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ORIGINAL ARTICLE

Autologous Th2-polarized lymphocytes induce atopic dermatitis lesions in non-atopic human skin xenotransplants

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Abstract

Background: The key signals that suffice to induce atopic dermatitis (AD) in human skin remain incompletely understood. Also, current mouse models reflect human AD only unsatisfactorily. Therefore, we have asked whether a humanized AD mouse model can be developed that reflects human AD more faithfully and permit to identify key signals that suffice to induce AD lesions in previously healthy human skin in vivo.

Methods: Healthy human skin from non-atopic donors was transplanted onto SCID/beige mice. After xenotransplant reinnervation by mouse sensory nerve fibers had occurred, mixed autologous human Th2 CD4+ and Tc2 CD8+ T cells that had been pretreated in vitro with IL-2, IL-4, and LPS were injected intradermally into the xenotransplants without skin barrier disruption.

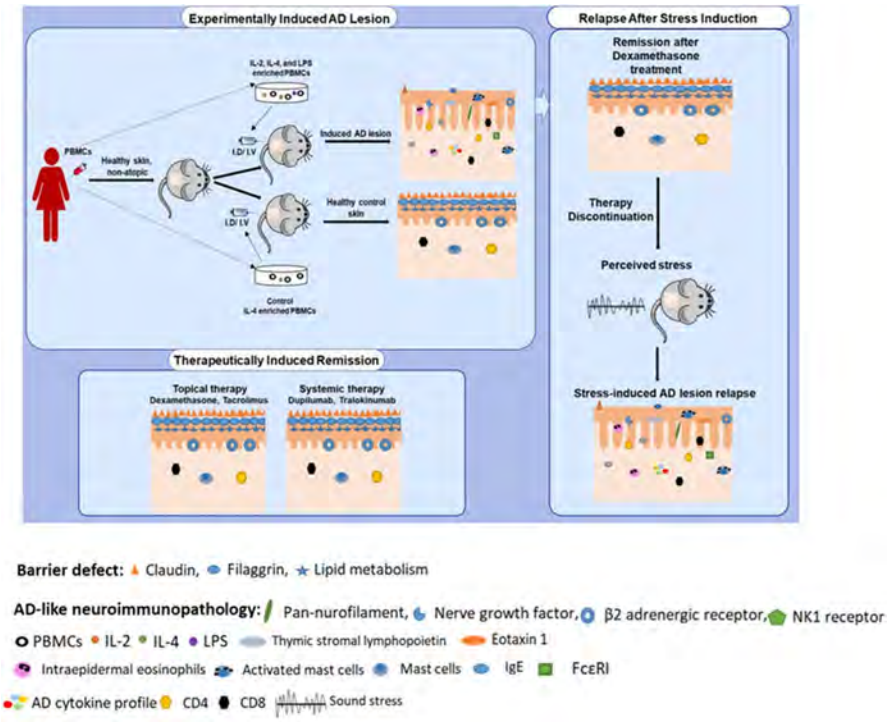
Results: Injected non-atopic xenotransplants rapidly developed a morphological, functional, and immunological phenocopy of human AD lesions regarding skin barrier defects, immunopathology including intraepidermal eosinophils, mast cell activation, increase of thymic stromal lymphopoietin, eotaxin-1 and type 2 cytokine circuits, and even showed characteristic neuroimmunological abnormalities such as ß2-adrenergic receptor downregulation. The experimentally induced AD lesions in human skin responded to standard AD therapy (topical dexamethasone or tacrolimus; systemic anti-IL-4Rα antibody [dupilumab]), and relapsed when neurogenic skin inflammation was induced by exposing mice to perceived stress.

Conclusions: This new animal model uniquely mimics the complexity of human AD and its clinical response to standard therapy and psychoemotional stressors in vivo, and shows that Th2-polarized lymphocytes associated with excessive IL-4Rα-mediated signaling suffice to induce human AD skin lesions, while atopy and epidermal barrier disruption are dispensable.

KEYWORDS
IL-2, IL-4, LPS, perceived stress, SCID mice

Abbreviations: TSLP, Thymic Stromal Lymphopoietin; hBD-2, Human beta –defensin-2; AD, Atopic dermatitis; AFM, Atomic Force Microscopy; DTI, dermal texture index; NGF, nerve growth factor; NK1, neurokinin 1 receptor; Ova, ovalbumin; PAR-2, protease-activated receptor 2; PBMCs, peripheral blood mononuclear cells.

Ralf Paus and Amos Gilhar have contributed equally.



GRAPHICAL ABSTRACT

Healthy human skin xenotransplants on SCID mice are injected with autologous PBMCs pretreated in vitro with IL-2, IL-4 (Th2 polarization), and LPS (to mimic TLR stimulation by *S. aureus*). This elicits characteristic abnormalities in previously healthy skin that strikingly mimic human AD. The model responds to AD therapy and shows AD-like neuroimmunopathology and AD lesion can be retriggered by applying perceived (sonic) stress.

1 | INTRODUCTION

Atopic dermatitis (AD) is one of the most common, as yet incurable chronic inflammatory skin diseases, which occurs in distinct endotypes and shows increasing prevalence.[1,2] Typically, AD exhibits eczematous, pruritic skin lesions that result from a complex interplay between abnormalities in innate and acquired immunity, keratinocyte and epidermal barrier function, skin microbiome dysbiosis, and neurogenic skin inflammation.[1–3] Yet, the key stimuli that suffice to induce AD lesions in previously healthy human skin remain unclear. Therefore, preclinical animal models are urgently needed that permit the identification of key AD-inducing stimuli in previously healthy human skin (a prerequisite for AD prevention and cure), and are reasonably predictive for clinical trial outcomes of candidate therapeutics tested in these models.

However, while currently employed AD mouse models can mimic isolated AD characteristics, overall they are unsatisfactory: They insufficiently reflect the complexity of human AD and its endotypes, including characteristic AD-associated neuroimmunological and neurophysiological abnormalities, and often use clinically irrelevant triggering stimuli (for detailed discussion, see[4]). Moreover, optimal AD mouse models should be predictive for clinical treatment outcomes, should permit to elucidate which key signals suffice to induce AD lesions in healthy human skin in vivo and to trigger AD lesions by psychoemotional (perceived) stress[4]—just as in many AD patients,[5,6] in whom psychological interventions can be beneficial.[7–9] These desirable prerequisites of clinically relevant AD mouse models[4] favor the use of human skin xenografts on immunocompromised mice. These xenografts are rapidly revascularized and sensorily reinnervated by the murine host and retain viable resident human skin immunocytes such as Langerhans, mast cells, and T lymphocytes.[4]

Therefore, we have asked in the current study (a) whether a phenocopy of clinical AD lesions can be induced in previously healthy human skin xenotransplants on SCID/beige mice in vivo by the intradermal injection of appropriately pre-activated, autologous human lymphocyte populations recognized as major drivers of disease, namely Th2 CD4+ and Tc2 CD8+ T cells (Figure 1)[10]; (b) which key signals suffice to induce AD lesions; (c) whether the experimentally induced AD lesions respond to clinical standard therapy and perceived stress associated with neurogenic skin inflammation; and (d) whether atopy and/or skin barrier disruption are indeed required for AD lesion induction, as is often postulated (see discussion in Appendix S1).[2,4,11]

2 | MATERIAL AND METHODS

The overall study design is summarized in Figure 1, and methodological details are provided in the Appendix S1.

