the skin. These MNA are delivery platforms that incorporate biologics in a biodegradable matrix. When the microneedles penetrate the skin they dissolve and release their cargo with anatomic precision and dosage control¹⁶⁻¹⁹. In mice, we found that co-delivery of NK1R antagonists and hapten or Ag by MNA prevented the development of CD and desensitized the already established disease, locally and systemically in Ag-specific fashion. Importantly, the translational aspects of our results in mice were validated in ex-vivo models of human skin.

METHODS

Supplemental information can be found in the Methods section of the Online Repository.

Study Approval

Mice

Male or female mice (7-12 week old) were randomly selected for the experiments in accordance with the National Institutes of Health scientific rigor policy. Mice were maintained in the pathogen-free animal facility of the University of Pittsburgh School of Medicine that provides with around the clock husbandry and veterinary services. Animal care and handling were performed in accordance with institutional guidelines and the procedures approved by IACUC protocol number 19014279.

Human tissue and blood samples

Human skin samples from cosmetic plastic surgeries were provided by the Health Science Tissue Bank, from the Department of Pathology of the University of Pittsburgh, School of Medicine. Skin samples procured anonymously and without identifiers were used according to Institutional Review Board (IRB) outlines (IRB approval number 0501138). Human blood samples from healthy donors were obtained anonymously and without identifiers from the Central Blood Bank (University of Pittsburgh, Pittsburgh, PA, USA) and used according to IRB guidelines (IRB approval number 0602095). Both human skin and blood samples are classified as non-human subjects.

Induction and assessment of cutaneous CHS

For induction of CHS by topical application of sensitizers, mice were treated once with either 2,4-dinitrochlorobenzene (DNCB) (1% in 4:1 v/v acetone / olive oil), oxazolone (1% in 4:1 v/v acetone / olive oil) (both from Sigma-Aldrich) or vehicle, on the shaved abdominal skin. Topical application of 100 µg of

174 DNCB was used in comparative studies with DNBS-MNA. When indicated, the NK1R antagonists
175 L733060 (10 nmols) or RP67580 (10 nmols), or the NK1R agonists (SarSP) (10^{-9} M), SP (10^{-9} M) or HK1
176 (10^{-8} M) (all from TOCRIS), were injected i.d. immediately before topical application of the sensitizer.
177 Five days after sensitization, mice were challenged with DNCB (0.5% in 4:1 v/v acetone / olive oil) or with
178 oxazolone (0.5% in 4:1 v/v acetone / olive oil) on the dorsal side of the right ear, and with vehicle on the
179 left ear (control). Ear thickness increase was measured before and after treatment using a digital mini-
180 micrometer (Mitutoyo). Ear thickness was assessed 24 h following sensitization to evaluate the innate
181 immune response and 24, 48 and 72 h after elicitation to evaluate the adaptive immune response. In some
182 experiments, ear thickness was analyzed up to 120 h following elicitation. Values are expressed as
183 percentage in ear thickness increase obtained by the formula: (thickness of treated ear – thickness of
184 untreated ear) / (thickness of untreated ear) x 100. As negative controls, mice were treated with vehicle and
185 challenged with DNCB (0.5%).

186 For induction of CHS with MNA, mice were sensitized on the right ear by application of one MNA
187 loaded with DNBS (50 μ g) (Sigma-Aldrich) or OVA (100 μ g) (Sigma-Aldrich) alone or with the NK1R
188 antagonists RP67480 (10 nmols) or L733060 (10 nmols) (TOCRIS). Elicitation was done by topical
189 application of DNCB (0.5% in 4:1 v/v acetone / olive oil) or OVA injected i.d (50 μ g in 50 μ l of PBS per
190 injection site), 5 days after sensitization. Mice untreated, sensitized with empty-MNA, and then elicited
191 with topical DNCB, were included as controls.

192 For induction of CHS in hu-Lang-DTR mice, depletion of LC was achieved by administration of
193 diphtheria toxin (500 ng, i.p., once), 72 h before sensitization²⁰. Controls included WT mice and hu-Lang-
194 DTR mice not exposed to diphtheria toxin. LC depletion was confirmed by fluorescence microscopy on
195 epidermal sheets dissected from the ear skin, fixed in cold acetone, and stained with goat-anti human
196 CD207 Ab (R&D Systems) and biotin-IA^b Ab, followed by Cy3-conjugated anti-goat IgG and Cy2-
197 streptavidin (Jackson ImmunoResearch).

Statistics

Statistical analysis was performed using the GraphPad Prism v8 software (San Diego, CA). Results are expressed as mean $\pm$ 1SD. Comparisons of two means were done by two-tailed Student's *t*-test. Comparison of multiple means on a single data set was done by 1-way ANOVA followed by *ad hoc* comparison between means by Student-Newman-Keuls test. A "*p*" value < 0.05 was considered significant.

RESULTS

Efficient CHS requires NK1R, SP and HK1

The role of the NK1R and its natural agonists, the neuropeptides SP and HK1, in adaptive cellular immunity of CD was investigated in a model of CHS to DNCB in wild type (WT), NK1R^{KO}, *Tac1*^{KO} (SP-deficient), *Tac4*^{KO} (HK1-deficient) and *Tac1/Tac4*^{KO} and *MrgprB2*^{KO} mice. DNCB was chosen because is a potent hapten that induces CHS in mice and has the potential to sensitize humans ²¹.

Mice were sensitized with topical DNCB on the skin (abdomen) and elicited 5 days later on the dorsal skin of the right ear. Compared to WT, NK1R^{KO} mice showed significantly reduced CHS as determined by lower ear thickness increase and less mononuclear leukocyte infiltrate at the elicitation site (Fig 1A and B and Fig E 1A and B). To rule out the possibility that low secretion of SP or HK1 in a global NK1R^{KO} mice was causing deficient CHS ²², we induced CHS in NK1R^{KO} mice injected i.d. with the agonist [Sar⁹Met(O₂)¹¹] SP (SarSP) during sensitization ²³. In these conditions, NK1R^{KO} mice did not show a significant increase in ear thickness or the histological changes of CHS compared to NK1R^{KO} mice sensitized without SarSP (Fig 1A and B and Fig E 1A and B). The CHS response in NK1R^{KO} mice remained minimal up to 5 days after elicitation, indicating that absence of NK1R prevents, instead of delaying CHS (Fig 1A). Comparative studies between *MrgprB2*^{KO} and WT mice showed that lack of *MrgprB2* does not decrease the CHS to DNCB (Fig E 1C)

Similar CHS experiments in *Tac1*^{KO} (SP-deficient), *Tac4*^{KO} (HK1-deficient) or *Tac1/Tac4*^{KO} mice demonstrated that lack of either SP or HK1 reduces significantly CHS to DNCB (Fig 1C and D and Fig E 2A and B). The capability of *Tac1/Tac4*^{KO} mice to develop CHS to DNCB was partially restored when either SP or HK1 was injected i.d. at the sensitization site immediately before topical application of DNCB, and the CHS response was fully restored following co-administration of both neuropeptides (Fig 1E and F and Fig E 2C and D). Thus, skin CHS to DNCB requires signaling of the NK1R by SP and HK1.

Skin co-delivery of hapten and NK1R antagonists integrated in MNA abrogates CHS

244 Development of an immunosuppressive approach based on skin delivery of hapten and NK1R antagonists
245 to prevent the neuroinflammation triggered by hapten penetration through the skin, requires a system that
246 administers simultaneously reliable doses of the biologics to a circumscribed skin area eliciting minimal or
247 no inflammation, properties that are characteristic of MNA¹⁶ (Fig 2A). When the MNA is applied to the
248 skin, the microneedles dissolve within 5-10 min (Fig 2B). Release of the MNA cargo in the skin layers was
249 analyzed using MNA tip-loaded with the fluorochromes Alexa Fluor-488 and Alexa Fluor-555 as
250 surrogates for the NK1R antagonist and the hapten applied to the ears of WT mice. Live-animal imaging by
251 fluorescence microscopy revealed that the MNA delivers both cargos simultaneously and efficiently
252 throughout the skin layers of mouse ears (Fig 2C and D). Following, we tested if the MNA substrate per se
253 was proinflammatory and if the hapten and NK1R antagonists co-delivered by MNA were functional. We
254 compared in a dose response experiment, the cutaneous CHS induced by sensitization with topical DNCB
255 (100 µg) to that induced by MNA loaded with 25, 50 and 100 µg of 2,4 dinitrobenzene sulfonic acid
256 (DNBS) (DNBS-MNA), selected as the water-soluble analog of DNCB suitable for MNA loading. After
257 elicitation with topical DNCB, mice sensitized with DNBS-MNA loaded with either 50 or 100 µg of
258 hapten developed a significantly superior CHS than that of mice sensitized with topical DNCB (Fig 2E).
259 Therefore, MNA loaded with 50 µg of DNBS were selected for the rest of the experiments. Importantly,
260 MNA without cargo (empty MNA) did not induce skin inflammation.

261 We investigated if sensitization with MNA containing DNBS and NK1R antagonists (DNBS-
262 NK1R^{antagonist}-MNA) affects skin CHS. Because in mice SP and HK1 exert their agonist effects by binding
263 the NK1R and MrgprB2, we compared the effects of RP67580 an antagonist that inhibits specifically the
264 mouse NK1R with those of L733060 that blocks both NK1R and MrgprB2.^{10, 15, 24-28}. The doses of the
265 NK1R antagonists (10 nmols per MNA) were based on previous publications^{6, 24, 25, 27, 29-31}. Co-
266 administration of NK1R antagonist at the sensitization site, incorporated with hapten in MNA or injected
267 i.d. reduced CHS (Fig 2F and Fig E 3A-C). This effect was more pronounced when the NK1R antagonists

and DNBS were co-administered via MNA, and both RP67480 and L733060 exerted similar CHS inhibitory effects (Fig 2F and Fig E 3A-C). Thus, the MNA co-deliver NK1R antagonists and hapten efficiently to the skin during sensitization, which prevents cutaneous CHS.

Skin delivery of hapten and NK1R antagonists by MNA restrains hapten-induced inflammatory environment in mouse and human skin

We investigated in mice and in an ex vivo model of human skin if co-delivery of hapten and NK1R antagonists via MNA affects the skin inflammation triggered by hapten penetration. WT mice were sensitized on the ear skin with DNBS-MNA, DNBS-RP67580-MNA, DNBS-L733060-MNA or empty-MNA (control). Twenty-four h later, DNBS-MNA application induced a robust ear thickness increase and leukocyte infiltrate at the sensitization site, effects that were reduced in mice exposed to either DNBS-NK1R^{antagonist}-MNA (Fig 3A-D). Next, we investigated if skin application of DNBS-NK1R^{antagonist}-MNA affects the synthesis and secretion of IL-1 β and IL-6, which are relevant for the inflammatory response of CD³²⁻³⁶. Sensitization with DNBS-MNA increased pro-IL-1 β and IL-6 mRNA contents and IL-1 β and IL-6 protein concentrations at the skin treated site, which did not occur or was substantially reduced when mice were sensitized with DNBS-NK1R^{antagonist}-MNA (Fig 3E and F).

