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Induction of skin-pathogenic Th22 cells by epicutaneous allergen exposure

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ABSTRACT

Background: Atopic dermatitis (AD) is a common inflammatory skin disease with dysfunction of the skin barrier, an abnormal immune response and frequent allergies to environmental antigens like food antigens. Clinical observations suggest that certain diets can influence the course of AD.

Objective: Here we compared the phenotype of food allergen-specific T cells activated through skin or gut allergen exposure to transfer skin inflammation into naïve recipients upon epicutaneous allergen challenge.

Methods: Ovalbumin (OVA) TCR-transgenic mice were treated epicutaneously with OVA or were fed OVA. CD4⁺ T cells from skin lymph nodes or mesenteric lymph nodes were transferred into naïve BALB/c mice, which were challenged with OVA epicutaneously. Skin inflammation was determined by histological parameters. In addition, we analyzed the phenotype of the immune response in lymphoid tissues and in skin tissue.

Results: TCR-transgenic T cells activated through epicutaneous or oral OVA exposure both migrate to skin lymph nodes after adoptive transfer and epicutaneous OVA exposure. AD-like skin inflammation could only be induced by the transfer of epicutaneously primed OVA T cells. Analysis of the immune phenotype demonstrated an IL-22/IL-17A-dominated immune phenotype of skin-pathogenic T cells.

Conclusion: IL-22 seems to be the critical cytokine for the development of AD and is induced in this model by epicutaneous sensitization with OVA.

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1. Introduction

Atopic dermatitis (AD) is an excessive reaction of the skin immune system towards the environment, aggravated by alterations within epidermal-barrier functions and sensitization to exogenous allergens [1,2]. AD skin lesions are typically infiltrated by CD4⁺ T cells and other immune cells like dendritic cells (DC), innate lymphoid cells and eosinophils. As reported, initial AD is dominated by Th2 cells expressing IL-4 and IL-13 and by Th22 cells expressing IL-22 [3–6]. In the chronic phase of disease, interferon (IFN)- γ ⁺ Th1 cells and IL-17⁺ Th17 cells expand the Th2/Th22-dominated immune phenotype [4,5,7]. A hallmark of AD is dry and itchy skin with a disrupted epidermal barrier function. Patients with AD have reduced filaggrin expression in the epidermis due to

the frequent presence of *FLG* mutations in Western countries [8]. The epidermal barrier alterations may increase the risk for additional contact sensitizations to allergens [9].

In general, the prevalence of food allergies in children with AD is much higher than in children without AD [2]. Food allergens can induce an IgE-mediated type I allergic response but can also be recognized by T cells, which are responsible for type IV allergic responses presenting as contact dermatitis. Of note, T cells infiltrating the skin in AD lesions can be reactive to self antigens but also to non-self antigens like environmental allergens [10–12]. Moreover, studies have reported on the existence of food allergen-specific Th cells in lesional skin of patients with AD [11]. Clinical observations indicate that exposure of the skin to food allergens but also oral exposure of patients to food allergens can exacerbate

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AD. Yet, it is still an open question if diets can really influence the clinical course of AD. In children with AD, the worsening of skin symptoms has been reported as reaction to oral food challenge but also as reaction to challenge with placebo when tested in double-blind placebo-controlled food challenge studies [13]. From the patients' perspective a clear link between skin inflammation and certain diets is assumed. Therefore it is of clinical interest to find out the immunological relevance of food allergen-specific T cells activated through the skin or the gut for AD pathology.

A few studies have tackled the capability of gut-derived allergen-specific T cells to home to the skin and to induce dermatitis. However, in most of these studies non-physiological conditions using additional adjuvants like complete Freund's adjuvant or cholera toxin have been used [14–16]. An alternative approach for mimicking AD in mice is epicutaneous (EC) sensitization with antigens and the use of tape stripping for the manipulation of the epidermal barrier. In this study, we aimed to characterize the functional interaction between allergen-reactive T cells activated in the skin- or gut-associated lymphoid tissue. Both organs have large epithelial surfaces with the environment and their immune cells are constantly exposed to different antigens. Here, we analysed the effects of EC and oral exposure of the food allergen ovalbumin (OVA) on T cell activation and the development of AD-like disease in the absence of additional adjuvants.

2. Methods

2.1. Experimental mice

Female BALB/c mice were purchased from Janvier or Charles River, transgenic DO11.10 mice obtained from The Jackson Laboratory and were bred in the animal care facilities of the University Medical Center Tübingen under specific pathogen-free conditions. Animal experiments were performed according to the institutional animal care guidelines and were approved by the Regierungspräsidium Tübingen (HT2/08).

2.2. Reagents and cell culture

OVA_{323–339} peptide (ISQAVHAHAHAEINEAGR) was purchased from EMC (Tübingen, Germany) and OVA protein grade V was obtained from Sigma-Aldrich (Munich, Germany). Cells from naïve mice or from mice after epicutaneous sensitization or oral exposure were isolated and cultured in complete Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Thermo Scientific, Langensfeld, Germany), penicillin and streptomycin. For *in vitro* experiments, single cell suspensions were cultured with OVA peptide (10 µg/ml) for 3 days. Cells collected for mRNA analysis and supernatant was harvested for analyzing cytokine secretion by ELISA.

2.3. Epicutaneous and oral OVA exposure

Epicutaneous (EC) sensitization was performed as described [17] with some modifications. Briefly, DO11.10 mice were anesthetized with ketamine/ronpum and the shaved back skin was treated with 70% ethanol before the stratum corneum was removed by tape stripping with FixoMull patch (BSNmedical, Hamburg, Germany). Saline (SAL) or OVA peptide (40 µg) in saline was applied on both flanks of the shaved back skin using Finn Chambers (SmartPractice, Reinbeck, Germany) and fixed with FixoMull (BSNmedical, Hamburg, Germany). The chambers were placed for a period of 7 days and then removed. After a 1-week break, the procedure was re-applied to the same skin site. Each mouse had a total of three 1-week exposures to OVA separated by

two 1-week intervals. One day after the last sensitization, mice were inspected and sacrificed for analysis of lymphoid tissue and skin. BALB/c mice received OVA-specific T cells (5×10^6 cells, i.p.) from DO11.10 sensitized mice and were challenged EC with OVA peptide (100 µg) for 7 days. Like in DO11.10 mice, OVA was exposed by Finn Chamber patches on each site of the shaved back skin of BALB/c mice. For oral T cell activation DO11.10 mice were fed daily with 100 mg OVA protein dissolved in 300 µl sterile water (SAL) or with OVA-free sterile water (controls) for 3 days using 20 G-1.5 "gavage needles (Braintree Scientific). One day after the last feeding mice were sacrificed for analysis of lymphoid tissues.

2.4. Subcutaneous OVA immunization

OVA-specific T cells (5×10^6) from naive DO11.10 donor mice were injected i.p. into BALB/c mice. On day 4 after adoptive transfer recipient mice were immunized s.c. with 100 µg OVA peptide in complete Freund's adjuvant (CFA) (Difco, BD Biosciences, Heidelberg, Germany). Control mice received either 100 µg SAL in CFA. Mice were sacrificed at the indicated time points and lymphoid organs were removed and analyzed for the presence of OVA TCR-transgenic T cells.