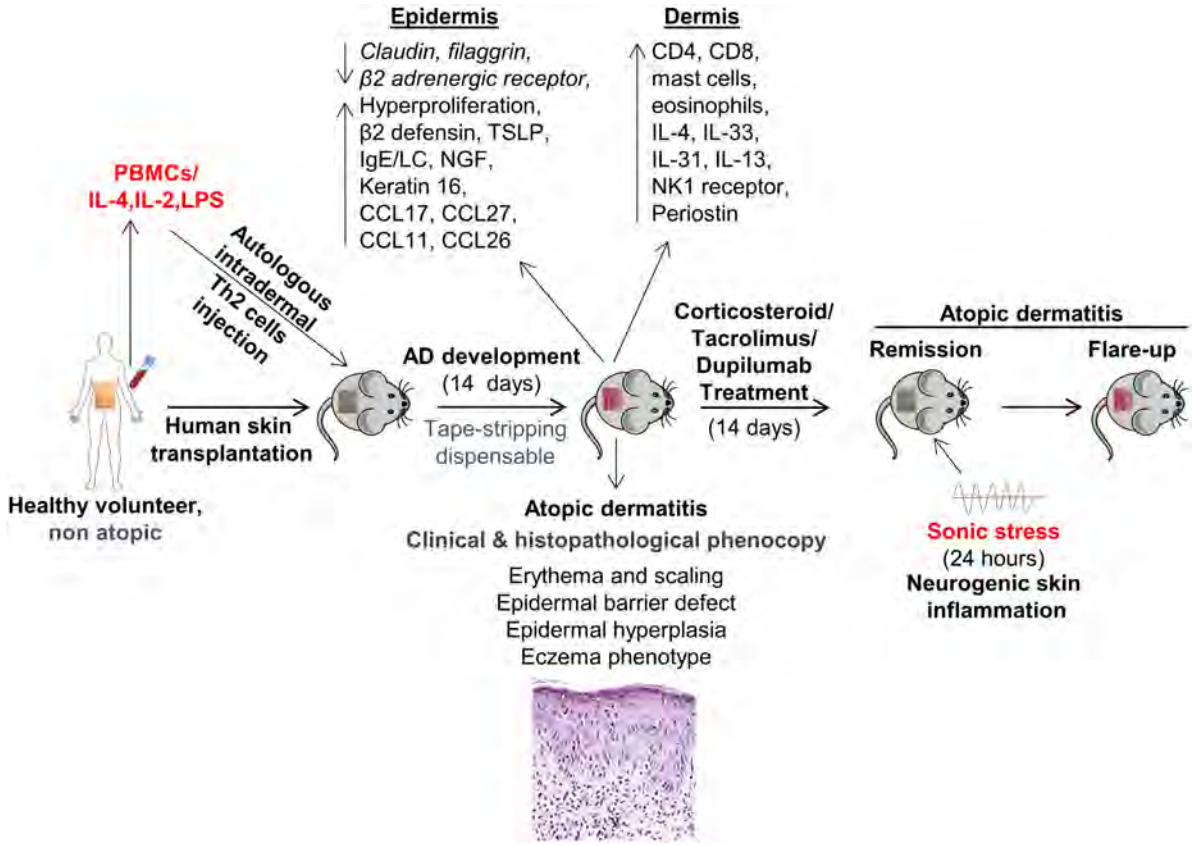


FIGURE 1 Overview of the humanized atopic dermatitis mouse model. Healthy skin specimens are obtained from donors without an atopic background. The skin is transplanted onto the back of a beige SCID mouse, and ~4 weeks after the transplantation, autologous PBMCs from peripheral blood from the same healthy donor are cultured with IL-2 (10 U/mL), IL-4 (200 U/mL), and LPS (1 μg/mL). When 1×10^7 of these Th2-polarized cells are injected intradermally into the human skin grafts, within 14 days, this leads to the development of skin lesions in the xenotransplants that are macroscopically, microscopically, and immunologically indistinguishable from spontaneous human AD. The AD-induced human skin grafts respond to treatment with topical tacrolimus or corticosteroids for 14 days, leading to the normalization of the lesions. Pretreatment of Th2-polarized cells or therapeutic treatment of xenotransplants with dupilumab both prevent and resolve AD lesions, respectively. When mice, whose induced AD lesions had responded to therapy, are exposed to perceived (sonic) stress for 24 h, the AD lesions relapse within 14 days after application of the perceived stressor along with the expected AD-associated read-out parameters, including cell-dependent neurogenic inflammation.

2.1 | Animals

A total of 131 female C.B-17/IcrHsd-scid-bg (beige-SCID, Harlan Laboratories Ltd., Jerusalem, Israel) mice were used and housed under pathogen-free conditions, compliant with institutional guidelines (animal permit: IL-037-03-2014).

2.2 | Patients

Clinically healthy abdominal skin was obtained from 10 healthy female volunteers (aged 46 ± 14 years) who underwent elective surgery and did not have a history of atopic disease, that is, AD, allergic rhinoconjunctivitis, or allergic asthma.[12] Thus, the model can be relatively easily reproduced by independent investigators with access to skin samples from cosmetic surgery of healthy donors. PBMCs were collected from 20 mL of venous blood from the same donor. The study was approved by the Rambam Health Care Campus Institutional Helsinki Committee (0182-14-RMB).

2.3 | AD lesion induction in vivo

Xenotransplantation of healthy human split-thickness skin was performed as described.[13] Briefly, one 1 cm^2/0.4 mm thick skin sample each from nine different, non-atopic human donors was transplanted onto female SCID/beige mice aged 8 weeks. One month after transplantation, that is, when the xenotransplants were fully revascularized and reinnervated,[4,14,15] the xenotransplants were injected intradermally or intravenously with autologous 1×10^7 Th2-polarized PBMCs (isolated from the relevant xenotransplant donor as described before[12]), after these had been pretreated for 14 days in vitro with different combinations of IL-4 (200 U/mL) a prototypical type 2 cytokine,[16] IL-2 (10 U/mL), a key T-cell expansion and activation

factor,[17,18] and lipopolysaccharide (LPS) (Salmonella enterica serotype enteritidis, Merck, 1 μg/mL). LPS was chosen since it is found in the outer membrane of most gram-negative bacteria known to aggravate human AD,[19,20] can trigger human skin inflammation,[21–23] and induces dermatitis in human skin.[22,24,25] In another set of experiment, 1 month following transplantation, the skin was tape-striped six times using a 3 M tape (3 M, USA), following by intradermal injection of 1×10^7 Th2-polarized PMBCs.

The aim was to develop a model based on minimal PBMCs stimulation, yet sufficient to transform healthy PBMCs in vitro into a pathogenic cell type whose intradermal injection, on its own, induces AD lesions. Therefore, the polarized PBMCs were injected together with the supernatant, which contains both IL-2, IL 4, and LPS and additional cytokines secreted by the polarized PBMCs cells.

Table 1 shows the controls that were run. Fourteen days after PBMCs injection, the xenotransplants were photodocumented and harvested for further analysis.

Two additional sets of pilot experiments were also performed:

A. In order to test the functional importance of IL-4 pretreatment in autologous Th2-polarized PBMCs, these were isolated and cultured in the presence of dupilumab (1.4 μg/mL; dupilumab, Sanofi Genzyme) or IgG4 isotype control (1.4 μg/mL; Jackson Laboratories).

Alternatively, autologous PBMCs were conditioned in vitro as before, but in the presence of tralokinumab (1.6 μg/mL/14 days) or of IgG2 isotype control antibody (1.6 μg/mL/14 days).

Following 2 weeks of culture, 1×10^7 cells were injected intradermally into the xenografts of 8 test and 6 control mice for the dupilumab experiment and 6 test and 4 control mice for the tralokinumab experiment, which were harvested 28 days later. For the dupilumab experiment and prior to cell injection, flow cytometric analysis of IL-17 and IL-22 gated on GATA3 was performed.

B. 14 days after injection of Th2-polarized PMBCs, we investigated how established AD lesions responded to three standard clinical AD therapeutics: topical dexamethasone (50 mg/mL), tacrolimus (90 μg), or topical vehicle; subcutaneous dupilumab injection (25 mg/kg).

2.4 | Human skin barrier assessment by nanotexture analysis

As a sensitive biophysical indicator of epidermal barrier function,[26] tape stripping of 8 test and control xenotransplants was performed in order to analyze characteristic AD-associated changes in the nanotexture of corneocytes by Atomic Force Microscopy (AFM).[26]

2.5 | Perceived stress exposure

Atopic dermatitis lesions were induced in human skin xenotransplants as described above and then treated with topical dexamethasone for 3 days, which normalized the human skin xenotransplant clinical phenotype after 4 days. On Day 5 after stopping dexamethasone treatment and 34 days after PBMCs injection, the tested mice were exposed to intermittent sonic stress for 24 h, a well-established model for perceived stress exposure that robustly induces neurogenic inflammation in mouse skin.[27–30] Sham-treated mice were housed in a cage with the sonic stress device turned off. In addition, six mice were exposed to perceived (sonic) stress alone, without the xenotransplants being injected with Th2-polarized PBMCs.

2.6 | IL-4 treatment ex vivo

In order to ask whether type 2 cytokines alone suffice to induce early AD-associated changes in healthy, non-atopic human skin ex vivo, full-thickness human skin was organ-cultured in serum-free medium[31] in the presence of rhIL-4 and rhIL-13 or vehicle for 24–120 h.

2.7 | Quantitative (immuno-)histomorphometry

Skin morphology and epidermal thickness were assessed by routine histochemistry and histomorphometryF. A carefully selected battery of key read-out parameters known to change in human AD lesions[32,33] (see Table S1) was assessed by quantitative immunohistomorphometry in defined reference areas in a sufficient number of xenotransplants and non-consecutive sections.[34,35] This method is best-suited to quantitatively assess multiple different read-outs in a limited number of available human skin xenotransplants and to precisely localize antigen expression patterns in situ.