To analyze if the inhibitory effects of NK1R antagonists on hapten induced inflammation also occur in human skin, we applied DNBS-MNA, DNBS-L733060-MNA, or empty-MNA on the epidermal side of human skin organ cultures composed of epidermis and dermis (4 cm² size, 1 MNA per cm²). The NK1R antagonist L733060 was selected because it blocks exclusively the human NK1R without affecting the function of the human MRGPR2X receptor that is also signaled by SP and HK1^{15, 26}. Six h after MNA application, human skin samples treated with DNBS-L733060-MNA contained less pro-IL-1 β and IL-6 mRNA compared to skin treated with DNBS-MNA (Fig 3G). Thus, in mouse and human skin, the NK1R is

key for triggering synthesis of pro-inflammatory IL-1 β and IL-6 and inflammation at the sensitization site, and these phenomena are minimized by co-administration of hapten and NK1R antagonists via MNA.

Our findings indicate that skin co-delivery of hapten and NK1R antagonists decreases the inflammatory milieu that promotes maturation of immunogenic DC within the skin and their subsequent mobilization to skin-dLN^{34, 37}. Thus, we next analyzed if blockade of NK1R signaling during hapten sensitization affects the mobilization out of the skin and the maturation of human skin DC, which express the NK1R (Fig E 4A and B). Forty-eight h after application of DNBS-L733060-MNA to the epidermis of human skin organ cultures, the DC mobilized out of the skin decreased in numbers (Fig 3H), exhibited lower expression of HLA-DR, CD86 and CD40 and increased PD-L1 surface content (Fig 3I and Fig E 4C). Additionally, we detected higher concentrations of IL-10 in 24 h culture supernatants of purified DC migrated from human skin organ cultures treated with DNBS-NK1R^{antagonist}-MNA than in supernatants of control DC mobilized from human skin explants treated with DNBS-MNA (Fig 3J). IL-12p70 was undetectable in the same DC culture supernatants, regardless of the skin treatment (Fig 3J). Thus, co-administration of hapten and NK1R antagonists via MNA impairs the maturation and migration out of the skin of human DC.

Skin CHS requires NK1R signaling in keratinocytes and DC

Different subsets of skin cells participate in the sensitization and effector phases of CHS³⁸⁻⁴¹. In mice, the NK1R or its mRNA were detected in skin keratinocytes, MC (dermal-resident and generated from peritoneal progenitors), epidermal and dermal DC, skin DC homed in skin-draining lymph nodes (skin-dLN), and bone marrow-derived DC generated in culture (Fig E 5A-D). Since skin keratinocytes, DC and MC have been shown to be involved in the innate and adaptive immune responses that causes CD^{6, 42-45}, we analyzed on each of these cell subsets the role of NK1R signaling on the sensitization and effector phases of CHS. We used B6 NK1R^{fl/fl} mice in which Cre driven recombination causes a deletion of exon 2 of the *Tac1r* locus that contains the initial ATG start codon and encodes a fragment of the 7tm_1 (PF00001) domain of the NK1R, which is critical for agonist binding and the functional structure of the

receptor. NK1R^{fl/fl} mice were crossed with K14-Cre, CD11c-Cre or Mcpt5-Cre mice to delete the functional NK1R in keratinocytes, DC or MC, respectively (Fig E 5E). NK1R^{fl/fl}, K14-Cre, CD11c-Cre, Mcpt5-Cre mouse parent lines developed skin CHS to DNCB similar to WT mice (Fig E 5F). K14-NK1R^{fl/fl}, CD11c-NK1R^{fl/fl} and Mcpt5-NK1R^{fl/fl} mice and respective control littermates were sensitized on the ear skin with DNBS-MNA or empty-MNA, and then elicited with topical DNCB. NK1R deletion in keratinocytes, but not in DC or MC reduced the inflammatory response induced by DNBS-MNA sensitization, based on decreased ear thickness and leukocyte infiltrate, as compared to littermate controls (Fig 4A and Fig E 5G and H). Accordingly, mouse keratinocytes stimulated in vitro with the NK1R agonist SarSP increased the content of pro-IL-1 β and IL-6 mRNA (Fig 4B). Likewise, the quantity of pro-IL-1 β and IL-6 mRNA in keratinocytes isolated from the ear skin of K14-NK1R^{fl/fl} mice sensitized with DNBS-MNA was less than that detected in keratinocytes isolated from the ear skin of control littermates exposed to DNBS-MNA (Fig 4C).

Mice sensitized with DNBS-MNA on the ear skin and with deletions of functional NK1R in keratinocytes or DC, but not in MC, developed a decreased effector phase of skin CHS against topical DNCB, compared to littermate controls (Fig 4D and Fig E 5I and J). Under the same conditions, mice lacking functional NK1R in keratinocytes exhibited fewer effector CD8 T cells at the elicitation site (Fig 4E and F). Thus, NK1R expression in keratinocytes is critical for the early inflammatory response induced by hapten sensitization and NK1R expression in keratinocytes and DC is key for development of the effector phase of CHS.

Effect of co-delivery of hapten and NK1R antagonists on mouse skin DC

Our findings indicate that NK1R antagonists restrain the pro-inflammatory skin microenvironment necessary for the activation of DC with potent T-cell stimulatory function required for the elicitation of CHS. Therefore, we investigated if NK1R antagonists affect migration, maturation and Ag transport of skin-resident DC. For these experiments, we used a mouse model of skin CHS to chicken ovalbumin

(OVA) after validating that skin application of MNA loaded with OVA (OVA-MNA) promote potent CHS, which is significantly decreased when NK1R antagonists and OVA are co-delivered by the MNA (Fig E 6A). To trace the migration of cutaneous DC exclusively from the skin area treated with the MNA, the experiments were done in mice expressing ubiquitously the Kikume Green-Red (KikGR) fluorescent photoconvertible protein ⁴⁶. The skin area sensitized with MNA was exposed to violet light immediately before MNA application, to shift the fluorescence of the photoconvertible skin cells from green to red. In this model, the photoconverted (red) DC that migrate from the skin area treated with MNA are distinguished from the non-photoconverted (green) DC that traffic from untreated skin or from DC that reside constitutively in LN. Using this model, we tested if co-delivery of OVA and NK1R antagonists via MNA alters the traffic of cutaneous DC to skin-dLN. Since the NK1R antagonists RP67580 and L733060 exerted similar effects on CHS to OVA (Fig E 6A), the following experiments were done with the selective mouse NK1R antagonist RP67580. OVA-MNA, OVA-RP67580-MNA or empty-MNA were applied to the ears of KikGR mice. Two days after MNA application, photoconverted DC mobilized from the MNA treated ear were identified by flow cytometry in the auricular skin-dLN as CD11c^{Pos} MHC class-II^{High} cells, which included CD207^{Pos} CD103^{Neg} Langerhans cells (LC), dermal conventional CD103^{Pos} (cDC1) and dermal conventional CD301b^{Pos} (cDC2) dendritic cells (Fig 5A and Fig E 6B). Treatment with OVA-MNA increased the number of LC and both dermal DC subsets in skin-dLN compared to skin application of empty-MNA. Addition of RP67580 to the OVA-MNA applied on the skin decreased the number of dermal cDC1 and cDC2 detected in skin-dLN, without altering significantly the number skin LC found in skin-dLN (Fig 5B). It also decreased expression of HLA-DR, CD86 and CD40 and augmented PD-L1 expression in photoconverted skin LC and dermal DC mobilized from the sensitization area to skin-dLN (Fig E 6C).

Next, we investigated if NK1R blockade during sensitization affects transport of Ag by cutaneous DC mobilized to skin-dLN. To track the Ag applied to the skin, we used MNA loaded with OVA conjugated to

the fluorochrome Texas Red (OVA•TR-MNA) alone or plus RP67580 (OVA•TR-RP67580-MNA). Since IL-10 has been associated with the immunosuppressive capacity of DC^{20, 47, 48}, these experiments were done in IL-10-enhanced green fluorescent protein (EGFP) reporter B6 mice in which EGFP expression is driven by the endogenous IL-10 promoter. OVA•TR-MNA, OVA•TR-RP67580-MNA or empty-MNA were applied to the ears of IL-10-EGFP mice, and cell suspensions of skin-dLN were analyzed by flow cytometry 2 days later. Following MNA treatment, OVA•TR was transported to skin-dLN mainly by skin LC and dermal cDC2 and to a less extent by dermal cDC1 (Fig 5C and D). Addition of RP67580 to the OVA•TR-MNA reduced the number of cDC1 and cDC2 with OVA•TR detected in skin-dLN without affecting significantly the number of migrating LC bearing OVA•TR in skin-dLN (Fig 5C and D). Following treatment with OVA-MNA, LC were the main subset of skin migratory DC containing IL-10 in skin-dLN and the number of LC containing OVA•TR and IL-10 in skin-dLN increased in mice treated with OVA•TR-RP67580-MNA (Fig 5E).

Our findings indicate that delivery of Ag and NK1R antagonist to the skin via MNA reduces the number of dermal DC transporting Ag to skin-dLN, without affecting the quantity of LC bearing allergen and IL-10^{20, 48}. Thus, we tested if LC have a role in the inhibitory effect of the NK1R antagonists in hu-Lang-DTR mice, a model that allows selective depletion of epidermal LC by administration of diphtheria toxin (DT)²⁰. Three days after DT injection, when epidermal LC were depleted (Fig E6 D), hu-Lang-DTR mice were sensitized with OVA-MNA, OVA-RP67580-MNA, or empty-MNA on the ear skin and elicited with OVA injected i.d., LC depletion abolished the inhibitory effect of the NK1R antagonist on the effector phase of CHS (Fig 5F). These results demonstrate that LC are required for the regulatory effect of NK1R antagonists.

Skin co-delivery of hapten and NK1R antagonists impairs the T cell response that causes CHS to DNCB

389 Since NK1R antagonists reduce the effector phase of skin CHS, we tested if co-delivery of hapten plus
390 NK1R antagonists via MNA affects the T cell response that causes the disorder. We used two
391 complementary relevant mouse models of CHS, one against DNBS that is mediated by a polyclonal T cell
392 response, and the other against OVA in which the Ag-specific T cell response can be tracked with TCR
393 transgenic OT-II CD4 and OT-I CD8 T cells.

394 In the CHS model against DNBS, following sensitization of WT B6 mice with DNBS-NK1R^{antagonist}-
395 MNA, analysis by flow cytometry revealed a higher percentage of activated (CD44^{High}) CD4 and CD8 T
396 lymphocytes undergoing cell death (Fig 6A) and a higher percentage of CD4 FoxP3 Treg (Fig 6B) in skin-
397 dLNs, compared to those detected in skin-dLNs of mice treated with DNBS-MNA. Next, we investigated if
398 the increase in the CD4 Foxp3 Treg in skin-dLN of mice treated with DNBS-NK1R^{antagonist}-MNA was due
399 to proliferation or recruitment of natural occurring Treg (nTreg), or de novo differentiation of induced Treg
400 (iTreg). To address that, untreated CD45.1 congenic B6 mice were injected i.v. with equal numbers of
401 nTreg (CD4^{Pos} Foxp3-EGFP^{Pos}) labelled with Violet Proliferation Dye (VPD) and naïve CD4 T cells
402 (Foxp3-EGFP^{Neg} CD25^{Neg}) labelled with Far-Red Proliferation Dye (RPD), both FACS-sorted from spleens
403 of CD45.2 congenic DEREK mice (Fig E 7A). Because in DEREK mice the DT-receptor-EGFP fusion
404 protein is under control of the endogenous *foxp3* promoter⁴⁹, this strategy allowed analyzing
405 simultaneously in skin-dLN the recruitment and proliferation of VPD labelled nTreg and the generation of
406 iTreg from RPD-labeled naïve CD4 T cells expressing de novo Foxp3 EGFP (Fig E7 A). One day after T
407 cell transfer, mice were sensitized on the ear with DNBS-MNA or DNBS-NK1R^{antagonist}-MNA. Four days
408 later, proliferation and cell number of nTreg and iTreg in skin-dLN was assessed by flow cytometry. The
409 results indicate that the increase in Treg numbers in skin-dLN of mice sensitized with DNBS-NK1R
410 ^{antagonist}-MNA was due to recruitment of non-proliferating nTreg (Fig E 7B).