2.5. Analysis of cell surface markers and cytokine production

Commercially available ELISA kits were used for the quantification of the cytokines IL-4, IFN-γ (BD Biosciences, Heidelberg, Germany), IL-17 and IL-22 (R&D Systems, Wiesbaden, Germany) from cell culture supernatants. Intracellular cytokine staining of FoxP3 was performed using a transcription factor staining kit (eBioscience, Frankfurt, Germany). The expression of cell surface markers was determined by using fluorochrome-labeled antibodies against CD4 (Gk1.5), DO11.10 TCR (KJ1-26) and CD62L (MEL-14, all BioLegend, Koblenz, Germany). Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany) and FCS Express software (De Novo Software, Glendale, CA, USA).

2.6. RNA isolation and gene expression

Total RNA was purified from tissue and reverse transcribed into cDNA using commercially available kits (Biozym, Hamburg, Germany). Relative gene expression levels were determined by quantitative realtime-PCR (qRT-PCR) using TaqMan probes (TIB MolBiol, Berlin, Germany) for *Bactin*, *Il4*, *Il17*, *Ifnγ*, *Foxp3*, *Il21*, *Il22*, *Tbx21*, *Gata3*, *Ccr4*, *Ccr6*, *Ccr10*, *Il23r* and the LightCycler480 system (Roche, Penzberg, Germany). The relative expression of the indicated genes was calculated relative to the expression of *β-actin*. Control conditions were set as 1.0 as indicated.

Bactin for: ACCCAGCTgTgCCATCTA, rev: gCCACAggATTCCA-TACCCA

TM: 6FAM-CATCCTgCgTCTggACCTggC-BBQ

Il4 for: CCAAACgTCCTCACAgCAAC, rev: gCATCgAAAAgCCC-gAAAg

TM: 6FAM-AgAACACCACAgAgAgTgAgCTCgTCTgTA-BBQ

Il17 for: AgggTgACgTggAACggT, rev: gAgAgCTTCATCTgTgTCTCT-gATgC

TM: 6FAM-TggACACgCTgAgCTTTgAggggATgAT-BBQ

Ifnγ for: CgAAAAAggATgCATTgATgTA, rev: gCTggTggAC-CACTCggA

TM-6FAM-TgCCAAgTTTgAggTCAACAACCCA-BBQ

Foxp3 for: gCAATAgTTCCTTCCCAgAgTTCTT, rev: CAAAg-CACTTgTgCaggCTC

TM: 6FAM-TTTCTgAAgTAggCgAACATgCgAgTAA-BBQ

Il21 for: CACCAAgATgTAAAggggCACTg, rev: CACgAggTCAAT-gATgAATgTCTTA

TM: 6FAM-TgTTTCCAgggTTTgATgCTTgATgT-BBQ

Il22 for: CTCCTgTCACATCAGcggT, rev: AgCAggTCCAgTTCCCA
TM: 6FAM-TCgCCTTgATCTCTCCAATCTCTCCAA-BBQ

Tbx21 for: ggTgTCTgggAAgCTgAgAg, rev: gAAggACAggAATgg-gAACA

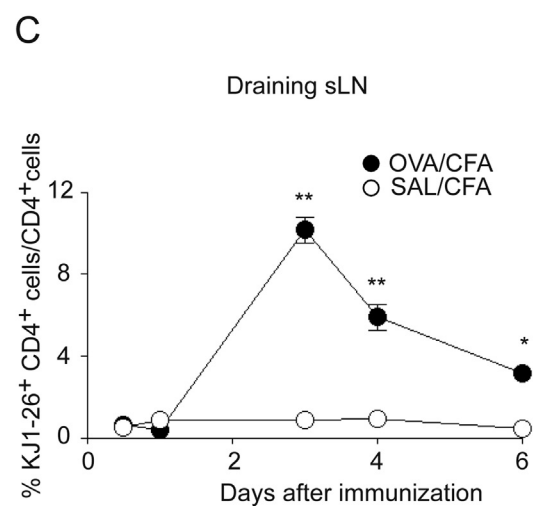
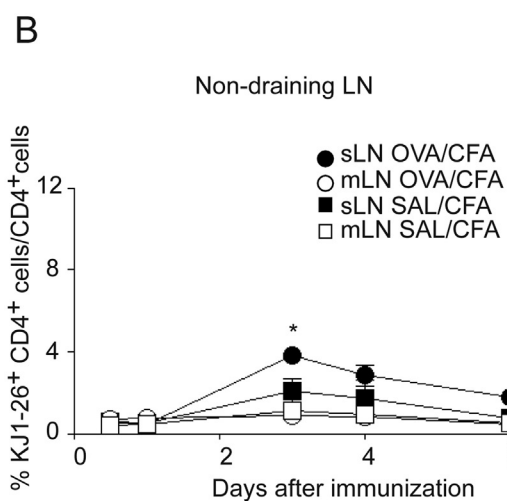
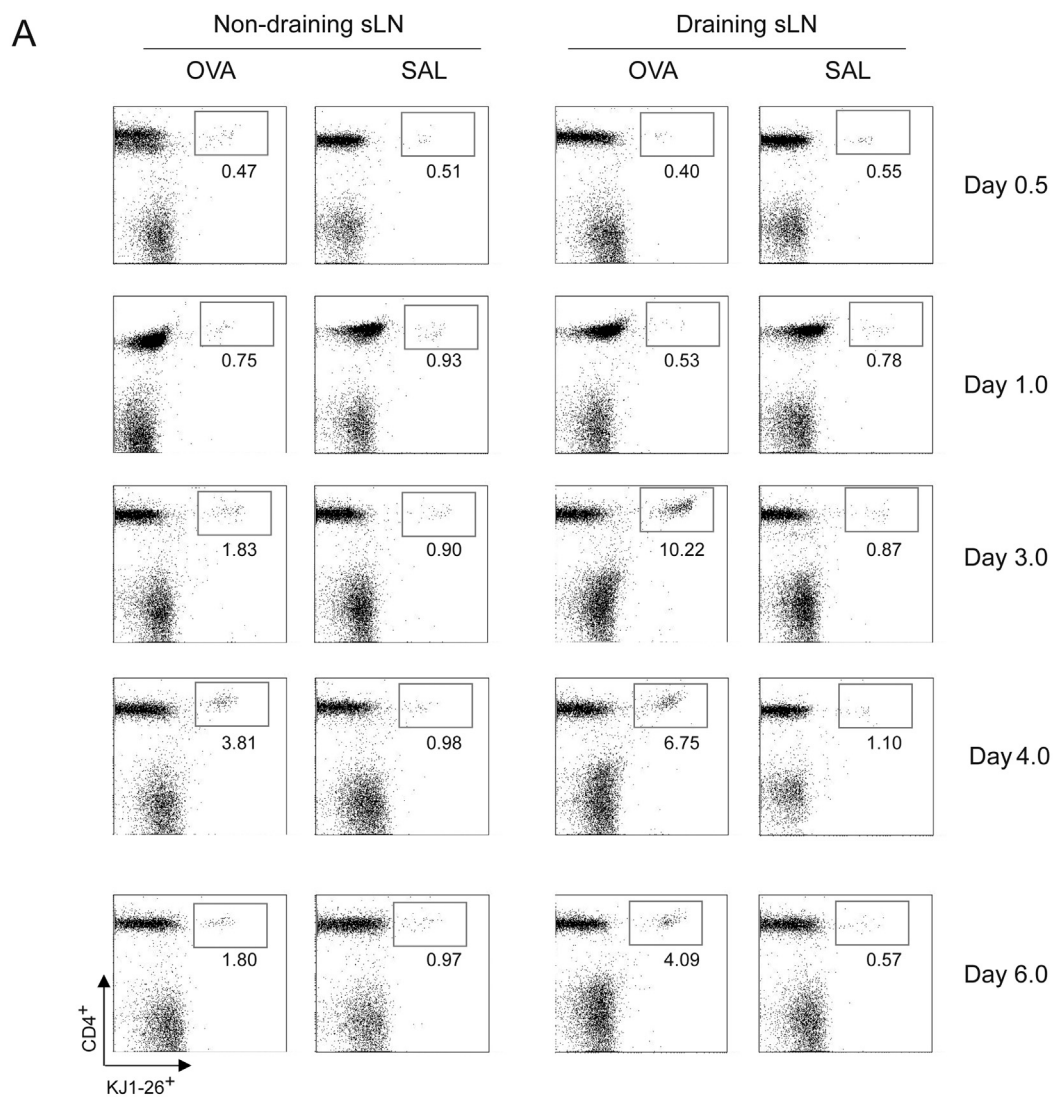