2.8 | Gene expression analysis

RNA was extracted from snap-frozen human skin xenotransplants or organ-cultured human skin and analyzed by qRT-PCR to determine steady-state transcript levels of *KRT1*, *TSLP*, *TSLPR*, *Involucrin*, *Claudin-1*, *IL1A*, *IL-31*, *IL-13*, *IL-4*, *IL-5*, *CCL17*, *CCL18*, and/or *CCL27* as indicated.

2.9 | Serum IgE levels

Total IgE levels were tested for each donor by Immunochemistry analysis (BN II prospec, Siemens) performed by the Clinical Biochemistry Department at the Rambam Health Care Campus. IgE serum levels were within the normal range in all donors enrolled for the current study. The average IgE level was 124 ± 89 IU/mL (reference range: 1–183 IU/mL).

2.10 | Analysis of ceramides

Tissues were cryo-crushed and then extracted overnight using an organic mixture chloroform/methanol. Several successive extractions

TABLE 1 Upregulation of characteristic AD markers in IL2/4-LPS-PBMC-injected human skin xenotransplants. AD read out parameters of normal skin xenotransplants on day 14 after intradermal injection of Th2 polarized cells as compared to PBMCs treated with IL-4 and normal skin xenotransplants, of PBMCs cultured with ovalbumin, PBMCs cultured with IL-2, with IL-2 and IL-4, PBMCs alone and not injected xenotransplants. Increased epidermal thickness and proliferation combined with decreased expression of filaggrin and claudin-1 in the Th2 polarized cells as compared to PBMCs treated with IL-4. Additionally, increased number of inflammatory cells including CD8,CD4, mast cells, cytokines, IgE and Fc epsilon RI, epidermal TSLP, hBD-2 and β-2adrenergic receptor are onoted in the AD induced xenotransplants. The pooled means were compared with: * student's t-test or § Mann-Whitney U test. Comparisons were made between Th2-polarized PBMCs versusIL-4-treated PBMCs, ova alone, ova cultured PBMCs, IL-2 cultured PBMCs, PBMCs treated with IL-2 andIL-4, PBMCs treated with LPS, untreated PBMCs and non-injected human skin xenotransplants. Except for markers in control groups marked with #demonstrated non-statistical significance versus Th2-polarized PBMCs.

	No' of grafts with histological features of dermatitis total of number mice									
Marker	Th2-polarized PBMCs 11/15	IL-4-treated PBMCs 0/10	Ova alone 0/8	Ova cultured PBMCs 0/4	IL-2 cultured PBMCs 0/10	PBMCs treated with IL-2 and IL-4 0/4	PBMCs treated with LPS 0/5	Untreated PBMCs 0/6	Non-injected human skin xenotransplants (following transplantation) 0/5	*p* value
Mean epidermal thickness (μm)[a]	647 ± 1*	404 ± 8	348 ± 3	323 ± 10	696 ± 25[f]	623 ± 14[f]	388 ± 9	320 ± 87	404 ± 55	*<.03
Epidermal markers										
Proliferation index (%)[b]	67 ± 10*	17 ± 3	11 ± 3	N.A[e]	62 ± 18[f]	71 ± 23[f]	9.8 ± 6	N.A[e]	13.3 ± 1.5	*<.02
Filaggrin (%)[c]	32 ± 17*	88 ± 4	75 ± 9.5		73 ± 11	79 ± 16	72 ± 8.5		87.6 ± 1.9	*<.01
Claudin (%)[c]	22 ± 7*	60 ± 28	N.A[e]		54 ± 8	N.A[e]	N.A[e]		N.A[e]	*<.05
Dermal cells[d]										
CD4	13 ± 5[§]	0.6 ± 0.1	0.4 ± 0.5		19 ± 8	17.5 ± 11	0.8 ± 0.7		0.2 ± 0.4	[§]<.05
CD8	14 ± 3[§]	1 ± 0.2	0.6 ± 0.6		21 ± 14	17 ± 9[f]	0.7 ± 0.7		0.1 ± 0.3	
Mast cells[d]										
Tryptase	14 ± 5.9*	7 ± 1	8.2 ± 2.1		N.A[e]	N.A[e]	7.5 ± 3.8		4.9 ± 1.3	*<.05
C-kit	28 ± 8[§]	12 ± 1	10 ± 2.7		26 ± 12[f]	N.A[e]	9.7 ± 2		6.75 ± 1.2	[§]<.001
Toluidine blue	8 ± 2*	3 ± 2.5	N.A[e]		N.A[e]	N.A[e]	N.A[e]		N.A[e]	*<.05
Cytokines[d]										
IL-33	8 ± 2*	0.3 ± 0.1	0.5 ± 0.7		1.8 ± 1.3	1.5 ± 0.9	0.5 ± 0.7		0.1 ± 0.3	*<.05
IL-4	5 ± 3[§]	0.25 ± 0.1	0.4 ± 0.6		0.9 ± 0.4	1 ± 0.3	1 ± 0.8		0.4 ± 0.5	[§]<.001
IL-22	8 ± 2.5*	1 ± 0.2	0.3 ± 0.7		10 ± 8[f]	N.A[e]	0.3 ± 0.5		0.8 ± 0.7	*<.001
IL-17	5 ± 1.2*	0.2 ± 0.1	1 ± 0.8		12.5 ± 6	N.A[e]	0.25 ± 0.4		0.1 ± 0.4	*<.01
IFN-γ	3 ± 2*	0.2 ± 0.1	N.A[e]		N.A[e]	N.A[e]	N.A[e]		N.A[e]	*<.001
Allergy markers[d]										
TSLP	37 ± 12*	9.6 ± 0.9	N.A[e]		17 ± 11	18.5 ± 13	N.A[e]		N.A[e]	*<.01
Eosinophils	5 ± 2.4*	0.4 ± 0.2	N.A[e]		N.A[e]	N.A[e]	N.A[e]		N.A[e]	*<.01
IgE	6.5 ± 3[§]	1 ± 1	1 ± 0.8		1.8 ± 0.7	2.4 ± 1.3	1.2 ± 1.1		0.7 ± 0.9	[§]<.001
Fc epsilon RI	17 ± 4*	4.7 ± 1.8	4.2 ± 3		9.4 ± 5.1	10.2 ± 3.3	5.2 ± 1.2		4 ± 0.7	*<.05
Langerhans cells[d]										
CD1a	7 ± 2.4*	2 ± 0.4	N.A[e]				N.A[e]		N.A[e]	*<.001
Antimicrobial peptides[d]										
HBD2	42 ± 18*	14 ± 8	2.4 ± 1.7				1.9 ± 1.4		3.3 ± 0.5	*<.01
β2-adrenergic receptor[d]	28.4 ± 7.4*	14 ± 4.6	6.8 ± 2.6				5.7 ± 2.3		6.6 ± 1.4	*<.001

[a]Only responder grafts (73%) were analyzed.

[b]Proliferating epidermal basal cells (% of total basal cells).

[c]Staining intensity (% as calculated by Image J software).

[d]Number of immunoreactive cells per 0.66 mm^2.

[e]Not analyzed.

[f]Non-statistical significance versus Th2-polarized PBMCs.

were carried out to purify the extract. A solid/liquid extraction was subsequently conducted. Ceramides fraction was then evaporated to concentrate the lipidic residue. Residue was resuspended in a mixture of chloroform/methanol. Ceramides were analyzed using an UltiMate 3000 liquid chromatography system (ThermoScientific, Sunnyvale, CA) coupled to a ISQ Mass detector (ThermoScientific, Sunnyvale, CA). Mobile phases [M1, methanol/water (50:50, v/v)] and [M2, methanol/isopropanol (80:20, v/v)] were eluted at a flow rate of 0.4 mL/min. Injection volume was 40 µL, and column temperature was maintained at 40°C. For MS detection, atmospheric pressure chemical ionization (APCI) was used as the ion source.

Statistical analysis was performed as indicated in the Appendix S1 and figure legends.

3 | RESULTS

3.1 | IL-2, IL-4, and LPS polarize healthy human PBMCs towards a Th2 phenotype in vitro

Given that Th2 cells are intimately linked with the acute phase of AD,[36] we first aimed to polarize autologous human PBMC-derived T cells isolated from healthy, non-atopic donors in vitro towards a mixture of T cells that prominently included CD4+ and Tc2 cells with a Th2 phenotype[10] by exposing them to different combinations of IL-4,[16] IL-2,[17,18] and LPS. FACS analysis demonstrated that the majority of T cells ($CD3^+$) from PBMCs cultured for 2 weeks without cytokines or only with IL-4 failed to express the hallmark Th2 transcription factor GATA3[37,38] (Figure S1A,B). Instead, after conditioning with IL-2, IL-4, and LPS, the CD3-gated cells prominently expressed GATA3, along with the Th2-associated chemokine receptor CCR4 and IL-4, IL-13, IL-5, IL-10, IL-22, and IL-17, that is, key cytokines involved in AD pathogenesis,[2,3,39–41] as measured by intracellular immunostaining (Figure S1A,B), attesting to their Th2-polarization. Incubation with IL-4 alone only modestly increased the concentration of IL-4, IL-13, IL-10, and IFN-γ in the supernatants of conditioned PBMCs, while IL-4 + IL-2 + LPS robustly induced secretion of these type 2 cytokines (ELISA, Figure S1C).