411 Following, we analyzed in the CHS model against DNBS if cutaneous co-delivery of hapten and NK1R
412 antagonists affects the number of effector T cells at the skin elicitation site (Fig 6C and Fig E 7C and D).

Mice were treated on the ear skin with DNBS-RP67580-MNA, DNBS-MNA or empty-MNA, and elicited with topical DNCB. Forty-eight h later, the elicitation sites of mice treated with DNBS-RP67580-MNA contained fewer CD8 and CD4 T cells with decreased secretion of IL-17A and IFN- γ , respectively (Fig 6C and D and Fig E 7D) and more CD4 Foxp3 Treg, than the skin area of mice sensitized with MNA without the antagonist (Fig 6C and Fig E 7C). Thus, blockade of the NK1R during hapten sensitization of the skin results in death of activated T cells and recruitment of nTreg in skin-dLN, and fewer effector T cells secreting IL-17A and IFN- γ and more CD4 Foxp3 Treg at the skin elicitation site.

Skin co-delivery of OVA and NK1R antagonists impairs the CHS-effector response in an Ag-specific fashion

The ability of the immunosuppressive MNA to abrogate the T cell response in an Ag-specific manner was validated in the mouse model of CHS to OVA. Since the NK1R antagonists RP67580 and L733060 inhibited to a similar extent skin CHS to OVA (Fig E 6A), the experiments were done with MNA loaded with RP67580. WT B6 mice (Thy1.2 congenic) were injected i.v. with CFSE-labeled OT-II naïve CD4 T cells specific for OVA₃₂₃₋₃₃₉-IA^b or with naïve OT-I CD8 T cells against OVA₂₅₇₋₂₆₄ (SIINFEKL)-H2K^b (in this system OT cells are Thy1.1). The next day, mice were treated on the ear with OVA-MNA, OVA-RP67580-MNA or control empty-MNA. Three days (for OT-I) or 4 days (for OT-II) after treatment we analyzed by flow cytometry the absolute number, proliferation, activation, cell death and content of IFN- γ and IL-17A in OT-I and OT-II cells in skin-dLN. Treatment with OVA-RP67580-MNA did not affect OT-I or OT-II cell proliferation (by CFSE dilution) or activation (based on CD44^{High} expression) but increased the percentage of cell death (by FVD incorporation) (Fig 7A and B). Proliferating OT cells from mice treated with OVA-RP67580 MNA underwent similar number of cell cycles than those OT cells from mice treated with OVA-MNA, which indicates that treatment with OVA-NK1R^{antagonist}-MNA did not induce T-cell anergy (Fig 7C). In addition compared to mice skin sensitized with OVA-MNA, sensitization with

OVA-RP67580-MNA reduced the content of IFN- γ and IL-17A in dividing OT cells in skin-dLNs (Fig 7D and E). In accord with the results obtained in the CHS to DNCB, we did not detect de novo generation of OT-II iTreg in mice treated with OVA-RP67580-MNA (Fig 7F). Thus, the model of CHS to OVA revealed that skin delivery of Ag plus NK1R antagonists via MNA leads to cell deletion and reduced IFN- γ -content in Ag-specific T cells in skin-dLNs.

442

Cutaneous co-delivery of hapten and NK1R antagonists by MNA limits the T-cell stimulatory function of human skin DC

To assess the clinical applicability of our approach, we compared the T cell stimulatory function of DC mobilized out of human epidermal-dermal organ cultures (4 cm² surface area) treated with DNBS-MNA, DNBS-L733060-MNA or empty-MNA (1 MNA per cm², 5 explants per donor). Forty-eight h after MNA application, DC harvested from skin cultures of each donor were used as stimulators of CFSE-labelled allogeneic T cells in 5 day-mixed leukocyte cultures that were analyzed by flow cytometry. DC mobilized from skin explants treated with DNBS-L733060-MNA induced similar proliferation but increased percentage of cell death in dividing CD4 and CD8 T cells, as compared to that elicited by DC migrated from explants treated with DNBS-MNA (Fig 7G and Fig E 8A). In both situations, proliferating T cells underwent similar number of cell cycles, which indicates that treatment with DNBS-L733060-MNA did not cause T cell anergy (Fig E 8B). Thus, DC mobilized from human skin treated with hapten in presence of NK1R antagonists trigger proliferation of T cells unfit for survival.

456

Hapten and NK1R antagonists integrated in MNA desensitize established CD

We tested if co-delivery of DNBS and NK1R antagonists to the skin desensitizes established CHS and prevents its relapses. To address that, skin CHS to DNCB was first induced in WT B6 mice by topical DNCB sensitization on the skin (abdomen) followed on day 6, by cutaneous elicitation (right ear) with

topical DNCB. Skin CHS to DNCB was confirmed in each mouse. Sixteen days after (day 22), mice were treated on the elicitation site (right ear) with (i) DNBS-L733060-MNA or DNBS-RP67580-MNA, (ii) L733060-MNA or RP67580-MNA (no hapten), (iii) DNBS-MNA (positive control) or empty-MNA (Fig 8A). A week later (day 29), mice were re-challenged with topical DNCB on the homolateral (right) ear to evaluate the effect of the treatment on the local immune response, or on the contralateral (left) ear to assess the effect on the systemic response (Fig 8A). Co-administration of hapten with either NK1R antagonist via MNA prevented the relapse of local (Fig 8B) and systemic (Fig 8C) CHS to DNCB. These effects were superior to those observed when CHS desensitization was induced by application of MNA loaded with NK1R antagonists alone (Fig 8B and C).

Next, we tested if desensitization by DNBS-NK1R^{antagonist}-MNA is hapten-specific in WT B6 mice that had developed CHS against oxazolone induced by skin (abdomen) sensitization and confirmed by cutaneous elicitation with topical oxazolone (right ear). After 16 days (day 22), mice were treated on the skin (right ear) with (i) DNBS-L733060-MNA or DNBS-RP67580-MNA or (ii) DNBS-MNA or empty-MNA. A week later (day 29), mice were re-challenged with topical oxazolone on the skin (right ear). MNA loaded with DNBS and either NK1R antagonist were unable to desensitize CHS to oxazolone (Fig 8D). Thus, co-delivery of hapten and NK1R antagonists into the skin results in hapten-specific desensitization of established CHS and prevents CD relapses locally and systemically.

DISCUSSION

We developed an MNA-based immunosuppressive approach that restrains skin inflammation during hapten-sensitization and impairs the adaptive immune response of type-1 biased CD. Specifically, we targeted the function of proinflammatory neuropeptides that signal via the NK1R, which are released in the skin immediately after exposure to contact sensitizers. To our knowledge, the potential of this Ag-specific therapeutic strategy has not been previously explored likely due to lack of appropriate delivery systems able to simultaneously overcome the cutaneous physical barrier and deliver multiple biologics in the skin without disturbing its quiescent state. In addition, the role of neuropeptide-induced inflammation in the pathogenesis of chronic inflammatory skin disorders is beginning to be elucidated and the understanding of its mechanisms in vivo required genetically-modified mouse models that were not available until recently. In this study, we tested the therapeutic potential of dissolvable multicomponent MNA to co-deliver hapten and NK1R inhibitors efficiently in the skin to hinder the immune response of CD in mice and in an ex vivo human skin system, the later a biologically relevant translational model⁵⁰.

We described the role that NK1R signaled by its endogenous agonists, SP and HK1, plays in the pathogenesis of CD. Although it has been established that signaling the NK1R by SP promotes CHS, the function of HK1, had not been previously investigated^{13, 51, 52}. Besides, SP and HK1 also bind the mouse MrgprB2 and human MRGPRX2^{14,15}. Therefore, the exclusive roles the NK1R in CHS needed further analysis. Here, we demonstrated in mice deficient in NK1R or its endogenous ligands that induction of CHS requires NK1R, HK1 and SP, but not MrgprB2. Indeed, the CHS response was not compromised in MrgprB2^{KO} mice, but was impaired in NK1R^{KO} mice. Likewise in mice, blockade of the NK1R alone by the antagonist RP67580 induced similar immuno-suppressive effects than those caused by blockade of the NK1R and MrgprB2 by the antagonist L733060. In our mouse model, SP and HK1 exerted additive effects on CHS, which could be due to binding to different receptor sites, induction of a distinctive conformation of the receptor, redundancy or partial agonistic function of these tachykinins⁵³⁻⁵⁷.

Our results demonstrated that delivery of hapten or Ag integrated in MNA induced robust CHS whereas inclusion of NK1R antagonists in the MNA exerted potent immunoregulatory effects at the sensitization and effector sites of the CHS. During CHS sensitization, proinflammatory neuropeptides trigger the release of IL-1 β and IL-6, which activate immunostimulatory skin DC that migrate to skin-dLN and initiate effector type-1 biased T cell responses³²⁻³⁶. In addition, in the context of ACD, IL-1 β and IL-6 are required for the induction of IL-17 secretion by activated T cells in the skin and skin-dLN and sustain the inflammatory skin microenvironment that facilitate the recruitment of leukocytes to the skin³². We showed that the NK1R is required for the skin inflammation induced by the hapten of Ag and that MNA administration of hapten and NK1R antagonists minimizes release of IL-1 β and IL-6 in the skin treated area and reduced the number of skin DC homed to skin dLN during the sensitization of the CHS. Thus, co-delivery of hapten and NK1R antagonists, integrated in MNA, prevents the skin inflammation necessary for the development of the adaptive immune response that sustains CD. Importantly, similar findings were obtained in an ex vivo model of human skin, which support the translational benefits of this therapeutic approach for future clinical application.

The cell types of the skin that express the NK1R and contribute to CD have not been previously identified. The lack of in vivo models allowing cell type-specific deletion of the NK1R precluded analyzing the relevance of NK1R-signaling in different skin cells that could be the targets of NK1R antagonist-based therapies for CD. We validated that NK1R is expressed by keratinocytes, skin DC and MC, which are all involved in the pathogenesis of CD^{32, 42, 43}. The use of cell type-specific NK1R^{KO} mice revealed that the NK1R-dependent inflammation triggered by the hapten was mediated mainly by keratinocytes, and that the adaptive immune component of CD required expression of functional NK1R in keratinocytes and DC. Our findings indicate that keratinocytes are early targets of hapten-induced neuroinflammation and they provide the ideal skin microenvironment for the activation of immunostimulatory DC. In addition, our data suggest that signaling DC via the NK1R was necessary for

533 their ability to sustain type-1 immunity an observation that was previously inferred by our laboratories ^{4, 5}.
534 By contrast, in our model, the absence of functional NK1R in MC did not decrease the severity of CD,
535 which seems to agree with previous observations that MC have a rather regulatory role in CHS against
536 potent sensitizers ^{40, 41, 58}.