Fig. 1. OVA-specific T cells are directed to the draining skin lymph node (sLN) after subcutaneous immunization with OVA in adjuvant. (A–C) Four days after adoptive transfer of OVA-specific T cells, BALB/c mice were immunized s.c. with OVA or SAL in CFA. Distribution of OVA-specific T cells in draining and non-draining LN of the skin (sLN) and gut (mLN) was analyzed by staining for the OVA TCR (KJ1-26) on the indicated days. Dot plots from single mice are depicted in (A), pooled data of 3 experiments ($n = 15$) are shown in (B) and (C) (* $P < 0.05$; ** $P < 0.01$; Wilcoxon test).

TM: 6FAM-ATCTCTgTCTggTgCTggTTgAACTT-BBQ
Gata3 for: gCTgACCACACgACgC, rev: CATgTggAgCagggCTCTg
 TM: 6FAM-TCggACCTCACCACCTTCCA-BBQ
Rorc for: CCgCTgAgAgggCTTCAC, rev: TgCaggAgTAggCCACAT-TACA
 TM: 6FAM-CCCTACTgAggAggACaggAgCCA-BBQ
Ccr4 for: gCttCATAgACTgTCCTCaggATCA, rev: TCTTgTATTTgAA-CaggACCgAACC

TM: 6FAM-ACACCACCCAggATgAAACTgTgTACAA-BBQ
Ccr6 for: AgTTACTCATgCCACCAACACTTg, rev: TgACCT-TACTgTgCgTCAGTgTTC
 TM: 6FAM-CTCCTggCCTgTATCAGCATggACC-BBQ
Ccr10 for: AAgCCACAgAgCaggTCT, rev: AggCAAAGTCagggC-CAATAA
 TM: 6FAM-ACggAggTgggAgATCgggTAGTT-BBQ

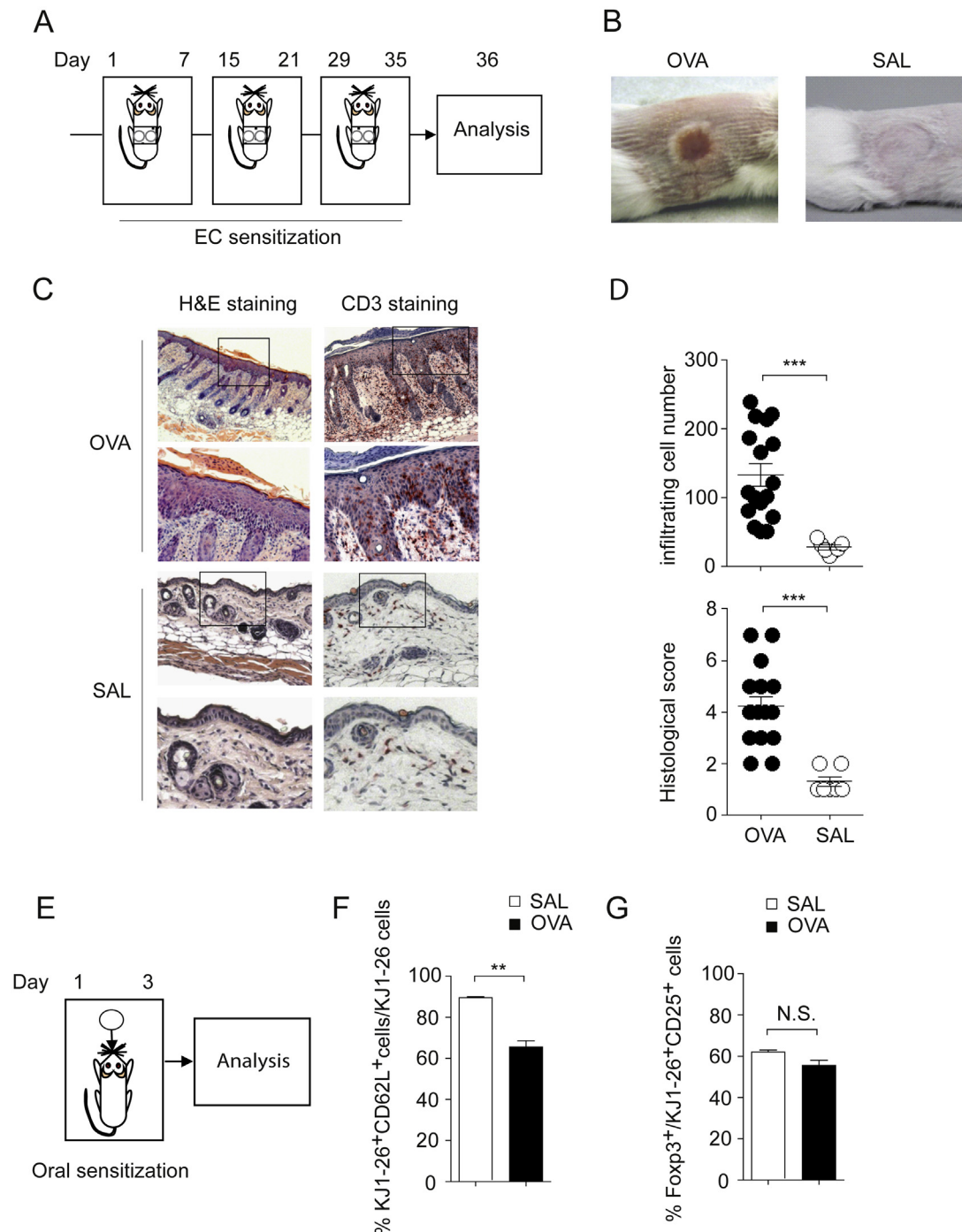


Fig. 2. Epicutaneous sensitization with OVA peptide induces AD in DO11.10 mice. (A) DO11.10 mice were sensitized three times with OVA peptide (OVA) or saline (SAL) on shaved back skin, each for a period of 7 days every other week. (B) Representative clinical presentation of mouse back skin after the first sensitization period is shown. (C) Representative histology (H&E) and CD3 immunohistochemistry of skin biopsies from sensitized areas of mice. (D) Skin histology sections as shown in (B) were further evaluated for leukocytic infiltration and histopathological scoring. Results in (D) represent 10 Sal and 15 OVA sensitized DO11.10 mice. (E–G) DO11.10 were fed with OVA or OVA free water (SAL) daily by intragastric gavage. After three rounds of oral sensitization mice were sacrificed, cells from mLN were isolated and the number of KJ1-26⁺CD62L⁺ (F) and regulatory FoxP3⁺KJ1-26⁺CD25⁺ T cells (G) were quantified. Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; ** $P < 0.01$; *** $P < 0.001$ Mann-Whitney test).