3.2 | Autologous Th2-polarized PBMCs from non-atopic donors promote AD lesions in healthy human skin xenotransplants in vivo

Next, we tested whether autologous Th2-polarized PBMCs were able to promote AD lesions in healthy human skin xenotransplants in vivo. As females have a higher AD incidence,[42] we only used skin and autologous PBMCs from healthy, non-atopic female volunteers with normal levels of IgE in peripheral blood and female SCID/beige mice. After transplantation, each human skin xenograft was intradermally (ID) injected once with 1×10^7 Th2-polarized, autologous PBMCs 4 weeks after transplantation. PBMCs pretreated only with IL-4 served as the core negative control (additional controls: see Table 1).

In another experiment, each mouse grafted with human skin was intravenously injected once with 1×10^7 Th2-polarized, autologous PBMCs 4 weeks after transplantation. These lesions strikingly mimicked the key AD readouts, including filaggrin and claudin-1, TSLP, keratin 16, Ki-67, TARC, CTCAK, Eotaxin-1 and 3. Periostin, CD8, mast cell (tryptase, c-kit), IgE and FcεRI cells, IL-4+, IL-22+, IL-33+ and IL-13+ cells (Figures S2 and S3) as compared to PBMCs pretreated only with IL-4 served as a negative control.

The majority (73%) of all xenotransplants injected ID with Th2-polarized PBMCs and 50% xenotransplants of the IV injected mice (3/6 xenotransplants), developed skin lesions that clinically and histologically closely mimicked the characteristic features of human AD lesions[4] at both, 14 or 42 days after cell injection, which was not seen in the xenotransplants injected with control PBMCs at either (Figure 2A,B and Figure S4), indicating that ID injections are more efficient in our model. Therefore, the experimentally induced lesions in previously healthy human skin are no transitory phenomenon, but persist relatively long in vivo. Notably, 42 days after PBMCs injections all injected xenotransplants showed AD lesions (see Figure S4). Macroscopically, responder grafts showed the erythema associated with epidermal hyperplasia, hyperproliferation, and increased keratin 16 protein expression (Figure 2A–C) expected of human AD lesions.[32,43–45] Th2-polarizing conditions (i.e., the combined in vitro preconditioning of PBMCs with IL-2, IL-4, and LPS) were necessary for AD lesion induction in vivo (Table 1). Since the IgE level of each donor was not elevated, correlations/associations of donor serum IgE levels with the few "non-responder" mice that did not develop AD lesions in the xenotransplants after injection of autologous PBMCs, could not be detected.

Although an increase in epidermal thickness was also observed in all xenografts injected with PBMCs conditioned only with IL-2 (Table 1), this did not induce the full range of AD-associated epidermal, cellular, and cytokine biomarkers induced by Th2-polarized PBMCs (Table 1). Additionally, when mice were treated with autologous PBMCs injections as above, that is, without tape-stripping of the epidermis, neither the number nor the quality or the time course of AD lesions differed from the in vitro-polarized PBMCs plus tape-stripped treated mice (Figure S5).

Importantly, atopy does not appear to be a prerequisite for AD lesion induction in healthy human skin by Th2-polarized autologous PBMCs.

3.3 | Induced AD lesions show the expected skin barrier defect, but primary barrier disruption is not required for AD lesion development

The expression of the key epidermal barrier proteins, filaggrin[46] and claudin-1,[47] is decreased in the epidermis of most AD patients due to local overproduction of type 2 cytokines.[48] Indeed, both

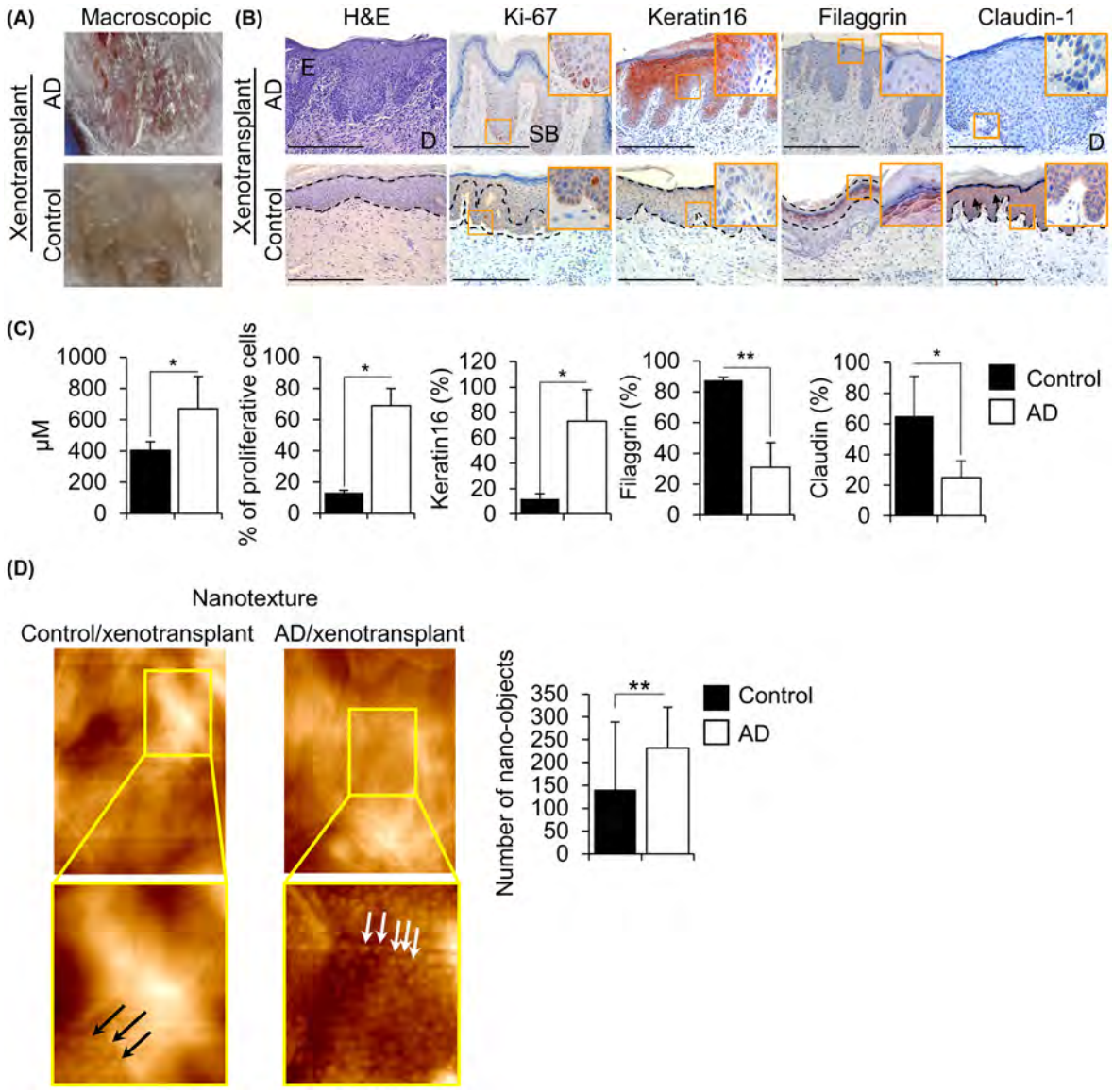


FIGURE 2 AD skin lesions are characterized by keratinocyte hyperproliferation and barrier dysfunction. (A–C) Macroscopic, histological, and immunohistochemical features. (B, C) Epidermal thickness ($N = 14$ mice) was determined by hematoxylin stain and eosin, and the intradermal expression of Ki-67 ($N = 14$ mice), keratin 16 ($N = 9$ mice), filaggrin ($N = 10$ mice), and claudin-1 ($N = 12$ mice) was analyzed by IHC from 3 independent donors. Four areas were evaluated per section, and 3 sections were analyzed per mouse. (D, E) Analysis of circular nano-objects protruding (CNOs) from the cell surface, whose average density is given as Dermal Texture Index (DTI) ($N = 4$ mice injected with Th2-polarized PBMCs and 4 control mice from 2 independent donors); 17 images were analyzed. (A, B) Representative images showing reference areas for the evaluation and (C, E) the pooled means of the indicated parameters. The pooled means were compared with Student's *t*-test: $^{*}p < .05$, $^{**}p < .01$. Scale bars: 50 µm. E: epidermis, D: dermis.

filaggrin and claudin-1 were significantly down-regulated in the epidermis of AD-induced xenotransplants compared to control grafts (Figure 2B,C), suggesting an impaired skin barrier function.