537 The DC subsets that are affected by cutaneous inflammation differ on their ability to prime and polarize
538 effector T cell responses. Epidermal LC exert a regulatory function ^{20, 59} whereas CD103^{Pos} cDC1 promote
539 type-1 immunity and CD301b^{Pos} cDC2 stimulate type-2 bias ⁶⁰⁻⁶⁵. Using the KikGR mouse model to
540 identify and quantify exclusively the DC from the skin treated area we demonstrated that the three DC
541 subsets transported Ag to the skin-dLN. Interestingly, delivery of NK1R antagonists during sensitization
542 led to a pronounced decrease of dermal DC without altering the influx of LC carrying Ag and IL-10 in the
543 skin-dLN. These data indicate that the immunosuppressive effects of NK1R antagonists in CHS depend at
544 least in part, on an imbalance between stimulatory and suppressive skin DC homed in skin-dLN ^{20, 48}. In
545 addition, our results from in vivo experiments in which LC were depleted confirmed that LC are necessary
546 for the effect of the NK1R antagonist-based MNA immunosuppressive approach. Experiments beyond the
547 scope of this study will determine the individual roles of specific NK1R-signaling in different subsets of
548 skin DC.

549 Abrogation of the immune response in CD requires expansion of Treg and deletion of hapten-specific
550 effector T cells ^{7, 66-72}. Here, we provide in vivo evidence that the NK1R antagonist-based
551 immunosuppressive approach for CD increased the percentage of nTreg and promoted cell deletion and
552 reduced IL-17A and IFN- γ -content in Ag-specific activated T cells within the skin-dLN. Likewise, this
553 approach reduced the number of effector T cells and increased the quantity of Treg at the skin elicitation
554 site, resulting in defective effector CHS response.

555 Although our data requires further confirmation in clinical trials, relevant for its clinical applicability,
556 the immunosuppressive MNA developed here prevented the relapse of established CD, locally and

systemically, in an Ag-specific fashion. Importantly, our findings in human skin support the translational relevance of this immunosuppressive approach.

In summary, our results validate the concept that systemic immune regulation is achievable through localized control of skin neuro-immune networks to treat CD and likely other skin diseases mediated by type-1 biased immune responses.

577 **AUTHOR CONTRIBUTIONS:** **Mohna Bandyopadhyay**, conducted in vitro and in vivo experiments,
578 analyzed data, and contributed with manuscript writing. **Adrian E. Morelli**, planned and conducted in vitro
579 and in vivo experiments, analyzed data, contributed with manuscript writing and intellectual input.
580 **Stephen C. Balmert**, performed and analyzed experiments with human skin samples. **Nicole L. Ward**,
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FIGURE LEGENDS

Fig 1. Efficient CHS requires NK1R, SP and HK1. A) Skin CHS to DNCB by ear thickness increase in WT and NK1R^{KO} B6 mice. **B)** Microscopic images of the ears of mice in (A) showing epidermal-dermal thickness (lines), hyperkeratosis, parakeratosis, acanthosis and leukocyte inflammatory infiltrate. **C)** Skin CHS to DNCB, in WT, *Tac1*^{KO}, *Tac4*^{KO} and *Tac1/Tac4*^{KO} B6 mice. **D)** Representative microscopic images of mouse ears in (C). **E)** CHS to DNCB in *Tac1/Tac4*^{KO} B6 mice injected i.d. with SP or HK1, or SP and HK1 during sensitization. **F)** Microscopic images of the ears of mice in (E) showing epidermal-dermal thickness (lines) and leukocyte infiltrate. In (A), (C) and (E), representative experiment of 3. (A) Six mice per group, (B) and (C) 5 mice per group. Means $\pm$ 1 SD analyzed by 1-way ANOVA followed by Student-Newman-Keuls test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS: not significant. Statistic comparisons among values obtained 48 h following ear skin elicitation are depicted. In (B), (D) and (F), images are from samples obtained 48 h after elicitation. H&E, X200, scale bars, 20 μ m.

Fig 2. Generation and testing of MNA integrating hapten and NK1R antagonists. A) Three-dimensional design of MNA and geometric parameters. **B)** Stereomicroscopy images of the microneedles before (left) and after (right) application to mouse ears. Scale bars, 200 μ m. Center, MNA-delivered cargo traces (dotted line square) on the mouse ear. Scale bar, 1mm. **C)** Live-animal fluorescence images of a mouse treated with MNA on the ear skin, showing delivery of Alexa Fluor-488 (left) and Alexa Fluor-555 (right). **D)** Cryosection of a mouse ear skin showing (left) two areas of MNA penetration (squares), and the corresponding images by fluorescence microscopy showing delivery of Alexa Fluor-488 (green) and Alexa Fluor-555 (red) to skin layers. Scale bar, 40 μ m X100. **E)** CHS induced by sensitization with MNA loaded with DNBS versus DNCB applied topically. Each dot represents one mouse. **F)** Comparative CHS in mice sensitized with topical DNCB plus NK1R antagonists injected i.d. or with DNBS-NK1R^{antagonists}-MNA. In (E) and (F), a representative experiment of 3 is shown, 5 mice per group. Means $\pm$ SD analyzed by one-

way ANOVA followed by Student-Newman-Keuls test. **** $p < 0.001$, ***** $p < 0.0001$, NS: not significant. Statistic comparisons among values obtained 48 h following ear skin elicitation.

Fig 3. DNBS and NK1R antagonists integrated in MNA restrain hapten-induced inflammation in mouse and human skin. A) Inflammation in WT B6 mice (by the ear thickness) after MNA sensitization. One experiment of 5. Five mice per group. **B)** Microscopic images of the ears of mice in (A) showing epidermal-dermal thickness (lines) and leukocyte infiltrate. H&E, X200, scale bars 20 μ m. **C)** Epidermal and dermal thickness of mouse ears in (A). Five mice / experimental group. **D)** Quantification of inflammatory cells in the skin sections of mice in (A). Means $\pm$ 1SD of 20 microscopic high-power fields (HPF, X40) per skin section. A representative experiment of 3. Five mice per group. **E)** Content of IL-1 β and IL-6 transcripts in the ears of mice. **F)** Content of IL-1 β and IL-6 in the ears of mice. In (E) and (F) One experiment of 3. **G)** IL-1 β and IL-6 transcripts in human skin treated with MNAs. Each dot represents a skin sample from a different donor. A representative experiment of 3. **H)** Quantification of DC migrated from the skin samples in (G). One experiment of 3. **I)** Expression of HLA-DR, CD86, PD-L1 and CD40 on DC mobilized from the human skin shown in (G). **J)** Concentration of IL-10 and IL-12p70 in supernatants of the migratory DC in (G). Means $\pm$ 1 SD analyzed by 1-way ANOVA followed by Student-Newman-Keuls test. * $p < 0.05$, ** $p < 0.01$. **** $p < 0.001$, NS: not significant.

Fig 4. Skin CHS requires NK1R signaling in keratinocytes and DC. A) Inflammatory response, by ear thickness increase, in K14-NK1R^{fl/fl}, CD11c-NK1R^{fl/fl}, or Mcpt5-NK1R^{fl/fl} and littermate controls. One experiment of 3. Five mice per experimental group. **B)** IL-1 β and IL-6 mRNA in COCA cells cultured alone or with SarSP. **C)** IL-1 β and IL-6 mRNA content in the ears of K14-NK1R^{fl/fl} mice and littermate controls treated with DNBS-MNA. One experiment of 3. Four mice per experimental group. **D)** CHS effector response in K14-NK1R^{fl/fl}, CD11c-NK1R^{fl/fl}, and Mcpt5-NK1R^{fl/fl} mice treated with MNA. One

835 experiment of 3. Five mice per group. **E)** Quantification of CD4 and CD8 T cells in the ears of K14-
 836 NK1R^{fl/fl} mice and littermate controls in (D) 48 h after elicitation. Numbers are percentages of cells per
 837 quadrant. **F)** Quantification of T cells in the ears of mice in (E). One experiment of 3. Means $\pm$ 1 SD
 838 analyzed by 1-way ANOVA followed by Student-Newman-Keuls test. * $p < 0.05$, ** $p < 0.01$, *** $p <$
 839 0.001 , **** $p < 0.0001$. NS: not significant. In (A) statistic comparisons among values obtained 24 h
 840 following ear skin elicitation are depicted. In (D) statistic comparisons among values obtained 48 h
 841 following ear skin elicitation are depicted.

842

843 **Fig 5. Effect of cutaneous co-delivery of hapten and NK1R antagonists on mouse skin DC. A)**
 844 Populations of mouse skin DCs analyzed by FACS in skin-dLN. **B)** Quantification of percentages (left) and
 845 numbers (right) of skin DCs homing in skin-dLN of KikGR mice treated with MNAs. One experiment of 4.
 846 **C)** Transport of OVA•TR and IL-10 content in DCs mobilized to skin-dLN of IL-10-EGFP-reporter mice
 847 treated with MNAs. In (B and C) Numbers in dot plots are percentages of cells per quadrants. **D and E)**
 848 Numbers of skin DC expressing OVA•TR (D) or with IL-10 content (E), in skin-dLN of mice. One
 849 experiment of 3. **F)** Effect of LC depletion on the inhibition of CHS to OVA assessed by ear thickness.
 850 One experiment of 3. Four mice per experimental group. Means $\pm$ 1 SD were analyzed by 1-way ANOVA
 851 followed by comparison between all means by Student-Newman-Keuls test. * $p < 0.01$, *** $p < 0.001$,
 852 **** $p < 0.0001$. NS: not significant.

853

854 **Fig 6. Co-delivery of hapten and NK1R antagonists via MNA impairs the CHS-T cell response to**
 855 **DNCB. A)** Quantification of cell death in activated CD4 and CD8 T cells in skin-dLN of mouse ears
 856 treated with MNA. One experiment of 4. Each dot represents a sample including 4 auricular LN from 2
 857 mice. Numbers in contour plots are percentages of cells per quadrant. **B)** Percentages of Treg cells in skin-
 858 dLN of mice treated with MNAs. In the diagram on the right, each dot represents a sample containing 4

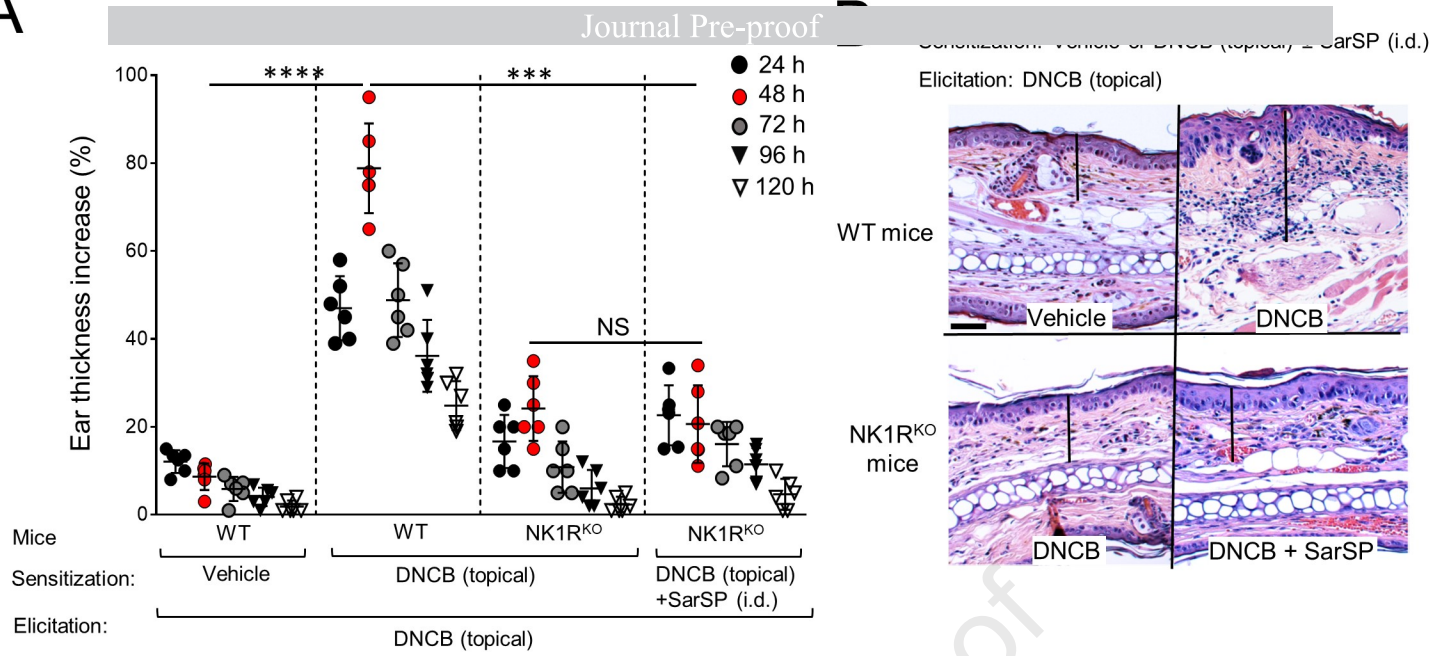
auricular LN pooled from 2 mice. One experiment of 3. **C)** Quantification of CD8, CD4 (Foxp3^{Neg}) and Treg cells in the ear skin of mice sensitized with MNA and elicited with topical DNCB. **D)** Percentage of CD4 (FoxP3^{Neg}) and CD8 T cells expressing IL-17 or IFN- γ at the elicitation site of mice in (C). One experiment of 3. Each dot represents a sample of skin pooled from 2 ears from the same mouse. Means $\pm$ 1 SD were analyzed by 1-way ANOVA followed by Student-Newman-Keuls test. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. NS: not significant.

