

Article Type: Regular Manuscript

Titel page

Titel:

Human atopic dermatitis skin-derived T cells can induce a reaction in mouse keratinocytes *in vivo*

Short title (max. 50 characteres incl. space): Humanized AD skin-derived T cell mouse model

Britta C Martel PhD.^{1,2,3}, Lars Blom PhD.¹, Beatrice Dyring-Andersen, MD. PhD.¹, Lone Skov, MD. PhD.¹, Kristian Thestrup-Pedersen, MD. PhD.¹, Søren Skov, PhD.², Kresten Skak, PhD.³, Lars K. Poulsen, PhD.¹

1 Department of Dermato-Allergology, Gentofte Hospital, University of Copenhagen, Denmark

2 Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

3 LEO Pharma A/S, Ballerup, Denmark

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/sji.12316
This article is protected by copyright. All rights reserved.

Corresponding author:

Britta C Martel

E-mail: brittapoulsen@hotmail.com

Address : LEO Pharma A/S

Industriparken 55, 2750 Ballerup

Denmark

Abstract

In atopic dermatitis (AD), the inflammatory response between skin infiltrating T cells and keratinocytes is fundamental to the development of chronic lesional eczema. The aim of this study was to investigate whether skin-derived T cells from AD patients could induce an inflammatory response in mice through keratinocyte activation and consequently cause development of eczematous lesions. Punch biopsies of lesional skin from AD patients were used to establish skin-derived T cell cultures and which were transferred into NOD.Cg-*Prkd^{scid} Il2rg^{tm1Sug}*/JicTac (NOG) mice. We found that subcutaneous injection of the human AD skin-derived T cells resulted in migration of the human T cells from subcutis to the papillary dermis followed by development of erythema and edema in the mouse skin. Furthermore, the human T cells induced a transient proliferative response in the mouse keratinocytes shown as increased numbers of Ki-67⁺ keratinocytes and increased epidermal thickness. Out of six established AD skin-derived T cell cultures, two were superior at inducing a skin reaction in the mice, and these cultures were found to contain >10% CCR10⁺ T cells compared to <2% for the other cultures. In comparison, blood-derived *in vitro* differentiated Th2 cells only induced a weak response in a few of the mice. Thus, we

This article is protected by copyright. All rights reserved.

conclude that human AD skin-derived T cells can induce a reaction in mouse skin through induction of a proliferative response in the mouse keratinocytes.

Introduction

Atopic dermatitis (AD) is a relapsing inflammatory skin disease appearing as eczematous lesions with intensely pruritic erythematous papules and an underlying immune response involving heavy influx of T cells to the dermis. The onset of eczematous lesions can be initiated by an intrinsic skin-barrier defect allowing increased penetration of irritants subsequently provoking a T helper 2 (Th2) associated inflammation, or may be initiated by an unbalanced immune response involving the Th2 associated cytokines IL-4, IL-5, and IL-13 leading to a defective skin-barrier [1, 2]. Irrespective of the primary cause, the inflammatory response in AD involves a vicious circle of self-perpetuating interactions between the skin-barrier and cells of the innate and adaptive immune response, including cross-talk between keratinocytes and T lymphocytes. Keratinocytes can secrete chemokines and cytokines that attracts T lymphocytes to the site of inflammation and stimulates an inflammatory response [3]. Likewise, cytokines secreted by the T cells activate the keratinocytes leading to enhanced production of cytokines, chemokine and other pro-inflammatory mediators, and also affect the physical properties of the skin-barrier [3]. A mouse model mimicking the interaction between keratinocytes and T cells found in human AD lesions, could provide a valuable tool for mechanistic studies and for evaluation of new treatments targeting these interactions. Some features of AD can be induced in mice through epicutaneous sensitization or genetic alterations. Also, spontaneous development of AD-like lesions has been observed in Nc/Nga mice, though, not under specific pathogen free conditions [4], highlighting the role of environmental stimuli in the development of AD, but limiting the possibility of using spontaneously induced mouse models. Recently, humanized mouse models based on xenotransplantation of *in vivo* educated human tissue and

This article is protected by copyright. All rights reserved.