Because transepidermal water loss (TEWL) measurement[49] could not be performed with standard equipment due to the small size of the human skin xenotransplants, we used atomic force microscopy (AFM) to quantify characteristic AD-associated changes in the corneocyte nanotexture, a very sensitive biophysical as a surrogate marker for human epidermal barrier integrity, namely in atopic skin.[50] This showed a significantly different dermal texture index (DTI) and an increased number of nano-objects in AD-induced compared to control xenotransplants (Figure 2D,E), thus mimicking the changes in corneocyte nanotexture seen in spontaneous human AD skin lesions.[50]

Thus, our new humanized AD mouse model recapitulates key epidermal barrier defects observed in AD patients. However, our new humanized AD mouse model may best reflect disease development in those AD patients in which the epidermal barrier shows no primary defect prior to lesion development (e.g., in AD patients without a constitutive filaggrin mutation) and where no atopy is present.[51,52]

3.4 | Experimentally induced AD lesions recapitulate the characteristic TSLP, chemokine, and cytokine milieu of spontaneous human AD

Keratinocyte-derived mediators, such as thymic stromal lymphopoietin (TSLP) and its dermal inducer, periostin, contribute to the initiation and amplification of the Th2 response in AD.[53–55] In addition, chemoattractant antimicrobial peptides[56] such as human β-defensin 2,[57] the chemokines TARC (CCL17) and CTACK (CCL27)[33] and eotaxin-1 and -3[58] are significantly upregulated in the epidermis of AD patients. Therefore, it is important to note that quantitative immunohistomorphometry revealed that the epidermal or dermal eprimary.

Expression of all of these AD biomarkers was significantly increased in the induced human AD lesions in vivo compared to control xenotransplants (Figure S6A,B). These quantitative in situ protein expression data were independently confirmed by qRT-PCR analysis of mRNA extracted from entire xenotransplants; this documented significantly increased *TSLPR*, *CCL17*, and *CCL18 mRNA* steady-state levels in AD-induced compared to control xenotransplants (Figure S7).

In addition, quantitative immunohistochemistry demonstrated significantly increased numbers of IL-4-, IL-17-, IL-22-, IL-33-, IL-13-, and IFN-γ-producing cells, most prominently in the papillary dermis and to a lesser extent also in the epidermis of induced AD lesions compared to control xenotransplants (Figure S8A,B). In addition, the epidermis of human AD lesions showed a significant increase of IL-31 compared to control xenotransplants (Figure S8A,B), thus reproducing the typical upregulation of type 2 cytokines in spontaneous human AD, namely of IL-4, IL-13, IL-31 positive cells and of Th17/Th22 cytokines IL-17, IL-22, and IFN-γ positive cells in more chronic AD lesions.[2,3,32,33,59,60] These protein changes in situ were mirrored at the transcriptional level: qRT-PCR showed *IL31*, *IL13*, *IL4*, and *IL5* transcript levels to be significantly increased in AD-induced skin xenotransplants compared to controls (Figure S7). Thus, the induced xenotransplant lesions represented a phenocopy of spontaneous human AD lesions regarding TSLP, chemokine, and cytokine expression pattern.

3.5 | AD-associated immune cells undergo expansion in induced human AD lesions

Compared to control xenotransplants, the induced AD lesions also showed a significantly higher number of intradermal human $CD4^+$ and $CD8^+$ T cells, especially in the suprapapillary dermis (Figure S9A,B). There were also significantly more intraepidermal and dermal eosinophils, and $tryptase^+$ or $C\text{-}kit^+$ dermal mast cells (Figure S9A,B). Furthermore, human IgE-bearing and FcεRI+ cells were also significantly increased in AD-induced human skin xenotransplants (Figure S9A,B), primarily due to higher numbers of human FcεRI-positive mast cells and $CD1a^+$ Langerhans cells (Figure S9C,D). Since species-specific antibodies demonstrated the infiltrating immune cells to be predominantly of human origin (see Table S1), this AD-like infiltrate resulted primarily from local immune cell expansion within the xenotransplants and further confirms a Th2-skewed signaling milieu, perfectly in line with spontaneous human AD.[61,62]

3.6 | Induced AD lesions display key neuroimmunological characteristics of human AD

Human AD shows important, but often ignored neurophysiological, neuroanatomical, and neuroimmunological abnormalities such as defecting β2-adrenergic signaling,[63] sensory hyperinnervation,[64] and neurogenic skin inflammation.[65] Yet, no currently available mouse model of AD has demonstrated these neuroimmunological abnormalities.[4] Interestingly, quantitative immunohistomorphometry revealed a higher density of neurofilaments (indicating hyperinnervation) and a lower expression of the β2-adrenergic receptor in the induced AD skin lesions compared to control xenotransplants (Figure 3A,B). This was associated with increased epidermal expression of nerve growth factor (NGF) (Figure 3A,B), well in line with the increased production of keratinocyte-derived neurotrophins thought to drive sensory hyperinnervation in AD.[66] We also observed an significantly increased staining intensity of protease-activated receptor 2 (PAR-2) and neurokinin 1 receptor (NK1), two key receptors involved in neurogenic skin inflammation and AD-associated pruritus (Figure 3A,B).[67,68]

Taken together with the observed increase in mast cells (Figure S9A,B), which operate as central switchboard cells in the regulation of neurogenic skin inflammation,[28,67,69] and the upregulation of pruritus-associated cytokines such as IL-31, IL-4, IL-13[70] (Figure S8) in the AD-induced xenotransplants, this demonstrates that the new humanized AD mouse model also recapitulates the characteristic "neurodermatitis" aspects of human AD.[4]

3.7 | Induced AD skin lesions respond to topical glucocorticosteroids and tacrolimus

Any credible AD animal should demonstrate responsiveness to standard topical AD therapeutics,[4] such as the glucocorticosteroid, dexamethasone, and the calcineurin inhibitor, tacrolimus (FK506).[71] This was the case in our new model: In contrast to vaseline-treated control grafts, both AD therapeutics decreased the intensity of erythema and scaling (Figure S10A), reduced epidermal thickness and hyperproliferation (Ki-67 and keratin 16) (Figures S10B,C and S11A,B), increased filaggrin and claudin-1 protein expression (Figures S10B,C and S11A,B), and attenuated the local Th2 response as illustrated by significantly decreased levels of IgE, TSLP, and periostin (Figures S10B,C and S11A,B). This was combined with significantly reduced PAR-2, TARC, CTACK, eotaxin-1, and eotaxin-3 protein expression (Figure S11A,B), reduced intraepidermal IL-33 and NGF expression, a significantly lowered number of $tryptase^+$ or $C\text{-}kit^+$ mast cells, dermal $CD4^+$ and $CD8^+$ T cells, FcεRI+ dermal cells, and of dermal immune cells positive for IL-4, IL-13, IL-17, and IL-22 (Figures S12A,B and S13A,B).