Fig 7. Co-administration of NK1R antagonists and Ag impairs mouse CHS to OVA and decreases the T cell-stimulatory function of human skin DC. A and B) Cell death in proliferating CD8 OT-I (A) or OT-II cells (B) in skin-dLN of mice sensitized with MNAs. **C)** Side-by-side comparison of the proliferation of CD8 OT-I and CD4 OT-II cells shown in (A) and (B). **D)** Content of IFN- γ in the CD8 OT-I cells shown in (A). **E)** Content of IFN- γ and IL-17 in the CD4 OT-II cells shown in (B). **F)** Percentages of Treg in CD4 OT-II cells of the mice in (B). **G)** Proliferation and cell death of human CD4 and CD8 T cells stimulated with allogeneic DCs migrated from human skin treated with MNA. In the dot diagrams on the right of (A), (B), (D) and (E), one experiment of 3. In dot plots of (A), (B), (D), (E) and (F), numbers are percentages of cells per quadrant. In (G), each dot is a sample of responding T cells from a different mixed leukocyte culture. In (A), (B), (D), (E) and (G), means $\pm$ 1 SD were analyzed by 1-way ANOVA followed by Student-Newman-Keuls test. $**p < 0.01$, $****p < 0.0001$.

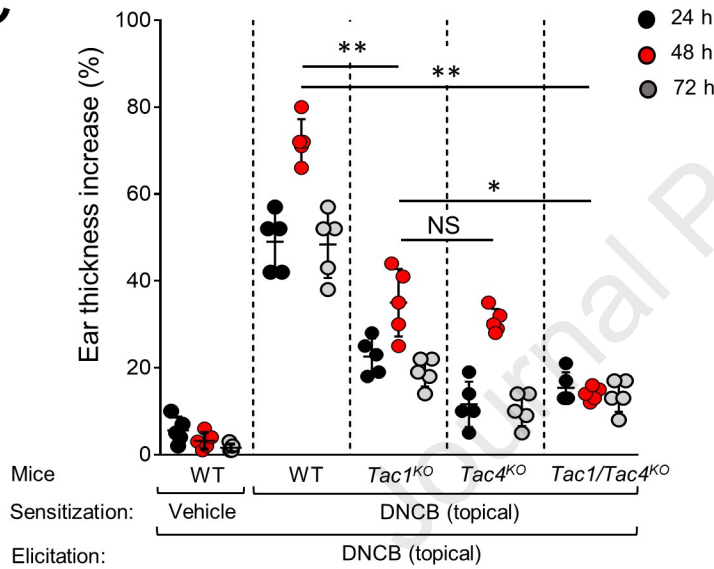
Figure 8. Co-delivery of hapten and NK1R antagonists in the skin desensitizes CD locally and systemically in hapten-specific fashion. A) Timeline describing the experiments of induction and desensitization of CHS in mice. **B and C)** Reduction of established CHS in mice, treated with MNA on day 22 and re-challenged on day 29, with topical DNCB on the treated homolateral (right) ear (B), or on the contralateral (left) ear (C). **D)** Mice with established skin CHS to oxazolone, treated with DNBS-

883 NK1R^{antagonist}-MNA on the homolateral (right) ear on day 22 and re-challenged with topical oxazolone on
884 the same ear on day 29. In (B), (C) and (D), a representative experiment of 3 is shown. Five mice per
885 group. Each dot represents one mouse. Means $\pm$ 1 SD were analyzed by 1-way ANOVA followed by
886 Student-Newman-Keuls test. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. NS: not significant.

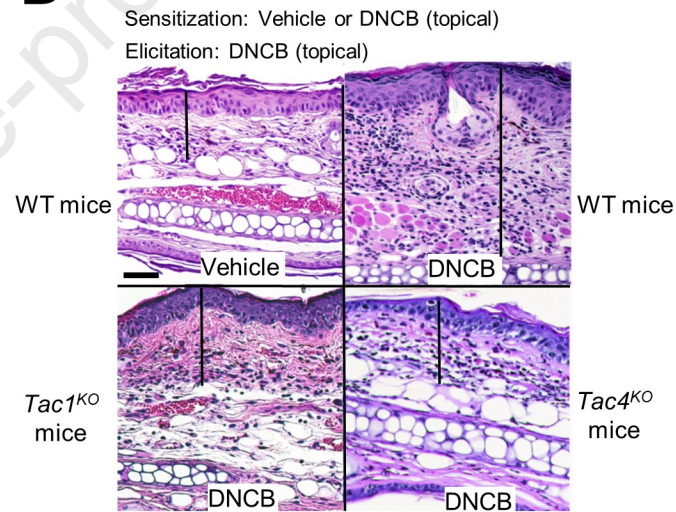
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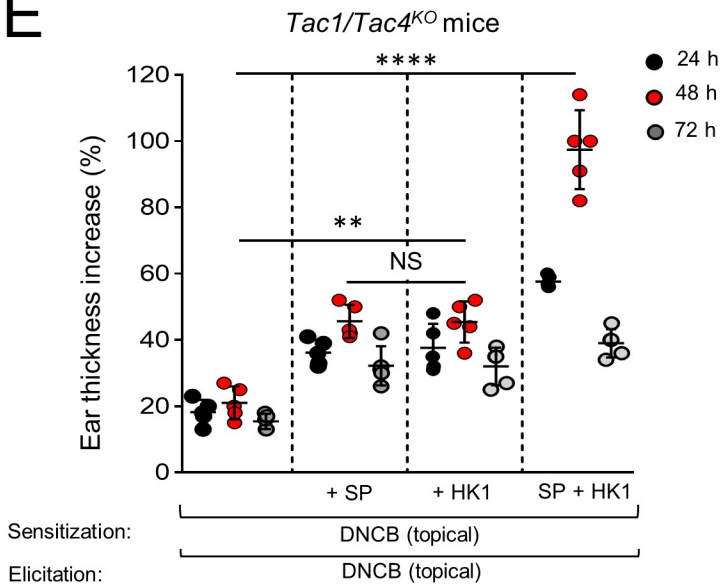
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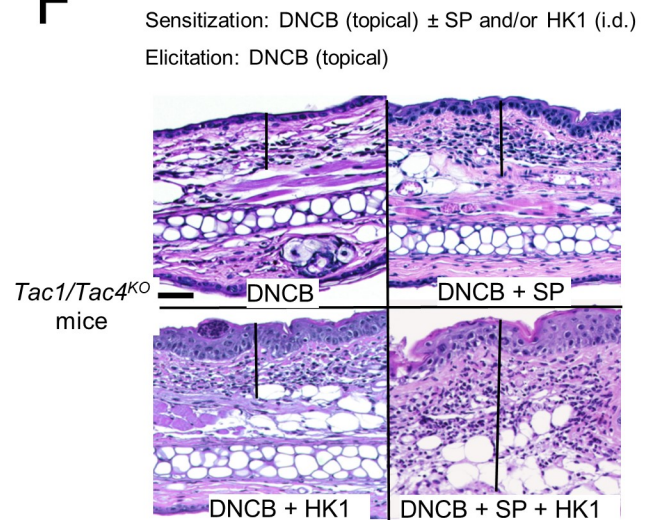