immune cells from patients into severely immunocompromised mice, have shown that human leukocytes can induce development of both clinical and immunological characteristics of the respective disease, and in some cases these mice even reflect the actual disease status of the human donor [5, 6]. Nolte *et al.* [7] found that PBMCs from patients with AD injected i.v. into immunocompromised mice could migrate into the mouse skin at the site of oxazolone challenge. This indicates that despite cross-species differences, human T cells can to some extent interact with mouse keratinocytes. Hence, we hypothesized that human T cells derived from lesional skin of patients with AD could induce an inflammatory response in mouse keratinocytes consequently causing development of eczematous lesions. To test this hypothesis we established skin-derived T cell cultures from biopsies taken from lesional sites of patients with AD and transferred them to immunocompromised mice. We further hypothesized that an inflammatory response induced in the mouse epidermis by the human skin-derived T cells would share similarities with that of human lesional AD skin. Thus we aimed at evaluating the induced response in the keratinocytes by assessing an increase in proliferation by staining for Ki-67 and induced expression of the inflammatory protein S100A8 known to be expressed by human keratinocytes in AD lesional skin.

Materials and Methods

Donors

For this study, 4mm lesional skin biopsies from six patients were used and are referred to as AD1-AD6. In total, seventeen patients diagnosed with AD according to the criteria of Hanifin and Rajka [8], were included in this study, and AD1-AD6 were chosen, because the skin-derived T cell cultures established from their skin biopsies, were the most actively proliferating cultures when thawed. In addition, the seventeen patients were also included in another study (submitted). Information of sex, age, disease severity, and Total IgE levels are presented in Table 1. No systemic or topical immunosuppressants were used one month and

This article is protected by copyright. All rights reserved.

two week prior to sampling, respectively. All patients gave written informed consent in accordance with the Declaration of Helsinki Principles for Human Tissue Research. The study was approved by the local ethics committee (H-04-2011-068) and the Danish Data Protection Agency. Buffy coat fractions of blood portions originated from anonymized donors, whose identity has been anonymized for the researcher by the Blood Bank of the National University Hospital Copenhagen (Copenhagen, DK).

Skin-derived human T cells

T cell cultures were established by culturing at 37°C in a humidified incubator a 4-mm punch biopsy in a 25 cm² culture flask containing 5 ml RPMI-1640 (Sigma, St. Louis, MO, US) with heat-inactivated 10% sterile filtered human AB serum (Invitrogen, Carlsbad, CA US), IL-2 (proleukin) (1,000 U/ml) (Novartis, Basel, CH) , IL-4 (250 U/ml) (eBioscience, San Diego, US), penicillin (100 U/ml), streptavidin (100 µg/ml), and L-glutamine (1 mM) (Sigma). T cells were observed to migrate out of the skin biopsy into the surrounding culturing media, beginning at day 7 and remarkable proliferation was observed after approximately 14 days of culture. More than 98.5% of the isolated cells were CD4⁺ and/or CD8⁺ T cells (results not shown). Fresh media including cytokines were added to the cultures three times a week, or as needed, to maintain a cell density of 0.5 x 10⁶ cells/ml. When a cell cultures reached 500 x 10⁶ cells the cells were frozen.

In vitro differentiated human Th2 cells

Due to anonymization, the skin anamneses of the blood donors whose buffy coat fractions were used for *in vitro* differentiated human Th2 cells is unknown to us. The cultures established from these donors will be referred to as BD1 and BD2.

This article is protected by copyright. All rights reserved.

Human naïve CD4⁺ T cells (>99%) were isolated using the naïve CD4⁺ Cell Isolation Kit II (Miltenyi, Bergisch Gladbach, DE) as previously described [9]. The naïve CD4⁺ T cells (0.25 x 10⁶ cells/ml) were co-cultured with mitomycin C (Sigma, St. Louis, MO, US) treated mouse fibroblastic L cells (ATCC, CRL-10680) at a ratio of one fibroblast per five T cells. The naïve CD4⁺ T cells were cultured in RPMI 1640 (Sigma) supplemented with penicillin (100 U/ml), streptavidin (100 µg/ml), L-glutamine (1 mM), β-mercaptoethanol (50 mM) (Sigma) and heat-inactivated 5% sterile filtered human AB serum (Invitrogen). The naïve CD4⁺ T cells were cultured under Th2 conditions with anti-CD3 (0.03 µg/ml, UCHT1, BD Biosciences, San Diego, CA, US) and anti-CD28 (0.03 µg/ml, CD28.2, BD Biosciences), IL-4 (250 U/mL) (eBioscience), IL-2 proleukin (1000 U/mL) (Novartis), day 0-9 anti-IFN-γ (10 µg/ml), day 9-17 anti-IFN-γ (5 µg/ml), and day 22-26 anti-IFN-γ (2 µg/ml) (MD-1, eBioscience). The media were completely replenished on day 5, 9, 13, 17, and 22.