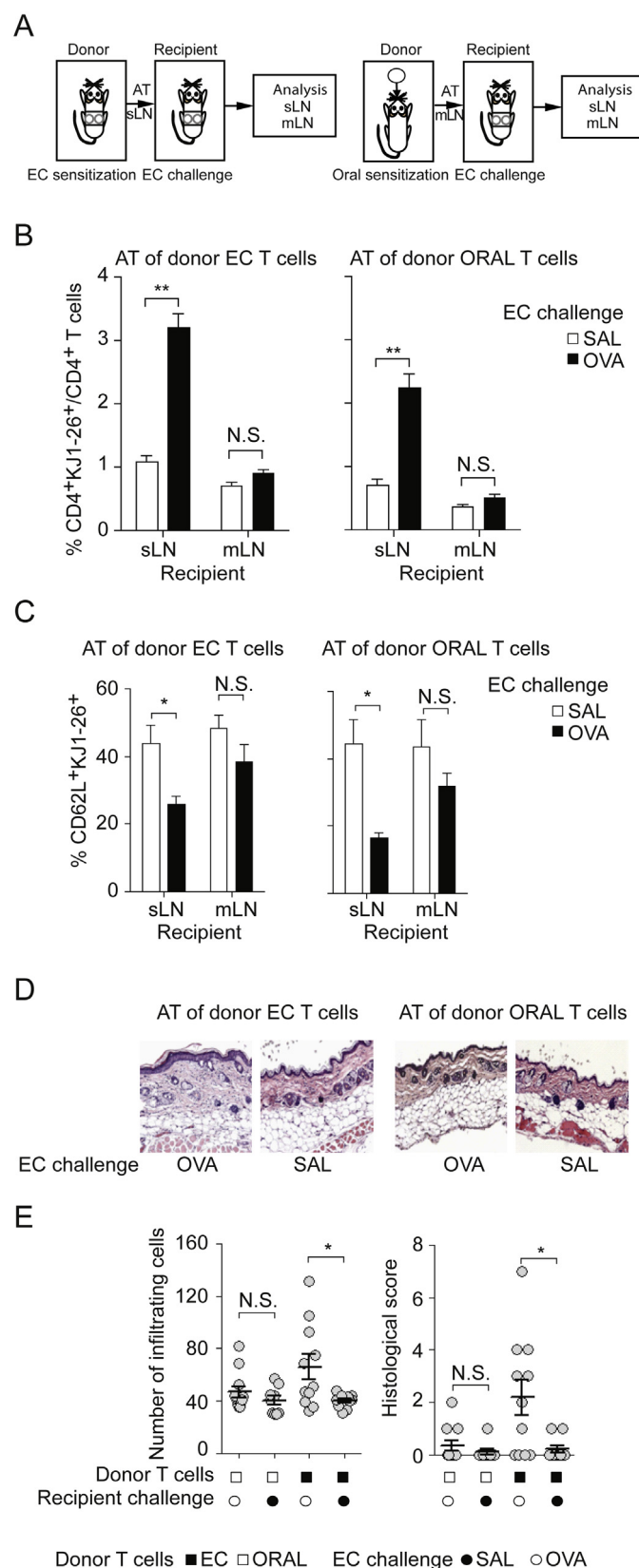


Fig. 3. Skin-primed OVA-specific T cells but not gut-primed OVA-specific T cells transfer AD. (A) OVA-specific T cells from draining sLN of DO11.10 mice as sensitized in Fig. 2A or OVA-specific T cells from mLN of OVA fed DO11.10 mice were transferred into naive BALB/c mice. Recipient mice were challenged EC for one week with OVA or SAL. (B) Enrichment of OVA-specific T cells in sLN and mLN was determined by staining for CD4 and KJ1-26. (C) Expression of CD62L by OVA-specific T cells in sLN and mLN of mice that received donor EC or donor ORAL T cells and EC

Il23r for: gTgATACCTCTgCgTCCATCATT, rev: CAgTTTCTgggAAGTTTggTg
TM: 6FAM-CCACgTTTggTTTgTTgTTTgT-TBBQ

2.7. Skin histology

Skin biopsies were fixed and processed through a standard paraffin embedding protocol. For hematoxylin and eosin (H&E) staining 5 μ m sections were used. For immunohistology skin sections were stained with anti-mouse CD3 (Dako, Hamburg, Germany). Tissue sections were examined with an Axiovert 200 microscope and histological features like parakeratosis, hyperkeratosis, hypergranulosis, necrosis, infiltration and presence of eosinophils were scored by a blinded dermatopathologist (ASY). Images were processed using an AxioCam MRc system and Axiovision software (all Zeiss, Jena, Germany).

2.8. Statistical analysis

All data were analyzed and plotted using GraphPad Prism 6 software. Statistical analyses were performed by using the Mann-Whitney test, the Wilcoxon test or the Tukey's *post-hoc* test after One-way ANOVA as indicated. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Subcutaneous immunization attracts OVA T cells into skin draining lymph nodes

To identify the ideal time points for studying OVA-specific T cells in draining skin lymph nodes (sLN), we first performed experiments using subcutaneous (s.c.) immunization. Naïve CD4⁺ T cells bearing a transgenic T cell receptor recognizing OVA peptide were adoptively transferred into BALB/c mice. On day 4 after adoptive transfer (AT) recipient mice were immunized s.c. into the left flank with OVA or saline (SAL) in CFA. The accumulation of OVA-specific T cells in draining sLN as well as non-draining sLN and mesenteric lymph nodes (mLN) was analysed by KJ1-26 staining and flow cytometry on day 0.5, 1, 3, 4 and 6 after immunization. At early time points (day 0.5 and 1) after immunization we observed a non-specific distribution of OVA-specific CD4⁺ T cells in sLN and mLN, independently from OVA or SAL immunization (Fig. 1A, B). Starting on day 3 we found a significant enrichment of the transferred OVA-specific T cells in the draining sLN node after immunization with OVA in CFA. An enrichment of transferred T cells in mice immunized with SAL in CFA was not observed (Fig. 1A–C). From these experiments we concluded that day 7 after adoptive transfer and OVA challenge is the ideal time point for T cell analysis in draining sLN.

3.2. Epicutaneous sensitization induces AD in DO11.10 mice

After defining the kinetics of specific T cell enrichment in draining sLN after AT of OVA-specific T cells and s.c. immunization, we next used a protocol for epicutaneous (EC) OVA sensitization with some modifications [17]. To avoid any immunological bias when using additional adjuvants like CFA we applied OVA peptide epicutaneously (e.c.) on the shaved back skin of OVA-TCR transgenic DO11.10 mice to generate skin-primed OVA-specific T cells (Fig. 2A). Control mice were treated e.c. with SAL. As illustrated in Fig. 2B, DO11.10 mice developed erythematous plaque at the site of OVA sensitization already one week after antigen exposure. In contrast, no erythema was visible when using SAL sensitization. After three rounds of weekly sensitization we performed skin biopsies for histological analysis. In agreement

challenge with SAL or OVA. Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; * $P < 0.05$; ** $P < 0.01$; One Way ANOVA, Tukey's *post-hoc* test). (D) Representative H&E staining and (E) quantitative analysis of skin infiltrating cells and histology score of biopsies from epicutaneously challenged skin of BALB/c recipient mice after transfer of donor EC or donor ORAL T cells and challenge with OVA or SAL e.c. Dots represent single mice (10 to 15 mice per group; mean $\pm$ SEM; N.S. not significant; * $P < 0.05$; One Way ANOVA, Tukey's *post-hoc* test).

with previous reports [17,18], EC OVA sensitization resulted in epidermal thickening associated with strong cellular infiltration of the dermis and epidermis (Fig. 2C, D). Importantly, we observed histological characteristics of eczema like acanthosis, spongiosis and hypergranulosis after sensitization with OVA. SAL sensitized skin showed no abnormalities (Fig. 2C, D). Immunohistochemistry showed marked infiltration of CD3⁺ T cells in skin biopsies of OVA-treated mice but not in the skin of control mice (Fig. 2C). Thus, EC OVA sensitization activates OVA-specific T cells to induce skin inflammation.