Fig. 1

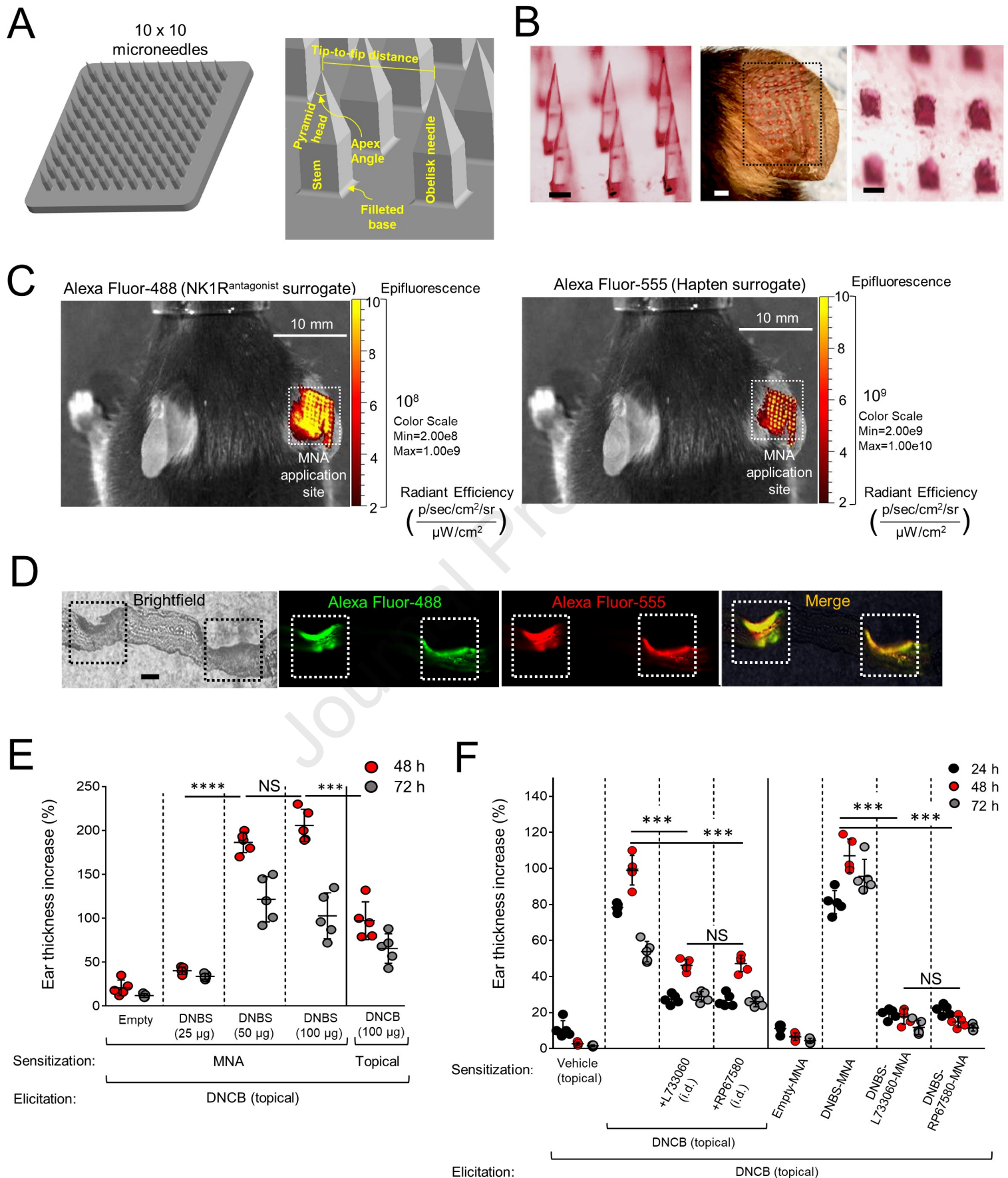


Fig. 2

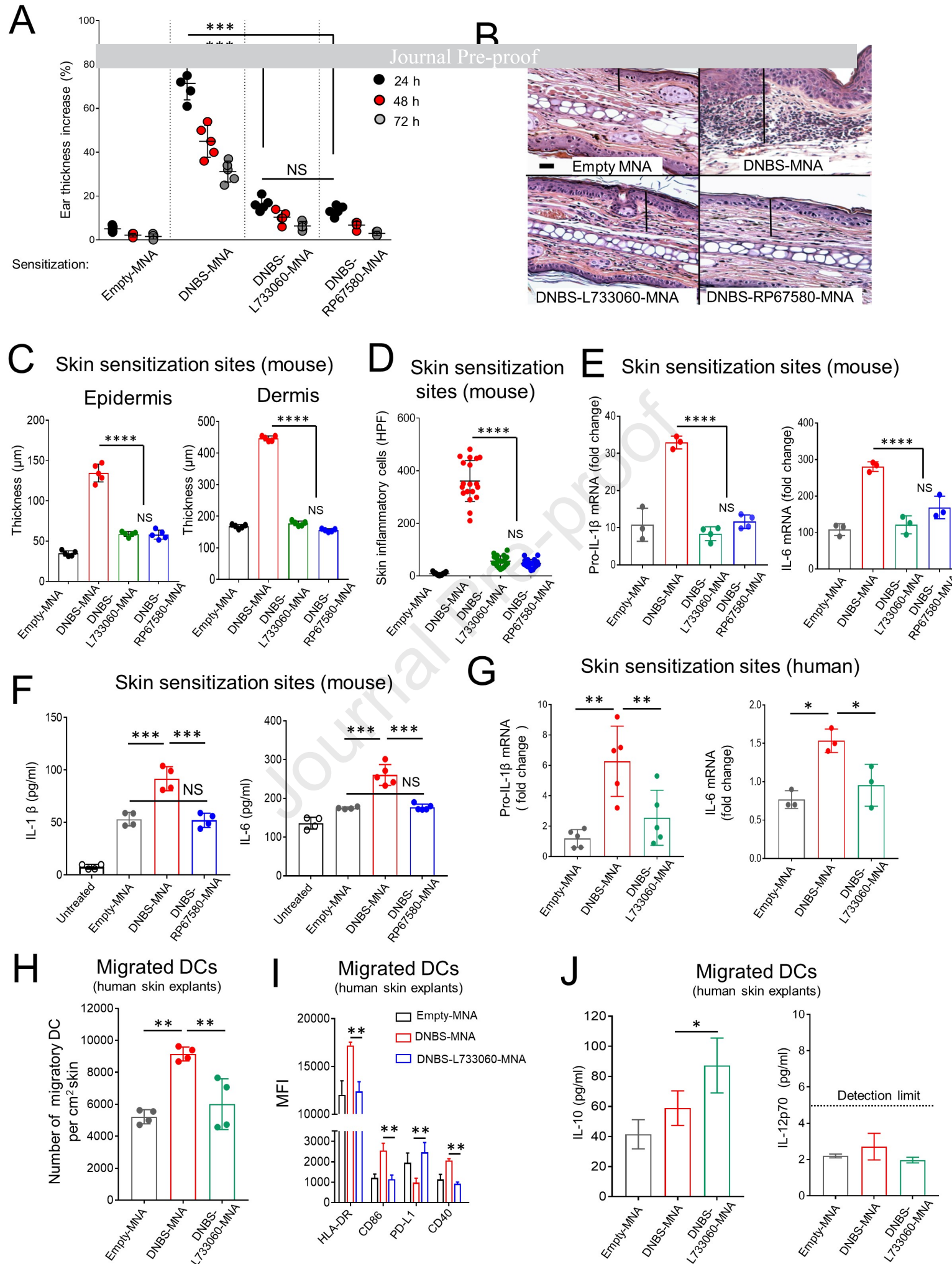
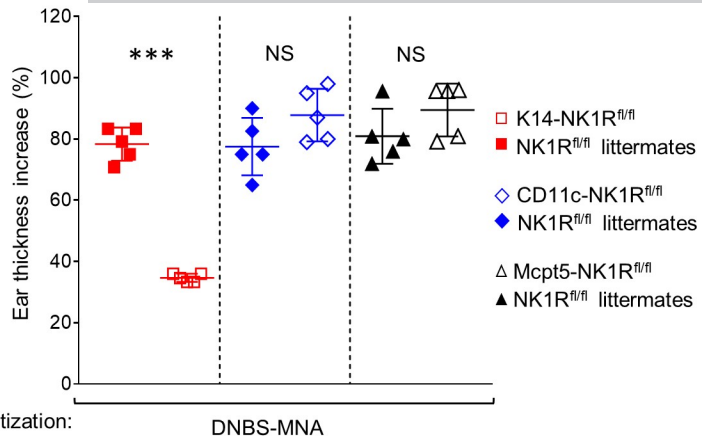
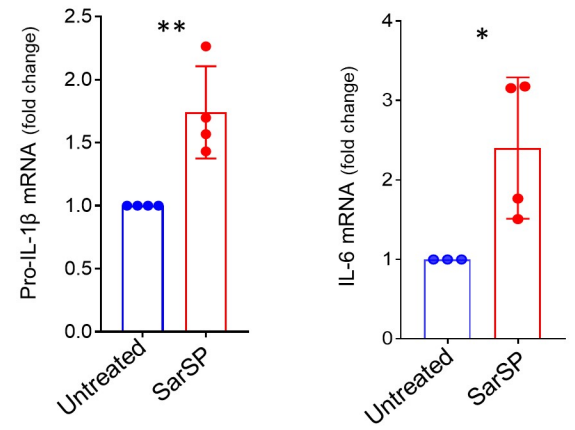


Fig. 3

A

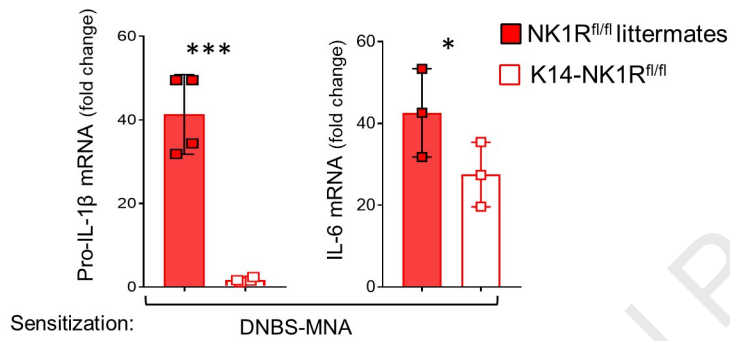


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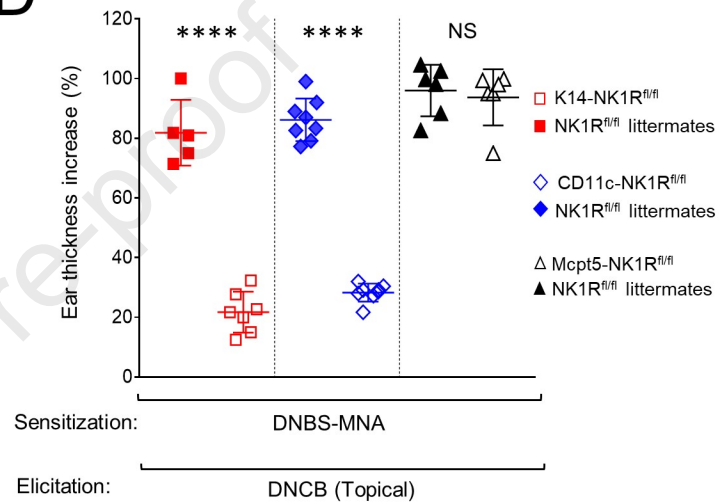


C

Skin sensitization sites (ear skin)

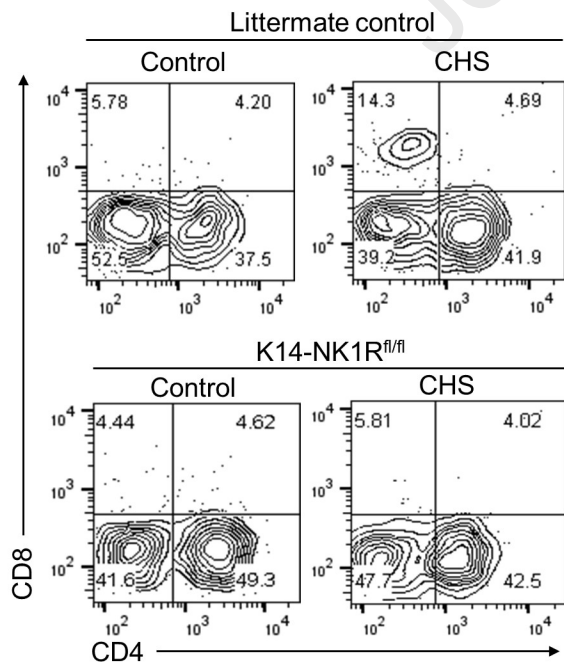


D



E

Skin elicitation sites (ear skin)



F

Skin elicitation sites (ear skin)

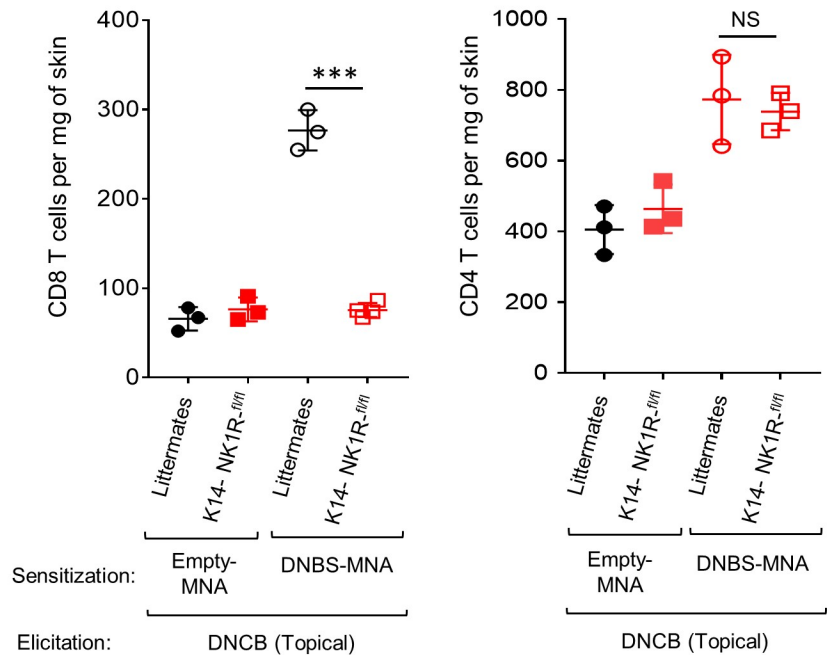


Fig. 4

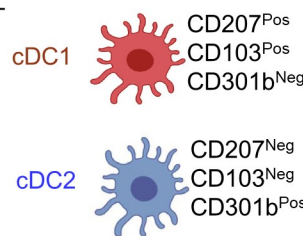
A

Mouse skin DC in draining lymph nodes
(CD11c^{Pos} and MHC-II^{High})