Mice

Female NOD.Cg-*Prkd^{scid} Il2rg^{tm1Sug}*/JicTac (NOG) mice from Taconic (Ry, Denmark) aged 8-12 weeks, were used for all studies. NOG mice are congenic nonobese diabetic (NOD) mice harboring a mutation leading to the severe combined immune deficiency (scid) defect and do not express functional common γ-chains and consequently lack T, B, and NK cells [10]. Mice were housed in the animal facilities at LEO Pharma, Ballerup, Denmark, in a specific pathogen free environment (20-23°C, relative humidity 30-60%, and 12h night/12h day cycle), with sterilized nesting- and enrichment materials and unlimited access to sterile water and feed (Altromin 1324, Brogaarden, Denmark). All the procedures performed on the mice were approved by the Danish Animal Experiments Inspectorate.

Transfer of human T cells to mice

Approximately five days prior to injection of human T cells, mice were tattooed (TT-101 Tech-Touch Advanced PMU System, Bella C., Taiwan) with four small dots to mark the injection site as a $<1\text{ cm}^2$ area on their lower right flank. At day 0, mice were injected either subcutaneously (s.c.) at the marked injection site or intravenously (i.v.) into the tail vein with 5×10^6 T cells in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) in a total volume of 100 μl . Subcutaneous injections at the marked injection site, with 10,000 IU and 2,500 IU of recombinant human IL-2 (Proleukin®, Novartis Pharmaceuticals, UK) and IL-4 (eBioscience, San Diego, US) respectively dissolved in PBS with 1% BSA (total volume 50 μl) were given on day 2, 4, 6, and 8. Subcutaneous injections with a human chemokine cocktail containing 0.5 μg of each of the recombinant human chemokines CCL5/RANTES, CCL17/TARC, and CCL27/CTACK (all Biolegend, San Diego, US) were given together with IL-2 and IL-4 (total vol 60 μl). After transfer of human skin-derived T cells, mice were weighed twice weekly and monitored daily for signs of reduced health e.g. reduced mobility, ruffled fur, and hunched back. Some mice developed an apparent clinical skin reaction (CSR) seen as erythema and in some cases additional edema at the s.c. injection site, but no scaling or scratching behavior was noticed. Heart blood was sampled under anesthesia with isoflurane followed by cervical dislocation. Spleens were collected in tubes containing cold PBS and stored on ice until further processing.

Flow cytometry analysis

100 μl of blood per animal was stained with the following mix of anti-human antibodies: PE-Cy7 conjugated CD45 (HI31, eBioscience, San Diego, CA, USA), FITC conjugated CD4 (RPA-T4), and PE conjugated CD8 (HIT8a) (both BD Biosciences, San Diego, CA, US). Following, the red blood cells were lysed with PharmLyse (BD) for 10 min at room temperature, the cells were washed, and resuspended in PBS with 1% fetal calf serum (FCS). Spleen cell suspensions were extracted by grinding the organs in sterile sives 40 μm . This article is protected by copyright. All rights reserved.

(BD falcon, cat.no.: 55352340) using a syringe piston. The spleen cell suspensions were lysed with PharmLyse (BD) for 15 min (37°C), washed, resuspended in PBS with 1% FCS, and subsequently stained with the same antibody mix as the blood. The T cell receptor variable region beta (TCRV β) analysis was performed using the IOTest[®] Beta Mark Kit (Beckman Coulter, Indianapolis, US) following the procedures of the manufacturer. The CCR expression of the T cell cultures was analyzed using the following anti-human antibody mix: BV711 conjugated CD8, BV421 conjugated CCR7, BV510 conjugated CD193 (CCR3), BV605 conjugated CD194 (CCR4), AF647 conjugated CD191 (CCR1), APC conjugated CD195 (CCR5) (all BD Biosciences, San Diego, CA, US), PC-Cy7 conjugated CD4 (eBioscience, San Diego, CA, US), and PE conjugated CCR10 (R&D Systems, Minnesota, US). Fluorescence minus one (FMO) controls of all CCR specific antibodies was used for compensation. 7-AAD (BD Biosciences) was added to all samples prior to analysis to stain dead cells. Cells were analyzed using a LSR-II or a LSRFortessa flow cytometer and FACSDiva software (BD Biosciences).