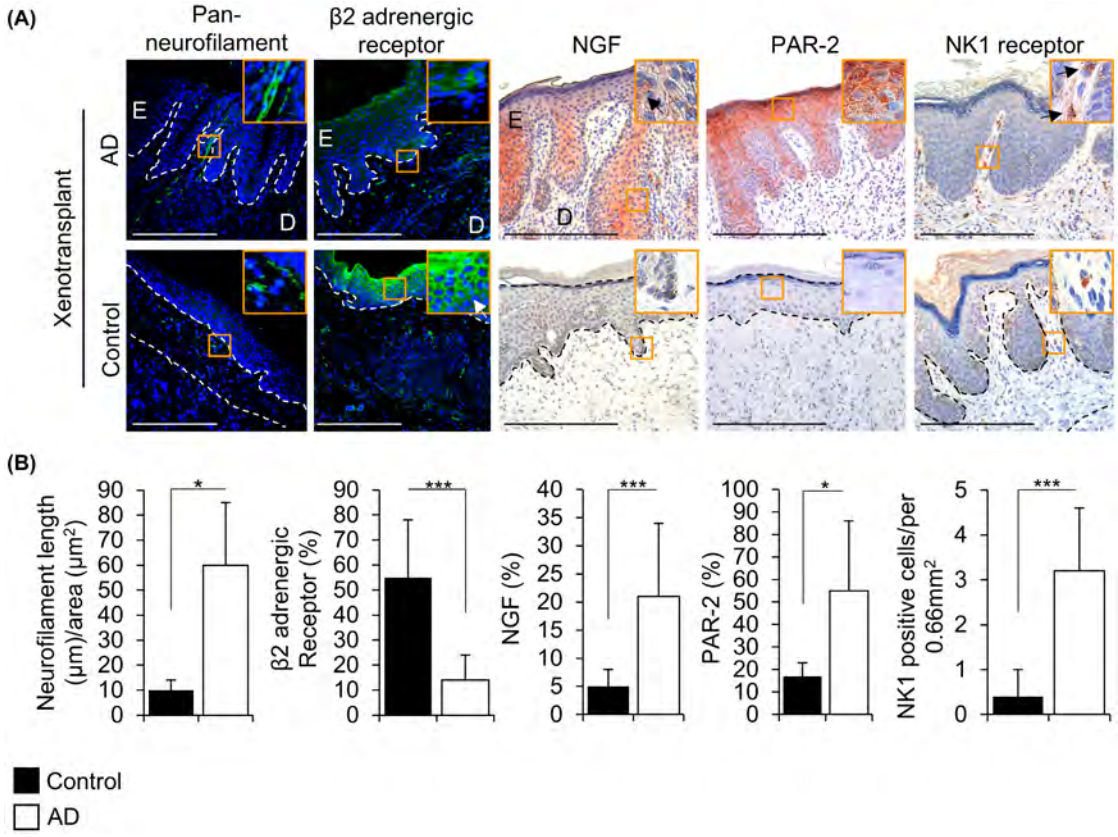


FIGURE 3 Expression of neurogenic markers in AD-induced xenotransplants. (A, B) Protein expression of pan-neurofilaments ($N = 12$ mice) and of β2 adrenergic receptor ($N = 11$ mice) were assessed by immunofluorescence, while that of NGF ($N = 12$ mice), PAR-2 ($N = 10$ mice), and NK1 receptor ($N = 12$ mice) was determined by IHC from 3 independent donors. Four to five areas were evaluated per section, and 3 sections were analyzed per mouse. (A) Representative images showing reference areas for the evaluation and (B) the pooled means of the indicated parameters. The pooled means were compared with a student's *t*-test: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Scale bars: 50 μm. E: epidermis, D: dermis.

3.8 | AD lesions can be re-invoked in human skin transplants by exposing mice to perceived stress

Many patients report triggering or aggravation of their AD lesions by psychoemotional stress, likely as a result of neurogenic skin inflammation.[69,72,73] Yet, this has not been recapitulated in any of the frequently used mouse AD models.[4,11] Human skin xenografts undergo rapid sensory reinnervation by host nerve fibers.[4] Also, after AD lesion induction, the xenografts overexpress PAR-2, NGF, NK-1R, and itch-associated cytokines, and show an increase in dermal mast cells number as well as reduced β2-adrenergic receptor expression (Figure 3A,B and Figure S9A,B). Furthermore, perceived stress induced by sound (sonic stress) induces substance P-/NK-1R-, NGF-, and mast cell-dependent neurogenic inflammation in murine skin.[28,29] Therefore, we finally tested whether perceived (sonic) stress can trigger AD lesions in human skin transplants in vivo.

To address this, we first treated PBMCs-induced human AD skin lesions with topical dexamethasone or vehicle for 3 days. As expected, dexamethasone rapidly reduced thickness, erythema and scaling of the induced AD lesions (Figure 4A) and normalized markers of skin barrier dysfunction (filaggrin), the number of IgE+ cells, immune cell infiltration (CD4, CD8, tryptase, and C-kit), and pro-inflammatory cytokine expression (IL-33 and IL-17) (Figure 4B,C and Figure S14A,B). One day after dexamethasone therapy was discontinued, we then exposed the mice to intermittent sonic stress for 24 h as described.[28,29]

Within 14 days, this induced a relapse of AD lesions in human skin xenotransplants, accompanied by a flare of all previously normalized read-out parameters towards levels seen prior to dexamethasone treatment (Figure 4A–C and Figure S14A,B). Since no relapse of AD lesions was seen in sham-treated mice, this excludes that the relapse reflected a glucocorticoid withdrawal phenomenon. As an additional control, we asked whether perceived stress alone suffices to initiate AD lesions in our model. This was not the case: In the absence of type 2 inflammation via PBMCs injection, sonic stress failed to induce AD lesions in human skin xenotransplants in vivo (Figure S15). This suggests that priming by Type 2 inflammation is required to make human skin susceptible to a perceived stress-induced development or relapse of AD lesions.

These observations strongly suggest that the (mouse) brain-(human) skin axis[74–77] is fully functional in our model is responsive to "psychoemotional" stress and is responsible for re-invoking AD lesions in the human skin xenotransplants in vivo.

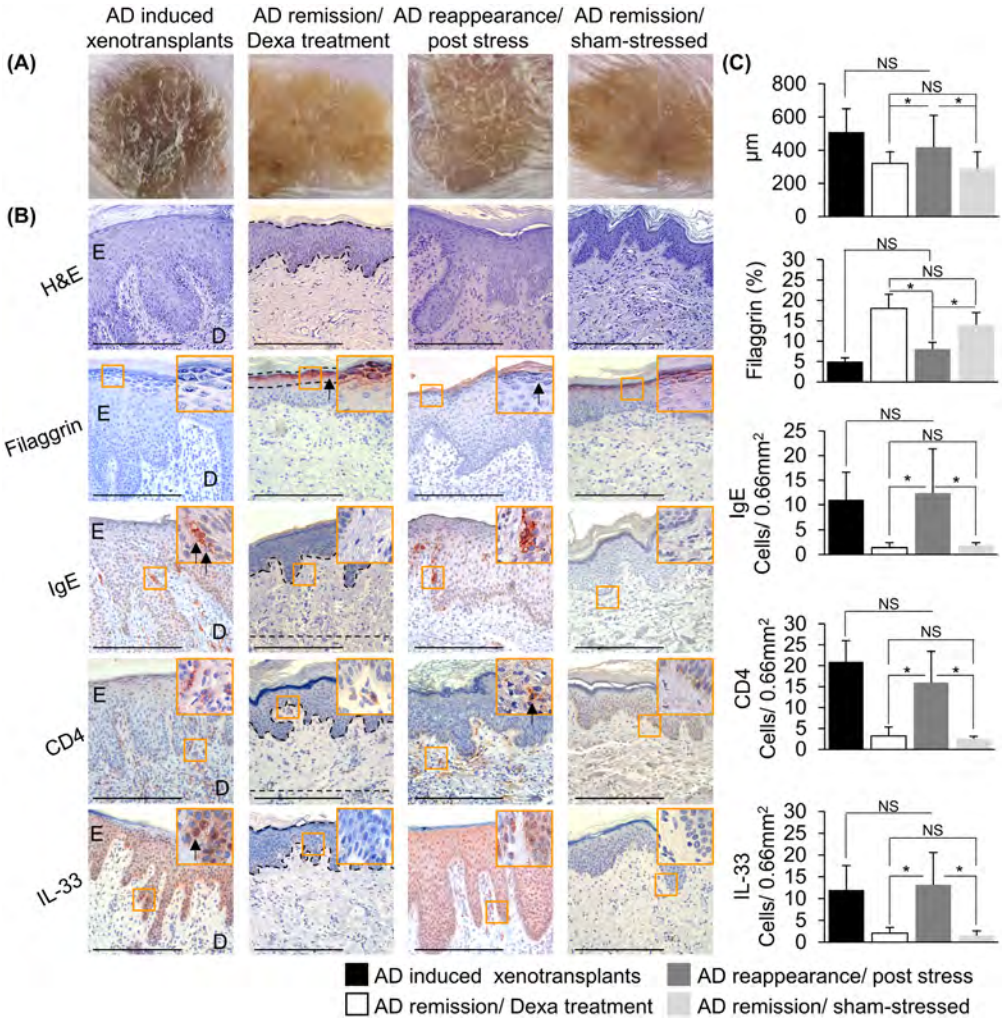


FIGURE 4 Perceived (sonic) stress promotes lesion relapse in treated AD-induced xenotransplants. Mice with AD-induced xenotransplants were treated and subsequently exposed to psychoemotional (sonic) stress. (A) Macroscopic appearance of xenotransplants, (B, C) Epidermal thickness ($N = 13$ mice) was determined by hematoxylin stain and eosin, and the intradermal expression of filaggrin ($N = 12$ mice), IgE ($N = 13$ mice), CD4 ($N = 13$ mice), and IL-33 ($N = 12$ mice) was analyzed by IHC. AD induced skin ($N = 4$ mice) and tracked throughout the remission (corticosteroid treatment; $N = 5$ mice) and relapse (stress induction; $N = 4$ mice) phases. To control for the procedure-induced distress, mice were also sham-treated in parallel ($N = 3$ mice). The final response was measured 14 days after the stress induction (or sham treatment); data were assessed by IHC from 3 independent donors. Four to five areas were evaluated per section, and 3 sections were analyzed per mouse. (B) Representative images showing reference areas for the evaluation and (C) the pooled means of the indicated parameters. The pooled means were compared with a one-way anova: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Scale bars: 50 µm. E: epidermis, D: dermis. Arrow- Filaggrin positive expression.