To generate gut-activated OVA-specific T cells without using adjuvants, DO11.10 mice were fed with 100 mg OVA for three consecutive days by intragastric gavage (Fig. 2E). This antigen dose has been shown to activate OVA-specific T cells without inducing suppressive immune responses [19]. Control mice received OVA-free water. Analysis of mLN showed an activation of OVA-specific T cells as determined by a significant decrease of CD62L expression in OVA-fed mice compared to SAL-fed control DO11.10 mice (Fig. 2F). T cell tolerance was not induced, since we did not observe any induction of FoxP3⁺ regulatory T cells by our OVA feeding protocol (Fig. 2G). In addition we could detect an increase in $\alpha 4\beta 7$ expression by OVA-specific T cells (data not shown). As expected, mice that were fed with OVA or SAL showed no erythema or other symptoms of the skin.

3.3. OVA-specific T cells activated by epicutaneous or oral routes both migrate to skin lymph nodes after adoptive transfer and epicutaneous OVA challenge

After we established the activation of OVA-specific T cells via EC and oral OVA exposure in DO11.10 mice, we decided to analyze the capability of *in vivo* activated OVA-specific T cells to transfer skin pathogenicity. Therefore we performed AT experiments and EC OVA challenge. We either transferred OVA-specific T cells from draining sLN of EC sensitized DO11.10 mice (donor EC T cells) or from mLN of OVA-fed DO11.10 mice (donor ORAL T cells) into naïve BALB/c mice. Recipient BALB/c mice were then challenged e.c. with OVA peptide (Fig. 3A). Based on the kinetics from experiments with s.c. OVA immunization (Fig. 1), we analyzed the sLN and mLN on day seven after transfer and EC OVA challenge. Challenge of recipient BALB/c mice that received donor EC T cells with OVA e.c. resulted in a selective and significant enrichment of CD4⁺KJ1-26⁺ T cells in sLN. No enrichment of OVA-specific T cells was observed in mice challenged e.c. with SAL (Fig. 3B). Donor ORAL T cells also accumulated into the draining sLN of recipient mice on day 7 after transfer and EC OVA challenge (Fig. 3B). In mLN, a random distribution independent from EC OVA or SAL challenge was found. By studying CD62L expression levels of OVA-specific T cells we could demonstrate that the EC OVA challenge was specific and effective. Only OVA-specific T cells in sLN of mice challenged with OVA peptide showed a further downregulation of CD62L expression (Fig. 3C). Thus, both donor EC T cells and donor ORAL T cells are capable to accumulate in draining sLN after AT in response to EC OVA challenge.

3.4. Skin-primed OVA-specific T cells induce AD after adoptive transfer

To determine the pathogenicity of skin- and gut-activated T cells, we took skin biopsies of the sites exposed e.c. to OVA or SAL

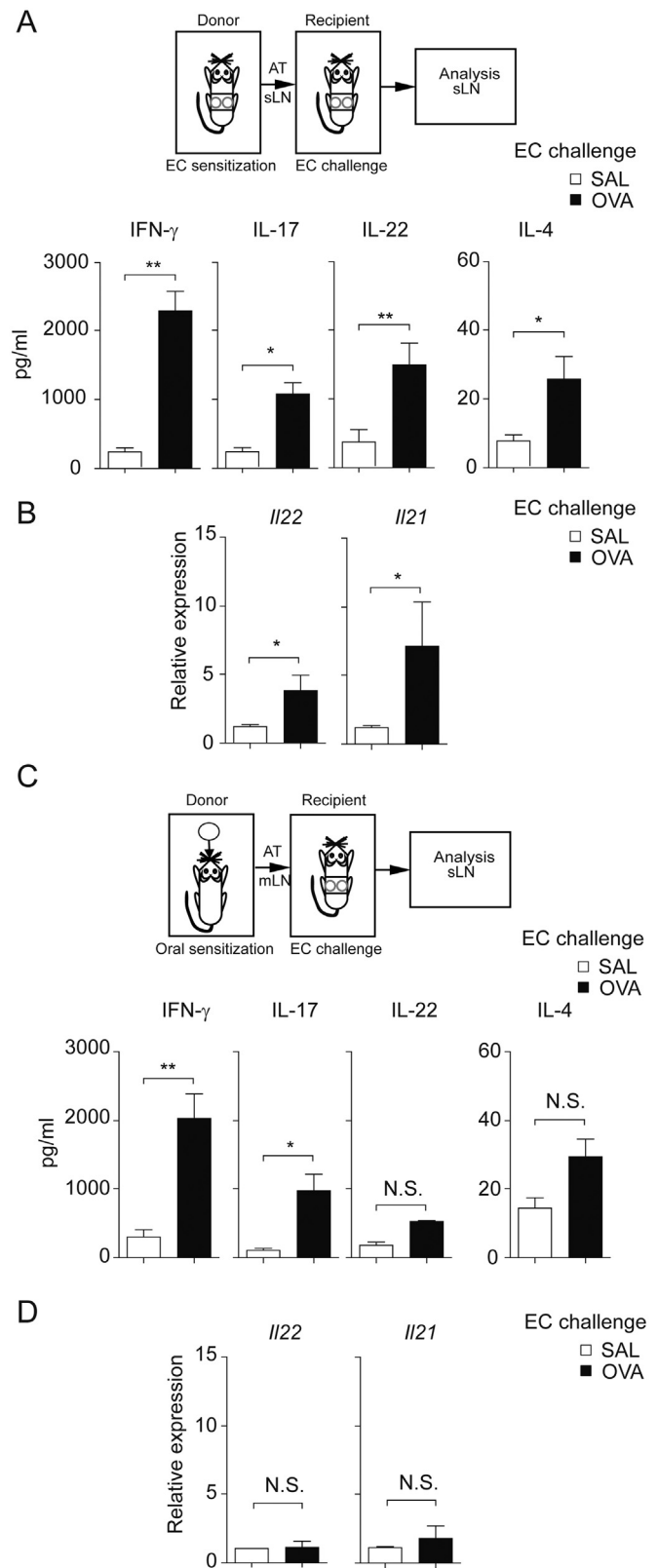


Fig. 4. Induction of IL-22 and IL-17 production after transfer of skin primed OVA specific T cells and epicutaneous OVA challenge. (A to D) Mice were sensitized as described and donor EC T cells (A, B) or donor ORAL T cells (D, C) were transferred. Recipient mice were challenged e.c. with SAL or OVA for one week. Thereafter sLN were isolated from each group and single cell suspensions were cultured with OVA peptide. Supernatants were collected and cytokine production was determined by ELISA (A, C) or by qPCR (B, D). Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; * $P < 0.05$; ** $P < 0.01$; Mann-Whitney test).

on day 7 after T cell transfer. A strong infiltration of the skin with lymphocytes was only found in biopsies from mice that received donor EC T cells and subsequent EC OVA challenge (Fig. 3 D, E). EC challenge with SAL did not result in skin lymphocyte infiltration. In contrast mice that received donor ORAL T cells showed less lymphocytes in the skin after EC OVA challenge. Histological scoring confirmed that only the skin of mice that received donor EC T cells and EC OVA challenge showed features of AD (Fig. 3E).