Supernatant analysis

For supernatant analysis, cells were cultured for 24 hours with IL-2 but without IL-4 and the supernatant was stored at -80°C. Supernatants were analyzed for the following cytokines: IL-4, IL-5, IL-10, IL-12, IL-13, IL17A, IL17F, IL-22, and IFN- γ by multiplex magnetic bead assay (BioRad, Berkeley, CA, USA), according to the manufacturer's protocol on a Bio-Plex 200 System using Bio-Plex Manager 5.0 (BioRad).

Immunohistochemistry

Skin punch biopsies (5-mm), were collected in tubes containing a 10% formalin solution and following embedded in paraffin and stained by immunohistochemistry (IHC) with either

This article is protected by copyright. All rights reserved.

mouse anti-human CD4 (clone 4B12, Dako, Denmark; 1:40), mouse anti-human CD8 (clone 144B, Dako, Denmark; 1:100), rabbit anti-mouse Ki-67 (clone SP6, abcam, Cambridge, UK, 1:150), or rabbit anti-mouse MRP8 (S100A8) (clone EPR3554, abcam, Cambridge, UK, 1:800). Negative controls excluding the primary antibody were conducted for all stainings. For further details of the staining procedure see methods S1. Presence of >10 human CD4 or CD8 positive T cells in the mouse papillary dermis will be referred to as papillary dermal human T cells. Stained slides were scanned using a NanoZoomer 2.0 HT (Hamamatsu Photonics, Germany). Slides stained with antibodies against Ki-67 were analysed using Visiopharm software (Visiopharm, Denmark). Epidermis was gated manually and the number of Ki-67 positive cells within the gate was counted by Visiopharm and the number was normalized to the length of the gated area.

For scoring of the epidermal thickness, skin sections were stained with hematoxylin and eosin (HE) and scanned. Dermis was concealed and epidermal thickness was arbitrarily scored by a trained pathologist blinded to the treatment of the animals. The epidermal thickness score (ETS) was as follows: (1) 2 cell layers; (2) 2-3 cell layers; (3) 3 cell layers; (4) 2-4 cell layers; (5) 2-5 cell layers.

Statistics

Statistics were calculated using GraphPad PRISM version 5. The level of human T cells in the blood and spleen of mice injected with T cells from the same donor was compared by unpaired, two-tailed t tests. Differences in number of mouse keratinocytes staining positive for Ki-67/length and epidermal thickness score for mice with and without presence of papillary dermal human T cells were compared using a Mann Whitney test.

Results

Human AD skin-derived T cell cultures differ in CD4:CD8 ratio and secrete Th2 associated cytokines

This article is protected by copyright. All rights reserved.

Skin-derived T cell cultures were established using biopsies from lesional sites of patients with AD. Skin-derived T cell cultures were frozen when they reached 500×10^6 cells, which took on average 30.5 (21-46) (median (min-max)) days. During this time the T cells were analyzed by flow cytometry, demonstrating that the skin-derived T cells were of memory phenotype ($CD45RO^+CD45RA^-$) (unpublished data). Over time, the majority of the cultures became dominated by one T cell subset CD4, CD8, or CD4CD8 double positive, respectively (unpublished data). Similar changes were also observed after thawing, and the CD4, CD8 profile of the T cell cultures from the individual donors at the time of injection are listed in Table 1. In addition, the distribution of T cell receptor variable region beta (TCRV β) was analyzed from established T cell cultures, and it was found that the T cell cultures from all donors except for AD5, were primarily characterized by expression of one or two T cell TCRV β -chain (Figure S1). The levels of secreted cytokines in the culture supernatants were measured for T cells cultured with IL-2 but without IL-4 in the media for 24 hours. This analysis revealed presence of secreted T helper 2 (Th2) associated cytokines IL-4, IL-5, and IL-13. In contrast, the Th1 associated cytokine IFN- γ was only moderately detected in supernatant from one culture, and IL-12 or the Th17, Th22 and regulatory T cell associated cytokines IL-17A, IL-17F, IL-22 and IL-10 respectively, were not detected (Table 1). Thus, the skin-derived T cells were characterized as Th2/Tc2 cells.