3.9 | IL-4 and IL-13 treatment can suffice to induce skin barrier impairment and intraepidermal TSLP production in healthy human skin ex vivo

In an additional pilot experiment, we also asked whether the type 2 cytokines IL-4 and IL-13 alone suffice to induce at least some early AD-associated changes in healthy, non-atopic human skin ex vivo, that is, in the absence of any confounding systemic or neural inputs (including from mice). To do so, we exposed organ-cultured human skin punches in serum-free, supplemented William's E medium[78–80] to IL-4 (50 ng/mL) and IL-13 (50 ng/mL).

qRT-PCR analysis after 24 h showed significantly increased mRNA steady-state levels for *IL1A*, *CCL27*, and *TSLP*, concomitant with a decrease in the level of the epidermal differentiation-associated keratin 1 (Figure S16A). Epidermal involucrin and claudin-1 protein expression were significantly down-regulated 72 h after IL-4 and IL-13 administration ex vivo, suggesting a compromised skin barrier function (Figure S16A,B). Incubating healthy organ-cultured human skin with IL-4 and IL-13 for 120 h also significantly upregulated the epidermal expression of TSLP mRNA and protein (Figure S16A,B). However, the above involucrin and claudin-1 response was seen only in 40% of the donors (2/5 total donors) ex vivo, while that of TSLP was seen in 2/3

donors, suggesting substantial interindividual differences in the response of healthy human skin to high levels of IL-4 and IL-13.

3.10 | Dupilumab and tralokinumab both prevent the induction of AD lesions in the humanized AD mouse model

Finally, we asked whether IL-4 and IL-13 are also required for the development of experimentally induced AD lesions in human skin xenotransplants in vivo. To address this, dupilumab, a monoclonal antibody that blocks signaling through IL-4 receptor-α and thereby targets both IL-4 and IL-13 pathways[81] was administered either during the Th2- polarization step in vitro ("AD prevention protocol"), which reduced GATA-3 expression (FACS) and IL-17 and IL-22 intracellular staining (Figure S17C), or after injection of the Th2-polarized PBMCs in vivo ("AD treatment protocol").

This showed that dupilumab both significantly inhibited AD lesion induction in the xenotransplants in vivo ("prevention protocol") (Figure 5 and Figures S17 and S18), and normalized key AD lesion parameters such as erythema and scaling (Figure 5A), epidermal thickness and keratinocyte hyperproliferation (Ki-67) ("AD treatment protocol") (Figure 5A–C). Filaggrin protein expression was also rescued by both, preventive and therapeutic dupilumab application, as were the Th2 immune response markers, IgE, TSLP, and periostin (Figure 5B,C and Figure S18A,B), TARC, CTACK, and eotaxin-1 and -3 expression (Figure S18A,B) and xenotransplant infiltration by tryptase$^+$ or c-Kit$^+$ mast cells, CD4$^+$ and CD8$^+$ T cells, and IL-4 and IL-13 expression (Figure S17A,B).

Next, we asked whether blocking of IL-13 alone possess preventive effect on the development of AD similarly to dupilumab, autologous PBMCs were co-cultured with LPS, IL-4 and IL-2, in the presence of tralokinumab, an anti-IL-13 humanized mAb, or of relevant isotype control antibody. AD phenotype was observed only in control mice, while AD key readouts vanished in the tralokinumab treated mice (Figures S19 and S20). More specifically, a significant downregulation of the key readouts (epidermal thickness, filaggrin TSLP, epidermal Ki-67, IgE, mast cell (tryptase, c-kit), TARC, CTCAK, Eotaxin-1 and 3, periostin, CD8+ cells, IL-4+ cells, IL-33+ cells and IL-13+ cells) was observed in a sharp contrast to the control group, which demonstrated the development of AD phenotype. This observation significantly enhances the validation of this model to solve key questions in the field. Therefore, IL-4 and IL-13 exert a dual role in the humanized AD model: They are critical for PBMCs Th2-polarization in vitro, and they represent key drivers of the experimentally induced AD development in the human xenotransplants, just as in spontaneously occurring human AD.[39,82]

3.11 | Analysis of lipid metabolism

The analysis revealed increase of short-chain ceramides in AD lesions and downregulation after treatment with dupilumab (31.11 ± 6.179 control, 49.00 ± 7.217 AD and 34.41 ± 4.555 dupilumab AU/mg of protein, Data±SEM), while there is no change in the long-chain ceramide in AD lesions but upregulation after treatment with dupilumab (3.096 ± 0.9867 control, 4.762 ± 1.434 AD, and 7.358 ± 1.655 dupilumab AU/mg of protein, Data±SEM). These results confirm impairment in the barrier in the AD lesions and re-establishment of the barrier upon treatment (Table S2).

4 | DISCUSSION

Here, we present the first humanized mouse model of AD, in which healthy, non-atopic human skin can be robustly induced to develop all major hallmarks of human AD lesions in vivo by either intravenous or intradermal injection of autologous human PBMCs that are preconditioned in vitro with just three factors: IL-4, IL-2, and LPS.

This combination was necessary for in vitro generated/expanded Th2 cells without antigen-specificity, suggesting that the model is most likely antigen-independent. In order to conclusively demonstrate antigen-indpendency in follow-up studies, we would require to deplete dendritic cells in the xenotransplanted human skin before and after cells injection.

Once primed PBMCs are injected into the skin, human resident T-cell activation and proliferation is possibly enhanced by the exogenous human IL-2 which is present in the injected cultured cells, or via an autocrine/paracrine response to IL-4/IL-13 released by injected Th2 cells. In addition, the response may be exacerbated by the interaction with injected and resident skin cells expressing co-stimulatory cell-surface molecules, or other alarmins, such as TSLP,[83] that drive the expansion of T cells and their differentiation into effector T cells.[84]

Atopic dermatitis phenotype was observed in 75% intradermal injected mice after 14 days and 100% after 42 days compared to 50% of intravenously injected mice after 14 days. This observation may indicate that intradermal injections overcome limitations related to skin homing and cutaneous viability following intravenous injections.[85]

These experimentally induced AD lesions secondarily develop a complex epidermal barrier defect that is characteristic of human AD, along with all other key features of spontaneously developing human AD including lipids metabolism abnormalities, respond to standard clinical AD treatment, and even relapse upon exposing the host animals to perceived stress, thereby mimicking also clinically important "neurodermatitis" aspects of human AD.[4,73,86–88] The crosstalk between Th2 inflammation, neuroimmune interactions, and skin barrier dysfunction is prominent feature of human AD pathobiology and may fuel a vicious circle that leads to AD chronification in humans, particularly in regard to pruritus and inflammation.[89,90] This permits to functionally dissect and pharmacologically target the key drivers of stress-induced neurogenic inflammation in human AD skin lesions in vivo.