3.5. Induction of IL-22 and IL-17 producing OVA reactive T-cells in recipient mice that received donor EC T cells

To explain the differences in the pathology of OVA-specific T cells to induce skin inflammation, we restimulated draining sLN of BALB/c mice that received donor EC T cells or donor ORAL T cells and subsequent EC challenge with OVA or SAL with OVA peptide. T cells isolated from sLN of mice that received donor EC T cells and OVA challenge showed a strong production of IFN- γ , IL-17, IL-22 and some IL-4 production (Fig. 4A). By qPCR significant levels of IL21 and IL22 were detected (Fig. 4B). T cells isolated from mice that received donor ORAL T cells and EC OVA challenge also produced high levels of IFN- γ , IL-17 and some IL-4 (Fig. 4C). Of note, no significant IL-22 production was observed, indicating that this cytokine is critical for the generation of inflammatory dermatitis in this model. We could confirm this by qPCR analysis (Fig. 4D).

3.6. Induction of IL-22-producing T cells by epicutaneous sensitization

We next analyzed the phenotype of the originally transferred donor EC T cells and donor ORAL T cells. DO11.10 mice were exposed to OVA by EC or oral routes as described in Fig. 2. Lymphocytes from sLN or mLN were restimulated with OVA peptide. Supernatants were collected and cytokine secretion was determined by ELISA. The OVA-specific response of donor EC T cells showed a significant production of IFN- γ , IL-4, IL-17 and IL-22 upon restimulation with OVA peptide (Fig. 5A–D). As expected, feeding OVA did not change cytokine production in sLN. In contrast, T cells from mice fed with OVA showed increased IL-4 and reduced IL-17 production in mLN (Fig. 5A–D).

3.7. Induction of proinflammatory cytokines in skin biopsies from DO11.10 mice after EC-sensitization with OVA

The analysis of donor EC T cells in Fig. 5 showed a predominant Th17/Th22 phenotype. To recapitulate this type of immune response at the sites of OVA-induced AD we analyzed cytokine expression in the skin. Biopsies from the Finn chamber location sites of the back skin of DO11.10 mice sensitized e.c. with OVA or SAL were used for qPCR analysis. The inflamed skin of OVA-sensitized mice showed a significant expression for the Th17/Th22 cytokines *Il17a* and *Il22* (Fig. 6). We also found high expression of *Il23a*. Analysis of the Th cell lineage-defining transcription factors showed enhanced expression of *Tbx21* and *Roryt* (Fig. 6). Thus, the Th17/Th22-dominated immune response to OVA in sLN is also present at the site of OVA-induced AD.

3.8. IL-22 and IL-17 expression in the skin of BALB/c mice after transfer of skin-primed OVA specific T cells and epicutaneous OVA challenge

Since only donor EC T cells with a Th17/Th22 phenotype were capable to induce dermatitis in BALB/c recipient mice challenged e. c. with OVA peptide, we finally tested the cytokine expression of the inflammation at the sites of skin OVA exposure. By quantitative PCR analysis of skin samples we found high expression of the Th17/Th22 cytokines *Il22*, *Il17a* and *Il21*. Moreover, *Roryt* expression and

Ifn γ expression showed a significant upregulation in the skin of mice challenged with OVA compared to SAL challenge (Fig. 7). In addition, we could detect a significant induction of *Ccr4*, while *Ccr6* or *Ccr10* expression in the skin were not affected, underlining the importance of the recruitment of pathogenic Th22 cells into the skin of OVA-induced dermatitis by EC allergen exposure.

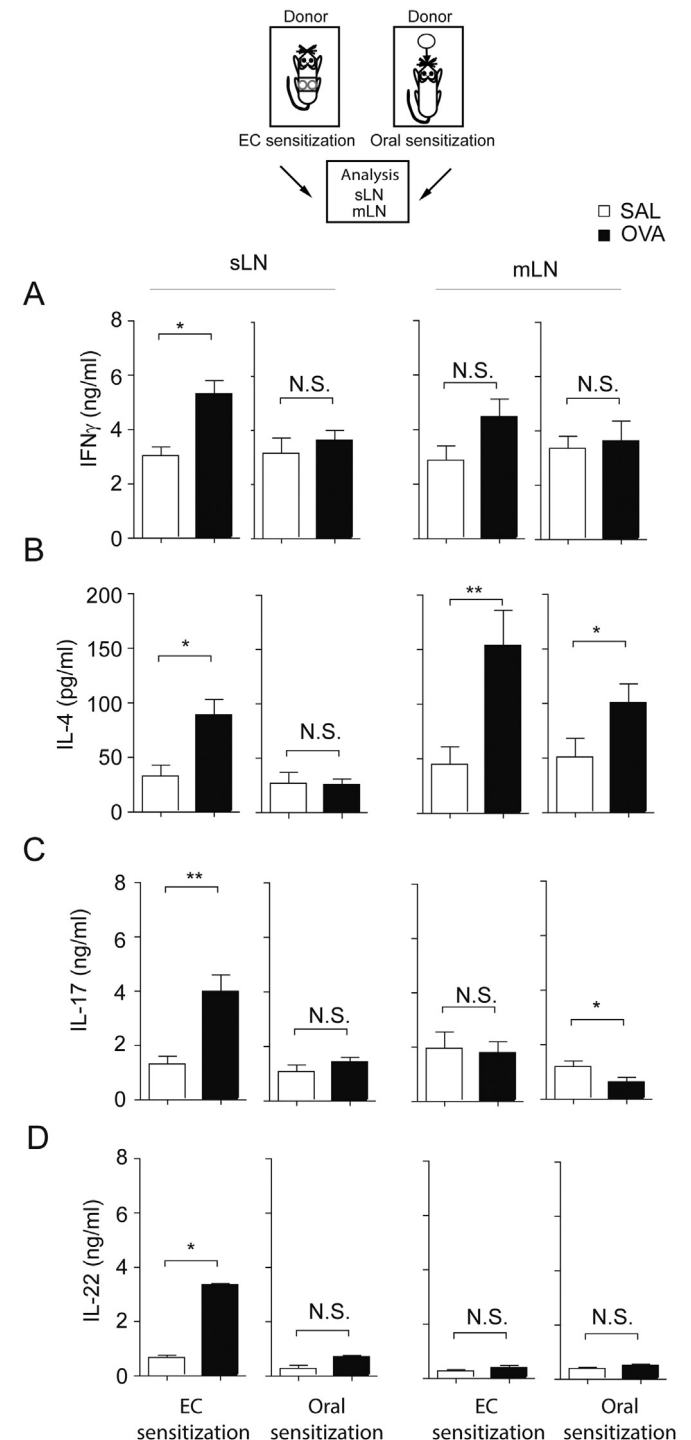


Fig. 5. Induction of IL-22 in skin draining lymphnode cells after EC-sensitization. DO11.10 mice were sensitized e.c. or orally with OVA as described. (A to D) cells from sLN and mLN were isolated and single cell suspensions were restimulated with OVA peptide. Supernatants were collected and cytokine production was determined by ELISA. Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; * $P < 0.05$; ** $P < 0.01$; Mann-Whitney test).