Induction of a clinical skin reaction in mice by transfer of human AD skin-derived T cells

The human AD skin-derived T cells were transferred to NOG mice to investigate if they could induce a skin reaction in the mice. An overview of the experimental set up is presented in Figure 1. On day 0, mice were injected with 5×10^6 human T cells or saline s.c. or i.v.. Our previous experiments had shown increased survival of human skin-derived T cells in the circulation of mice receiving supplemental injections of human IL-2 and IL-4. Thus, s.c. injections with human IL-2 and IL-4 or saline were given on day 2, 4, 6, and 8. In addition, a

chemokine cocktail of human recombinant CCL5, CCL17, and CCL27 which are known to be elevated in AD lesional skin [11-13] was injected s.c. simultaneously with IL-2 and IL-4 in mice that had received i.v. injected human T cells from AD1. None of the mice showed any signs of xenogeneic graft versus host reaction (X-GVHR), such as weight loss, decreased mobility, or ruffled fur. At day 1, 59% of the mice injected s.c. with human T cells from AD1 or AD2 had a CSR at the site of injection. Additionally four mice developed a CSR from day 1 to day 3, after which the erythema and edema subsided. No CSR was observed in the control group of s.c. injected mice with human IL-2 and IL-4. In the skin of i.v. injected mice, human T cells were not detectable by immunohistochemical staining, and no signs of CSR were seen regardless of s.c. chemokine injections. Immunohistochemical staining of skin biopsies from mice with a CSR terminated day 1 or 3, revealed presence of papillary dermal human T cells (Figure 2). At day 6, papillary dermal human T cells were seen in all mice with a previously noted CSR, whereas at day 10 papillary dermal human T cells were only found in three of eight mice (Figure 3). Only a few of the mice injected with skin-derived T cells from the donors AD3-6 showed signs of a CSR or presence of papillary dermal human T cells (Figure 3). Unexpectedly, several of the mice receiving regular injections with saline instead of IL-2 and IL-4 developed a CSR and had papillary dermal human T cells, thus, the additional cytokine injections were not essential to induce a skin reaction in the mice. In summary, these results show, that human T cells derived from lesional AD skin can induce a skin reaction in NOG mice when injected s.c.. In addition, the development of a skin reaction was not restricted to CD4⁺ or CD8⁺ T cells subsets, as >90% of T cells from AD1 were CD8⁺ and >99% of the T cells from AD2 were CD4⁺.

Human AD skin-derived T cells induce transient keratinocyte proliferation but not expression of S100A8

To investigate if the skin reaction in the mice induced by the human T cells shared some of the characteristics found in human AD lesional skin, the epidermal thickness was scored, and the expression of the proliferation marker Ki-67 [14, 15], and the inflammatory protein S100A8 [16] in the epidermis of the mouse skin biopsies was analyzed by IHC. Only skin from mice injected s.c. or i.v. with skin-derived T cells from AD1 or AD2 and control mice not injected with human T cells were included in these analyses. A significant ($p<0.05$) difference in epidermal thickness was found comparing mice with papillary dermal human T cells and those without, but only at day 3 (Figure 4). In line with this finding, a significant ($p<0.05$) increase in the number of Ki-67⁺ keratinocytes/length of biopsy was found for mice with papillary dermal human T cells compared to those without, also at day 3. However, expression of S100A8 was only seen in dermal cells and no positive staining was detected in the mouse keratinocytes. Thus, the s.c. injected human skin-derived T cells did induce a transient proliferative response in the mouse keratinocytes, whereas they did not induce expression of the inflammatory protein S100A8.

Th2 associated cytokines may be involved in development of a clinical skin reaction

We further wanted to investigate if the development of a CSR in the mice was induced by the Th2 associated cytokines. Human naïve (CD45RA⁺CD45RO⁻) CD4⁺ blood T cells were differentiated *in vitro* into Th2 cells as previously described [9]. The culture time from purification to transfer into mice was 27 days and the Th2 phenotype was confirmed by flow cytometry analysis prior to injection (Figure S2). In addition, IL-5 and IL-13 have previously been measured in levels >1 ng in the supernatant of Th2 cultures differentiated *in vitro* using the same protocol [9]. When transferred s.c. to NOG mice a CSR was visible as low erythema in three out of 48 mice at day 1, whereas eight of the 48 mice had presence of papillary dermal human T cells at the day of termination (Figure 3). This indicates that the human Th2 associated cytokines could be involved in the induction of a CSR in the mice.