Epidermal DC



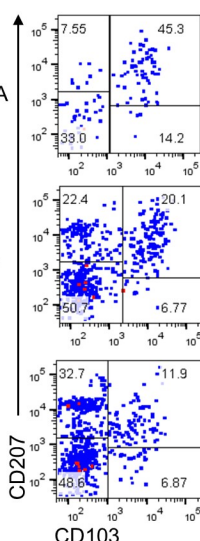
Dermal DC



Empty-MNA

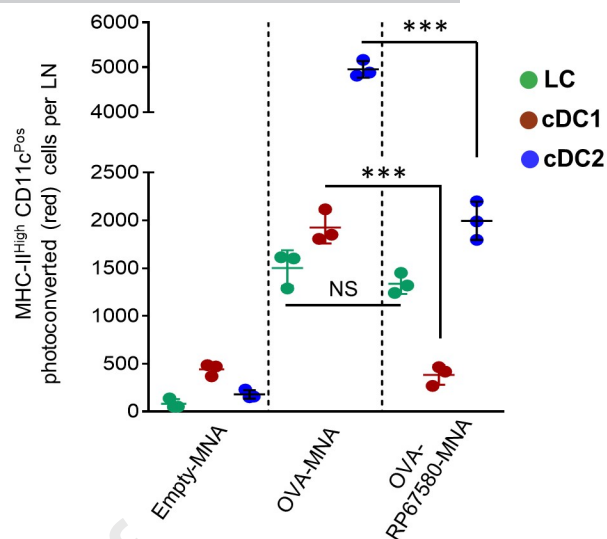
OVA-MNA

OVA-
RP67580
MNA



Journal Pre-proof
photoconverted (red) DC

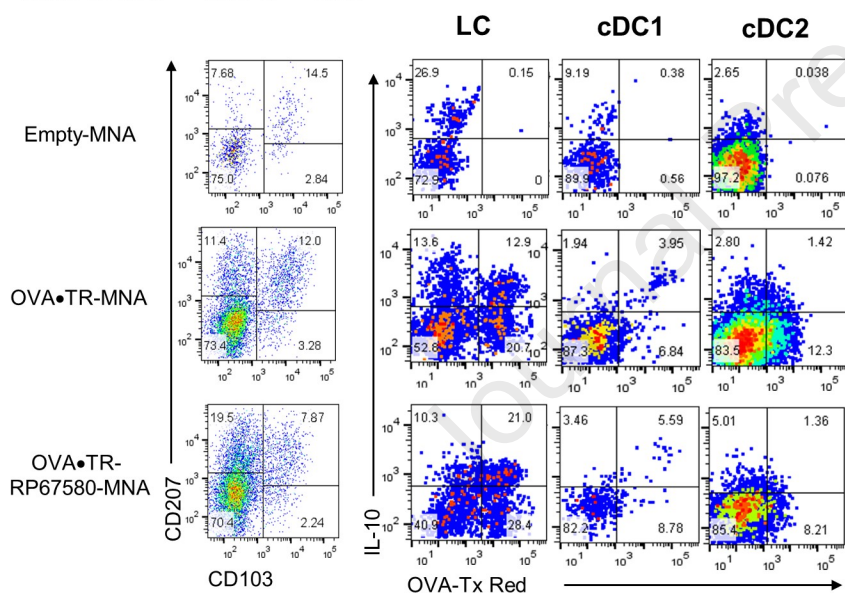
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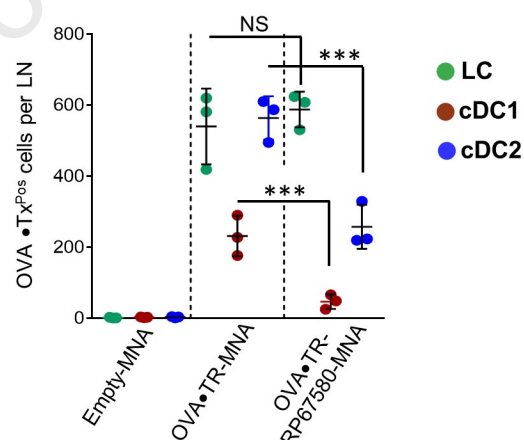
Skin-draining (auricular) lymph nodes