4. Discussion

Numerous immunological mechanisms are involved in the pathogenesis of inflammatory skin diseases like AD. A hallmark of AD is a dry and itchy skin with defects in skin barrier function [1,2]. From a clinical perspective, food allergens have been proposed to influence the clinical course of AD [20]. We aimed to use a mouse model that allows study the pathogenicity of food allergen-reactive T cells activated either through the skin or by oral uptake for the development of AD.

We decided to use OVA as allergen. As main protein in hen's egg white, OVA is part of the daily nutrition but can also act as allergen. IgE-mediated reactions to OVA are among the most frequent food allergies in childhood [21]. Cellular T cell responses to OVA have also been described in children with egg allergy or AD [22,23]. In experimental mice, OVA is frequently used as model antigen for T cell responses. The use of OVA TCR-transgenic DO11.10 mice allows identify allergen-specific T cells [24].

After AT of OVA TCR-transgenic T cells BALB/c mice were immunized s.c. with OVA in CFA. By this, we could follow the allergen-specific *in vivo* distribution of T cells (Fig. 1). The highest enrichment of OVA-specific T cells in draining sLN was found on day seven after adoptive transfer (day three after immunization). For studying the role of food allergen-specific T cells in OVA, it is not ideal to use artificial adjuvants like CFA, which is known to influence T cell polarization [14]. To avoid such a bias and to use an experimental setting that is close to AD in humans we decided to use a protocol of EC sensitization as published previously [17]. Several experimental models for AD have been reported. Spontaneous AD develops in Nc/Nga mice that are housed in non pathogen-specific conditions. The inflammation in this model is dominated by Th2 responses [25]. Transgenic mice that over-express *IL4*, *IL31* or *TSLP* develop pruritus and dermatitis with features resembling human AD [26–28]. In other models EC application of sensitizers induces AD-like disease [17,29,30]. EC sensitization can also be applied to mice with genetic barrier dysfunction, e.g. filaggrin-deficient mice [31].

Our aim was to study the role of skin-activated and gut-activated T cells specific for a food allergen in AD pathogenicity. To generate skin-activated T cells we used a modified skin sensitization protocol performing three rounds of EC OVA treatment of DO11.10 mice. To induce gut-activated T cells we fed OVA intragastrically to DO11.10 mice (Fig. 2). No additional adjuvants were used in either protocol. Sensitization of epithelial surfaces with allergens typically requires DC mobilization and activation of T cells in draining lymph nodes. Therefore we isolated skin-activated OVA-specific T cells from draining sLN and gut-activated OVA-specific T cells from mLN. Continuous feeding of mice with OVA in drinking water as well as high OVA doses have been reported to induce T cell tolerance with decreased production of inflammatory cytokines and an enhanced regulatory T cell response [19,32,33]. To prevent tolerance induction, we fed OVA protein at a concentration of 100 mg/day over a period of 3 days using gavage needles. We were able to induce CD4⁺KJ1-26⁺ T cells with decreased expression of CD62L selectively in the gut-draining mLN, when feeding OVA but not SAL (Fig. 2F). Importantly, we did not induce anergy or regulatory T cells (Fig. 2G) [19].

In the following we transferred OVA-specific T cells from sLN of EC sensitized mice or from mLN of OVA-fed mice into naïve BALB/c mice and challenged the recipient mice with OVA. Mice that received donor OVA-specific T cells from sLN of mice treated EC developed AD on day 7 after transfer and EC challenge with OVA (Fig. 3D, E). With this modified protocol we were able to induce AD with histological features as described in the original EC sensitization protocol [17,18].

The immune response in the OVA EC sensitization model was initially thought to be mainly Th2 and Th1 dominated [17,18]. In addition, the tape stripping procedure had been reported to be responsible for cutaneous Th2 responses [30]. In agreement with these earlier reports we also found a significant induction of IL-4 and IFN- γ production by Th cells from sLN of BALB/c mice that received donor OVA T cells from sLN and EC OVA challenge (Fig. 4). AD only developed after transfer of donor EC OVA T cells and EC OVA challenge and showed increased *Ifn γ* expression but no increased *Il4* expression in the skin (Fig. 7). Likewise, the analysis of sLN of donor DO11.10 mice sensitized e.c. with OVA also showed significant production of IL-4 and IFN- γ in response to OVA (Fig. 4). Interestingly, oral OVA feeding selectively induced the production of IL-4 by OVA-reactive T cells from mLN (Fig. 5B). The transfer of OVA-specific donor ORAL T cells and EC challenge with OVA induced IFN- γ production in sLN (Fig. 4C).

In addition to Th1/Th2 cytokines, we also found strongly elevated levels of Th17/Th22 cytokines in this model. In general, classical Th17 cytokines like IL-17A are expressed at lower levels in human AD than in other inflammatory skin diseases like psoriasis [34]. In contrast the Th17 cytokine IL-22 is highly expressed in AD and T cells with selective IL-22 production can be found in patients with AD [4]. Thus, human AD pathogenesis seems to be dominated by a Th2/Th22 response [5]. Recent studies in the OVA EC

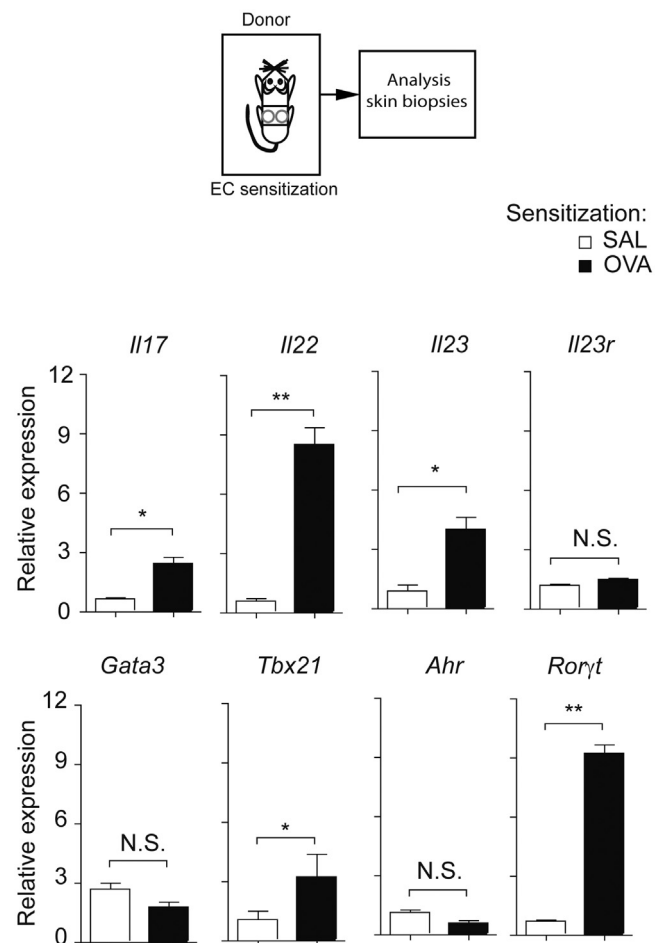


Fig. 6. Immune signature in AD skin of DO11.10 mice after EC-sensitization with OVA. Donor DO11.10 mice were treated as in Fig. 2A and mRNA expression in skin biopsies after EC-sensitization was quantified by RT-PCR. Data were normalized to β -actin, and levels of investigated genes in control naïve mice were set as 1.0. Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; * $P < 0.05$; ** $P < 0.01$; Mann-Whitney test).