Regular injections of IL-2 and IL-4 increased the number of human T cells in the circulation. To detect human T cells present in the blood and spleen of mice, cells were stained with anti-human CD45, CD4, and CD8 and analyzed by flow cytometry. The gating strategy is presented in Figure 5, together with the results from blood analyzed at day 6. These showed that s.c. injection with human recombinant IL-2 and IL-4 every second day, did increase the number of human T cells in the blood. The same tendency was found for the level of detected human T cells in blood and spleen at day 1, 3, and 10, and for both AD skin-derived human T cells, and human *in vitro* differentiated Th2 cells. Hence, repeated s.c. injections with human IL-2 and IL-4 prolonged the presence of s.c. injected human skin-derived T cells in the circulation. In addition, the percentages of CD4⁺, CD8⁺ T cell subsets of the T cell culture at the time of injection was comparable to that of the T cells detected in blood and spleen of mice at day 6 by flow cytometry (Figure S3). In general, the level of s.c. transferred human T cells detected in the circulation of mice increased from day 1 to day 6, followed by a decrease from day 6 to day 10. The level of human T cells in the circulation was not increased in animals with a CSR and/or papillary dermal human T cells, compared to mice without any skin response.

Skin-derived T cell cultures inducing a clinical skin reaction in mice have more CCR10⁺ cells. Differences in chemokine receptor (CCR) expression between the T cell cultures were investigated in search for an explanation as to why the skin-derived T cells from AD1 and AD2 were superior at inducing a CSR in the mice. Expression of CCR1, CCR3, CCR4, CCR5, CCR7, and CCR10 was analyzed by flow cytometry, and showed that none of the T cells in any of the cultures were positive for CCR1, CCR3, CCR5, or CCR7. The majority of the T cells in the skin-derived cultures expressed CCR4, and ≥13% (average: 14.5%) of the T cells derived from AD1 and AD2 expressed CCR10, whereas ≤2% (average: 1%) of the skin-derived T cells from AD3-6 expressed this receptor (Table 2). This indicates, that the

capability of the s.c. injected T cells to induce a pronounced CSR in mice is dependent on CCR10 expression.

Discussion

We found that human T cells derived from lesional AD skin, expanded *in vitro*, and secreting Th2 associated cytokines can induce a CSR seen as erythema and edema in NOG mice when injected s.c.. The skin-derived T cell from AD1 and AD2 containing CCR10⁺ cells were superior at inducing a marked CSR in mice, most often accompanied by presence of papillary dermal human T cells. In contrast skin-derived T cells from AD3 – 6 and blood-derived *in vitro* differentiated Th2 cells from BD1 and BD2 all containing <2% CCR10⁺ cells only induced weak signs of CSR in a minority of the mice. The presence of papillary dermal human T cells was associated with a transiently increased proliferation of the mouse keratinocytes and an increased epidermal thickness at day 3, whereas epidermal expression of the inflammatory protein S100A8 was not induced in any of the mice.

All T cells derived from AD lesional skin were found to be of memory phenotype, and T cell receptor stimulation was not necessary to induce proliferation of these cells during *in vitro* culture. The *in vitro* culture time varied considerably between cultures with the T cells being the most efficient at inducing a CSR in mice were these having the shortest culture times, indicating that a more rapid *in vitro* culture-expansion increases the likelihood of successful induction of a CSR *in vivo*. Both donor AD1 and AD2 had low EASI scores and low total IgE levels compared to some of the other T cell donors, indicating that the donors overall disease severity was not a decisive factor for the ability of the respective skin-derived T cells to induce a CSR in the mice.