Gate: MHC-II^{High} CD11c^{Pos} cells



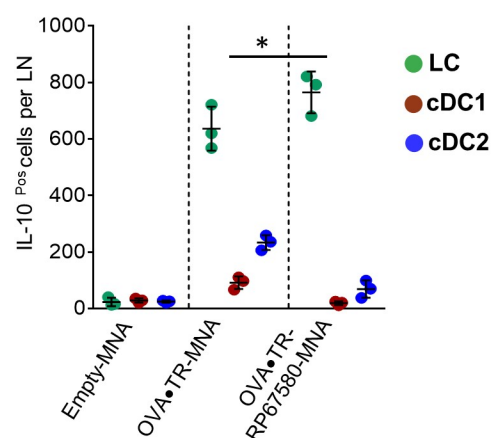
D

Skin-draining (auricular) lymph nodes



E

Skin-draining (auricular) lymph nodes



F

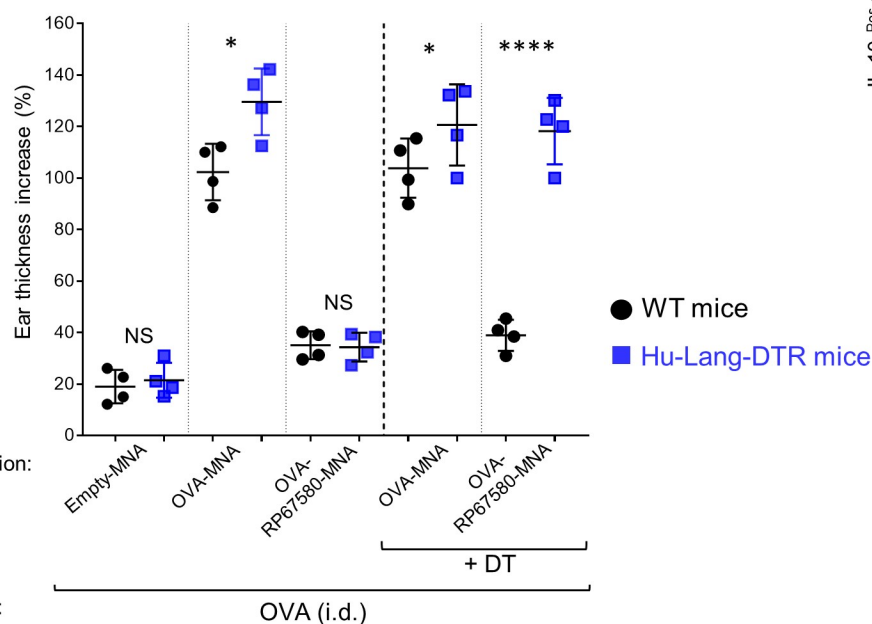


Fig. 5

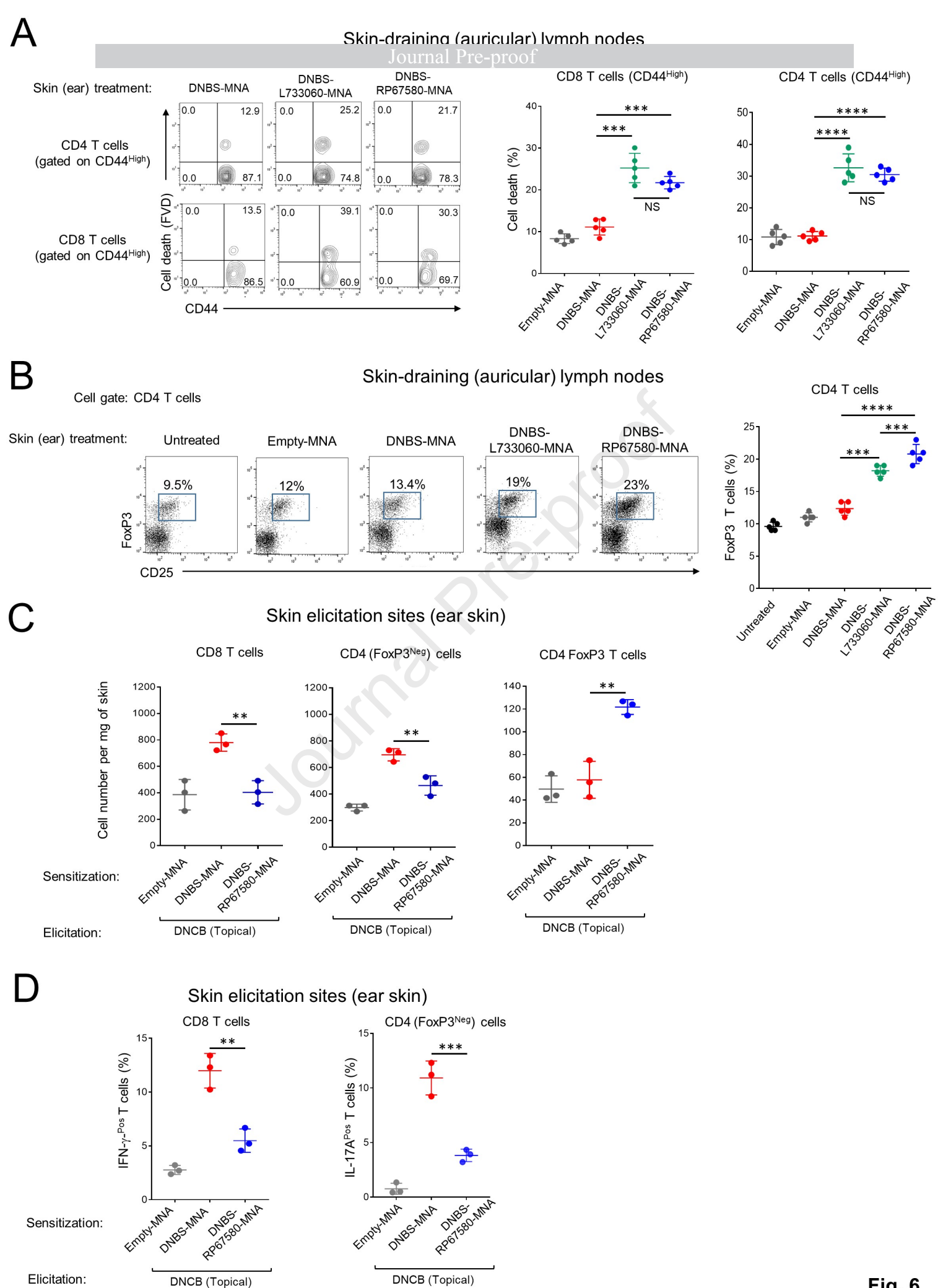


Fig. 6

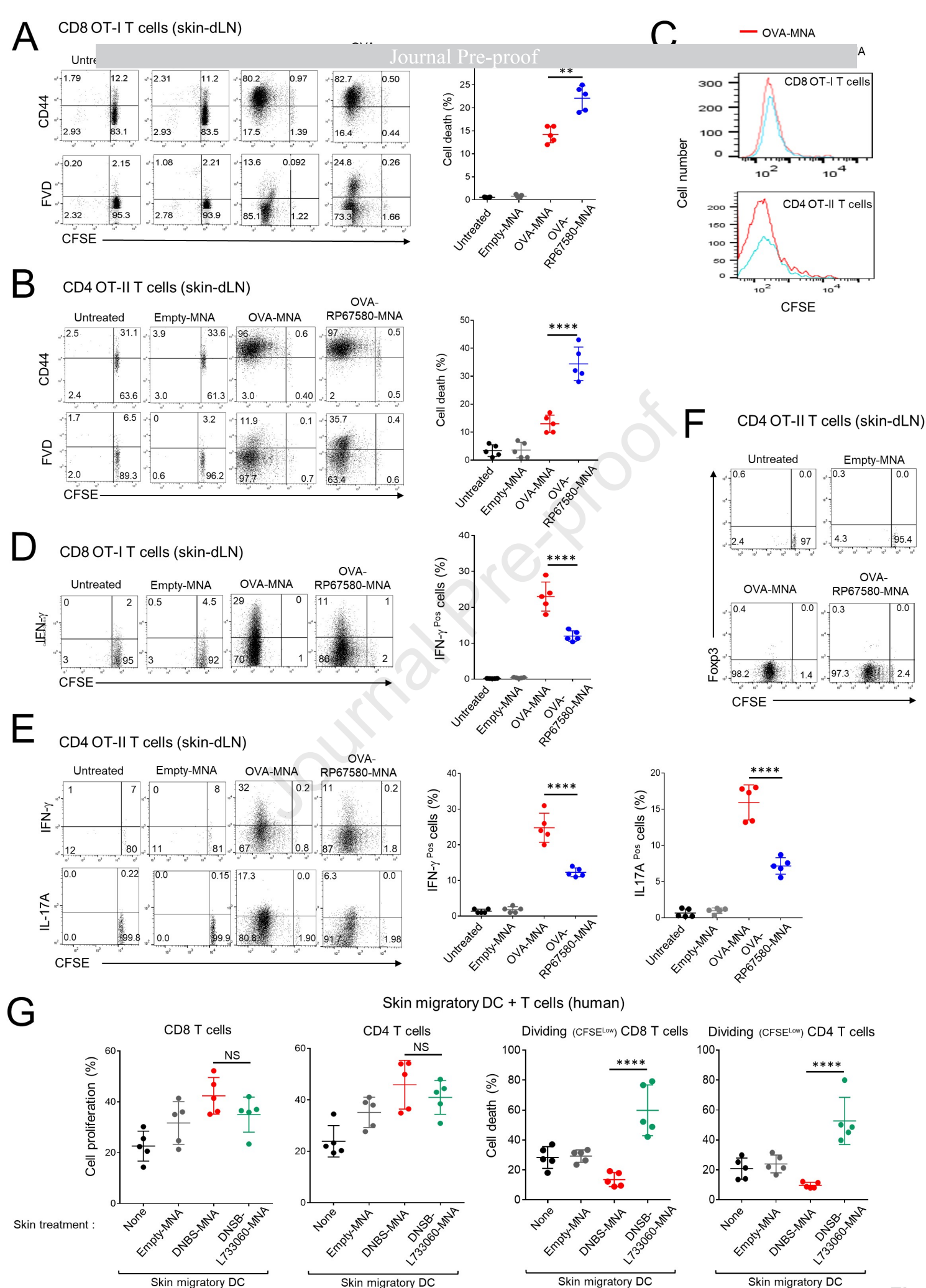


Fig. 7

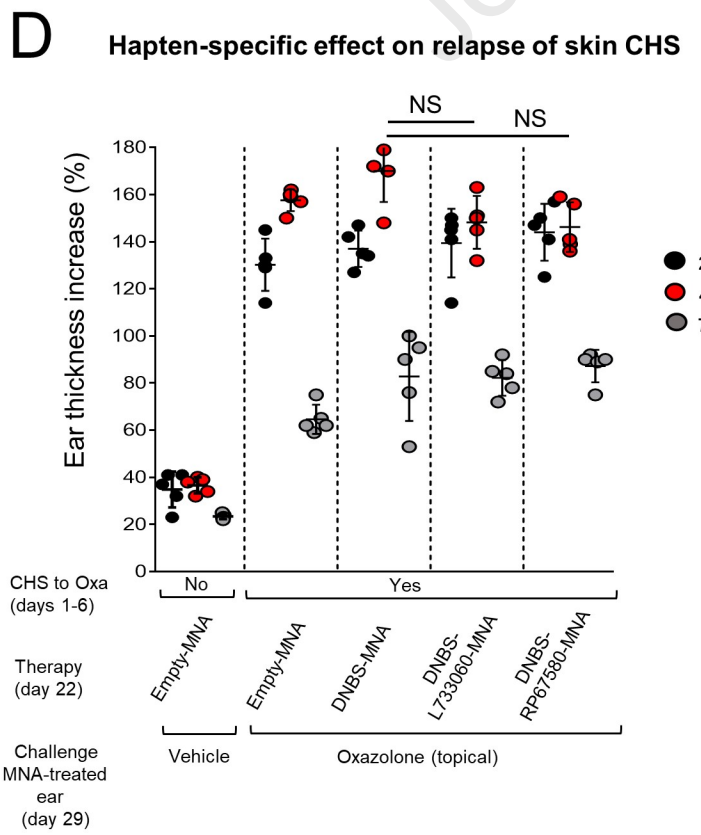
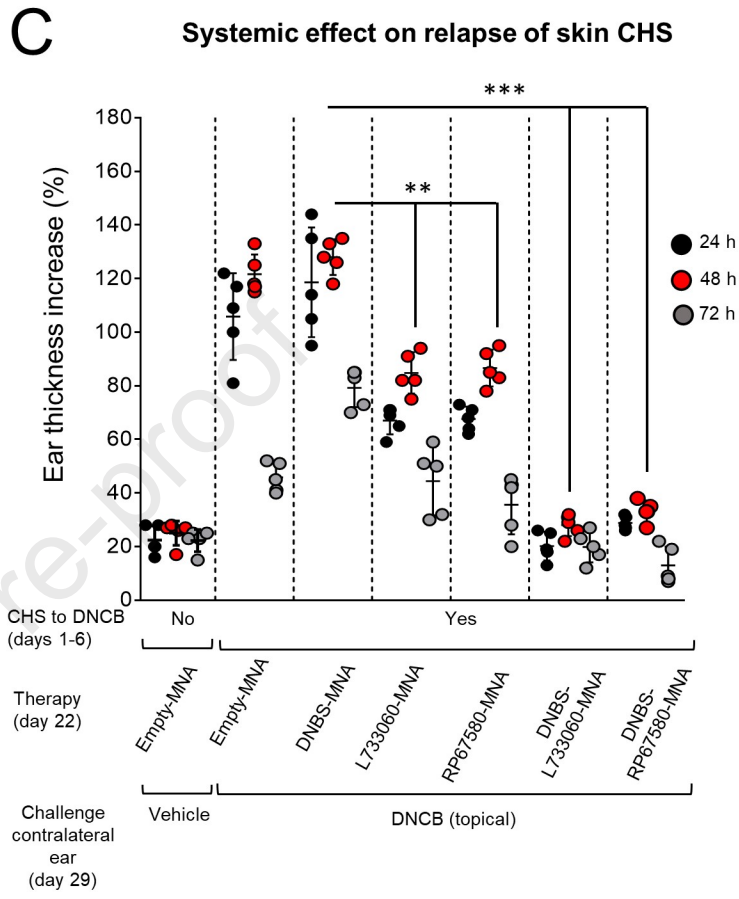
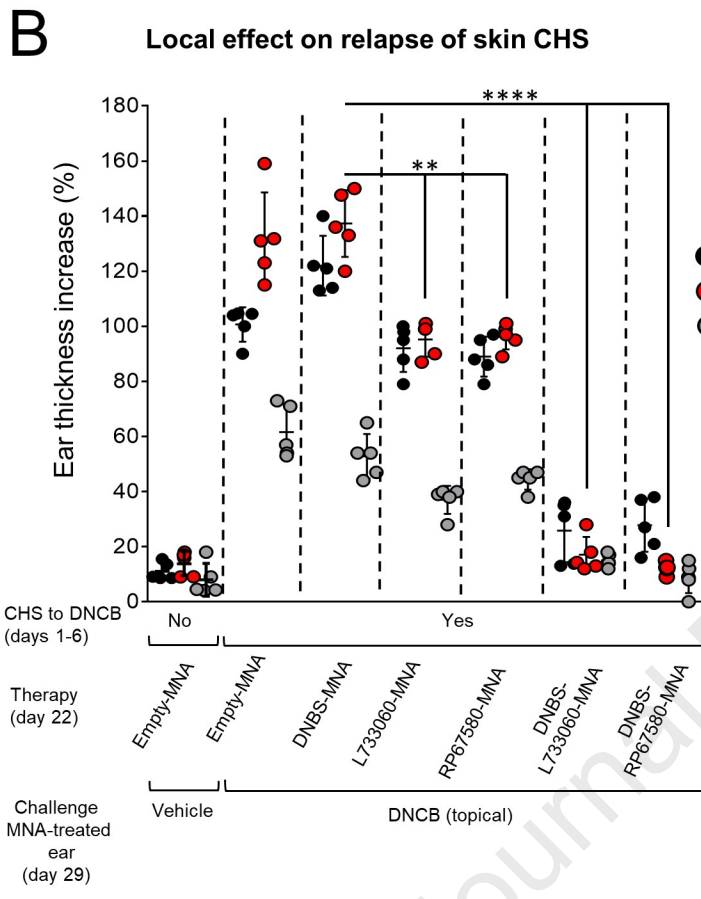
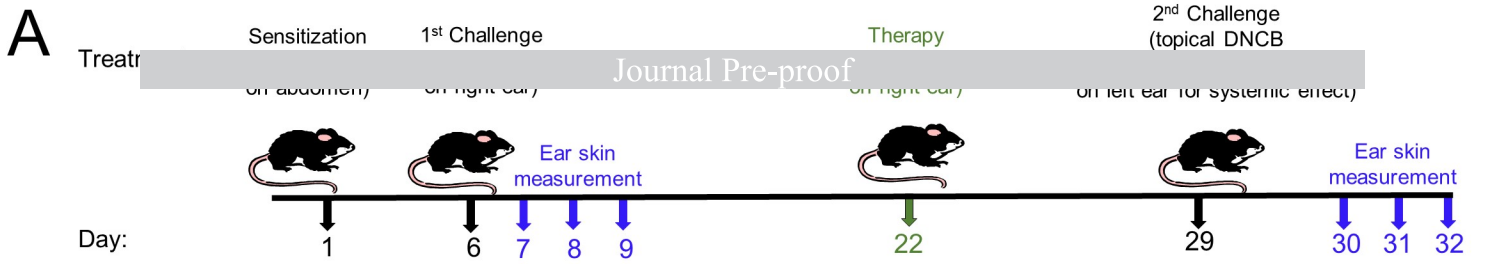


Fig. 8