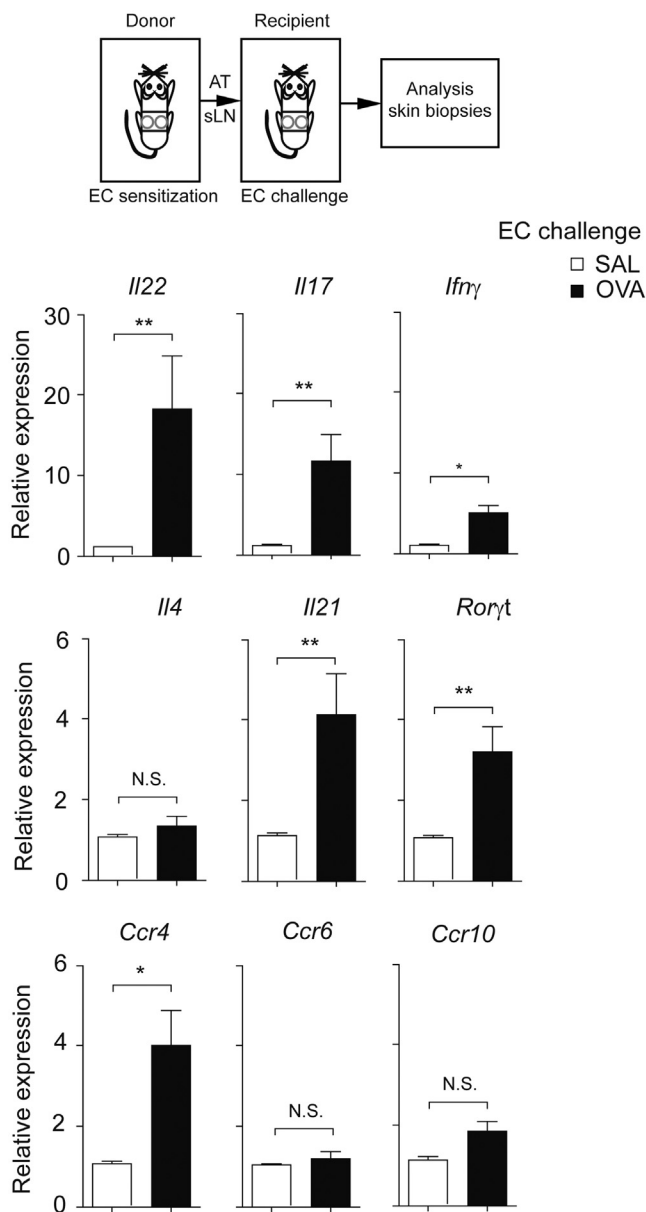


Fig. 7. Immune signature in AD skin of BALB/c mice after transfer of donor EC T cells and epicutaneous OVA challenge. Quantitative RT-PCR analysis of cytokines, transcription factors and chemokine receptors. Data were normalized to β -actin, and levels of investigated genes in control naïve mice were set as 1.0. Bars represent mean $\pm$ SEM of 3–5 independent experiments (N.S. not significant; * $P < 0.05$; ** $P < 0.01$; Mann-Whitney test).

sensitization model showed that the inflammation also involves high amounts of IL-17A expression and such a Th17 response is even enhanced in the absence of the Th2 cytokine IL-4 [35]. This is not surprising, since IL-4 is a negative regulator of Th17 responses and Th17-mediated skin inflammation [36]. Another Th17 cytokine that is implicated in the pathogenesis of human AD and in the OVA EC sensitization model is IL-21 [37]. IL-21 is upregulated upon tape stripping and influences DC migration [37]. In BALB/c recipient mice, a significant expression of IL-21 was only observed after transfer of donor EC T cells and OVA challenge, but not in mice that received donor ORAL T cells and OVA challenge (Fig. 4). Likewise, high *Il21* expression was also found in the skin of recipient mice that developed AD upon OVA challenge (Fig. 7). Neither SAL challenge nor transfer of OVA donor ORAL T cells had an influence

on *Il21* expression, indicating that skin-activated T cells are the source of IL-21 in our model.

Activation of food allergen-specific T cells by EC antigen exposure upon tape stripping induces an IL-22-dominated phenotype in draining sLN that is skin-pathogenic even after T cell transfer (.). The major cytokine driving IL-22 production by T cells and innate lymphoid cells is IL-23 [38,39]. More recently, it has been shown that tape stripping in the EC OVA sensitization model induces the release of IL-23 by keratinocytes and this augments IL-23 production by IL-23R⁺ skin DC [40]. Of note, EC sensitization of donor DO11.10 mice showed a significant induction of *Il23* expression in the skin (Fig. 6). Moreover, the expression of the Th17/Th22 cytokines *Il17A* and *Il22* as well as the transcription factors *Tbx21* and *Roryt* were significantly induced in OVA-treated skin. This immune phenotype resembles the characteristics of pathogenic Th17 cells in mouse models of inflammatory CNS disease. Such pathogenic Th17 cells have been shown to transfer encephalomyelitis in recipient mice [38].

As demonstrated in this study, oral exposure to antigen in the absence of additional adjuvants does not induce a Th22 phenotype and food allergen-specific T cells from draining mLN do not induce skin inflammation upon EC antigen challenge. In a published model using BALB/c mice and oral OVA immunization EC OVA challenge resulted in AD, even when T cells from mLN were adoptively transferred and the recipient mice were challenged e.c. with OVA [16]. Like in our model, gut-activated donor OVA T cells could home to the skin following EC OVA challenge. The migration of skin-homing T cells has been reported to depend on CCR4 expression by T cells. This is in agreement with our results. We also found a selective upregulation of *Ccr4* but not *Ccr6* or *Ccr10* in the skin of recipient mice that developed AD after transfer of donor EC T cells and OVA challenge (Fig. 7). However, our donor ORAL T cells were not pathogenic upon transfer and OVA challenge. The different outcome by our study and the study by Oyoshi et al. can be explained by the different protocols for oral activation. We did not use additional adjuvants and a short period of OVA feeding and different antigen dose. Oyoshi and colleagues used cholera toxin, a 8-week period of OVA sensitization and a low antigen dose [16]. Another recent study fed BALB/c mice with OVA in drinking water and then performed EC sensitization with OVA as described by Spergel and colleagues [17,32]. They found that OVA in drinking water inhibits *Il5* and *Il13* expression and impairs the infiltration of the skin with Th2-like cells [32]. The impact on Th22 responses has not been studied by the authors.

In the present study, we found that tape stripping and EC OVA sensitization induces the generation of an IL-22-dominated T cell response with additional expression of IL-17A, IFN- γ and some IL-4 that resembles the T cell phenotype observed in the skin of humans with AD. The responsible factors for this type of immune response seem to be local IL-23 and IL-21, which both have been shown to be induced by mechanical injury to the skin. In contrast, oral feeding of OVA induces IL-4 and IFN- γ production but suppresses IL-17A in mLN. In addition OVA feeding had no impact on IL-22 production. As a consequence, adoptive transfer of orally activated OVA-specific T cells did not induce AD upon EC OVA challenge. Taken together, the model presented here can be used in further studies to unravel additional environmental, cytokine or chemokine factors that may determine the protective or pathogenic role of EC or orally-activated food allergen-specific T cells in the development of AD.

Conflict of interest

The authors have no conflict of interest to declare.

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