No other animal model currently reflects human AD features at this level of completeness and complexity. For example, the current

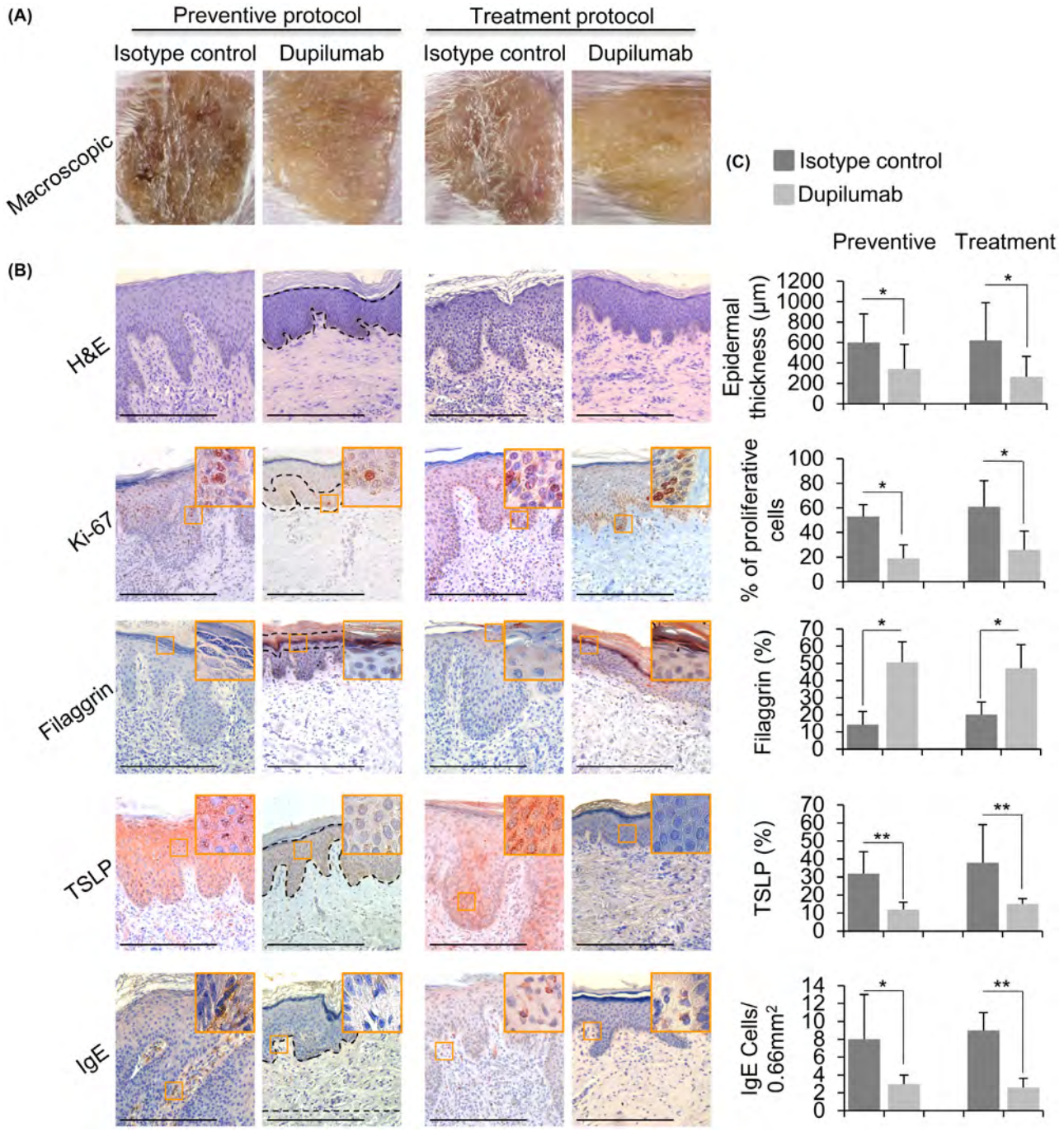


FIGURE 5 Preventive and therapeutic effects of dupilumab in AD-induced transplants. (A–C) The effects of dupilumab were assessed during the PBMCs polarization step prior to injection into the skin xenotransplants ("preventive protocol") or after injection of polarized PBMCs ("treatment protocol"). For the prevention protocol, autologous PBMCs were conditioned in vitro as before, but in the presence of dupilumab (1.4 µg/mL/14 days) or of relevant isotype control antibody (1.4 µg/mL/14 days). For the treatment protocol, dupilumab was administered subcutaneously once daily from Day 15 after injection of Th2-polarized PBMCs, until Day 28. For both protocols, we analyzed 6 mice treated with isotype control and 8 with dupilumab, in xenotransplants from 2 independent donors. (A) Macroscopic appearance of baseline and drug-treated AD-induced xenotransplanted skin. (B, C) Epidermal thickness ($N = 13$ mice) was determined by hematoxylin stain and eosin, and the intradermal expression of Ki-67 ($N = 13$ mice), filaggrin ($N = 12$ mice), TSLP ($N = 12$ mice), and IgE ($N = 12$ mice) was analyzed by IHC from 2 independent donors. Four to five areas were evaluated per section, and 3 sections were analyzed per mouse. (A, B) Representative images showing reference areas for the evaluation and (c) the pooled means of the indicated parameters. The pooled means were compared with a Student's *t*-test: $^{*}p < .01$, $^{**}p < .01$, $^{***}p < .001$. Scale bars: 50 µm.

model meets 8/8 essential and 6/7 optimal criteria recently suggested by an international panel of immunodermatology experts for clinically relevant murine AD models.[4] The observed upregulation of Th1, Th22, Th17, and Th2 cytokines suggests that our humanized AD mouse model reflects an intermediate state between acute and chronic AD[89,91,92] If this is confirmed in follow-up studies, the

model will be particularly valuable for the preclinical testing of new candidate therapeutics that aim to block the critical AD progression towards the eczema stage during which AD becomes increasingly difficult to manage.[45,93]

The pragmatic experimental design of the current model facilitates assay set-up and does not distinguish between classical Th2 lymphocytes, that is, CD4+ T cell with a phenotype skewed towards IL-4 and IL-13 production, and Tc2 cells, that is, CD8+ cells which also produce IL-4 and IL-13.[10] Thus, the autologous Th2-polarized cells which induce AD lesions in healthy human skin xenotransplants arguably reflect the heterogeneous T-cell immunopathology associated with human AD reasonably well.[94–96]

While our dupilumab and tralokinumab results demonstrate a dominant role of Th2 cytokines in the de novo induction of AD lesions in healthy human skin in vivo, there is increasing insight that Th1 and Th17/Th22 axes, namely IFN-γ, IL-17, and IL-22, are also involved in AD pathobiology, notably in its eczematous stage.[89,97,98] Therefore, it is interesting to note that, in our model, AD is induced by PBMCs that also produce cytokines other than Th2, such as IL-17, IFN-γ, and TH22 (Figure S8). Our limited human skin organ culture data suggest that IL-4 and IL-13 alone can suffice, at least in some individuals, to induce skin barrier impairment and production of the Th2 alarmin, TSLP, by epidermal keratinocytes even in the absence of systemic or neural inputs, atopy, and epidermal barrier disruption. Together with our dupilumab and tralokinumab, experiments, which show that IL-13 alone and IL-4Ra-mediated signaling is both, required for experimental AD lesion induction in human xenotransplants and is therapeutic in this model in vivo, this further supports the recognized key role of IL-4 and IL-13 in human AD pathobiology.[64,99]

Since both β2-adrenergic receptor and filaggrin are downregulated by injecting Th2-conditioned PBMCs, the dysregulated immune response appears to drive the manifestation of other key AD pathobiology biomarkers. This pathobiology chain may be accelerated in individuals who exhibit a function-impairing filaggrin and/or BAR2 mutation,[26,63,100–102] which could lower the threshold for AD to manifest clinically. Finally, in our model neither primary epidermal barrier disruption[2,103] nor a pre-existing "atopic" condition[13] are essential preconditions for AD lesions. While it is often assumed that atopy and epidermal barrier impairment play a key role in the initiation of the disease, at least in selected AD patients,[102] the importance of an epidermal barrier defect as a primary event in AD lesion development has been questioned before.[104] Our data show that both an epidermal barrier defect and atopy are not an essential prerequisite for developing AD lesions in previously healthy human skin in vivo. Together with increasing epidermal dysbiosis, a secondary, immunologically induced epidermal barrier defect could result in a vicious circle that promotes AD progression and chronicity. The current humanized AD mouse model now enables one to functionally and mechanistically probe this scenario directly in the human target organ in vivo.

AUTHOR CONTRIBUTIONS

A.G. and R.P. conceived and A.G. supervised the project. A.K. performed most of the experiments. C.R. performed AFM analyses. M.B. provided ex vivo data. Y.U. provided human skin samples. A.G. and R.P. designed the experiments and interpreted the data. K.R. provided critical input into data interpretation, literature synthesis, and text editing. A.K., A.G., and R.P. wrote the manuscript. All authors edited the final manuscript.

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CONFLICT OF INTEREST STATEMENT

A.G., A.K., R.P., and Y.U state no relevant conflict of interest. For the record, A.G., A.K., M.B., and R.P. perform dermatological contract research or consult for various industry clients, including in the context of AD research. C.R. has served as advisor and/or paid speaker for and/or participated in clinical trials sponsored by Abbvie, Almirall, Amgen, Boehringer Ingelheim, Bristol-Myers Squibb, Celgene, Forward Pharma, Gilead, Galderma, Janssen-Cilag, Kyowa Kirin, LEO Pharma, Lilly, Medac, Novartis, Ocean Pharma, Pfizer, Sanofi, UCB; Professor Reich is co-founder of Moonlake Immunotherapeutics.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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