Even though the culture condition may have affected the *in vivo* phenotype of the skin-derived T cells, their status as Th2 cells secreting IL-4, IL-5, and IL-13 is in accordance with that found in AD lesions [16]. When exposed to IL-4 and IL-13, *in vitro* cultured human keratinocytes have shown intraepidermal edema (spongiosis) and decreased expression of *FLG* [17, 18]. Also, there is significant cross-species reactivity between mouse and human IL-5 [19] and IL-13 [20, 21], but not IL-4 [22]. This provides a potential role for the Th2 associated cytokines in the development of a CSR in the mouse skin which was assessed by use of naïve T cells purified from buffy coats and activated and differentiated into Th2 cells *in vitro*. When injected s.c. into mice, slight erythema was observed in a few of the mice at day 1 whereas none of the mice only receiving regular injection with IL-2 and IL-4 developed any signs of a CSR. This indicated that IL-5 and IL-13 might be involved in the induction of a CSR, although the skin-derived T cells from AD1 that was the most efficient at inducing a CSR secreted the lowest levels of both IL-5 and IL-13 of all the cultures, revealing that the production of cytokines did not correlate positively with a short *in vitro* culture time. Originally, the CD4⁺ T cells have been the main focus for studying the inflammatory response in AD. However, a recent study found CD8⁺ T cells to be essential for development of an allergen-induced skin inflammation in a house-dust mite sensitized mouse model [23]. Furthermore, the same group performed atopic patch test with house-dust mite extract on house-dust mite sensitive patients with AD and found an early influx of CD8⁺ T cells prior to influx of CD4⁺ T cells and the development of clinically visible symptoms [24], underlining the importance of CD8⁺ T cells in AD. In our studies, the skin-derived T cell cultures from AD1 and AD2 which most potently induced CSR in the mice had a predominance of CD8⁺ and CD4⁺ cells, respectively. Thus, in this model the ratio of CD4:CD8 T cells did not seem to be crucial for inducing a CSR in the mice. However, if CD8⁺ T cells are merely essential for the initiation of the inflammatory response then in accordance with our results they would not be mandatory for the pre-activated CD4⁺ T cells to induce a CSR in the mice.

The CSR could only be induced when the skin-derived human T cells were injected s.c., and not i.v.. As an attempt to induce migration of human T cells to mouse skin, a chemokine cocktail was injected s.c. in mice receiving skin-derived human T cells i.v.. Earlier experiments in mice have shown that i.d. injections of CCL27 induced migration of mouse T cells to the site of injection [25], however, the most effective concentration used in above mentioned study was 4 times higher than the one used in these studies for the individual chemokines. Nevertheless, we did not detect any human T cells at the chemokine injection site in any of our mice, even though the majority of the i.v. injected T cells were positive for the CCL17 ligand CCR4 and 13% were positive for the CCL27 ligand CCR10. It is possible that the chemokine gradient created upon s.c. injection was lost due to diffusion before the chemokine receptor expressing T cells in the blood would have had time to migrate to the site.

We found that the major factor discriminating between the ability of the skin-derived T cells to induce a CSR was the percentages of CCR10⁺ cells in the cultures. CCL28 is also a ligand for CCR10, and both CCL17, CCL27, and CCL28 possess cross-species reactivity between mouse and man [25-27]. CCL27 and to a lower degree CCL28 is constitutively expressed by keratinocytes, whereas CCL17 seem to be primarily expressed during inflammation [11, 25, 28-30]. It could be speculated that the levels of CCL27 and CCL28 secreted by mouse keratinocytes varies between individuals. Consequently, migration of the human CCR10⁺ T cells from subcutis to papillary dermis would be limited to mice with keratinocytes secreting CCL27 and/or CCL28 above a certain level. This could provide an explanation as to why papillary dermal human T cells were not seen in all mice injected s.c. with CCR10⁺ T cells. The CCL27- and CCL28- CCR10 interaction would place the skin-derived T cells in close proximity of the murine keratinocytes thereby increasing the possibility that human T cell secreted inflammatory mediators would actually reach the

This article is protected by copyright. All rights reserved.

murine keratinocytes. Thus, secretion of cytokines could play a central role in the induction of the observed CSR, though, for the cytokines to exert their function the secreting cells should preferably express CCR10 that will place them in close proximity of the keratinocytes. However, a critical role for CCR10⁺ is only indicated by this study, and should be addressed in further larger studies. The proliferation marker Ki-67, have been found to be up-regulated in keratinocytes in human AD lesional skin [14-16]. Mice with papillary dermal human T cells had a significantly increased epidermal thickness score, supported by an increased number of keratinocytes positive for Ki-67. This shows that the skin-derived human T cells can induce proliferation in the murine keratinocytes, although, transient as the response was only detectable in mice terminated day 3.

Finally, we did not observe any indications of an X-GVHR in our mice injected with skin-derived human T cell. It has been found that human T cells injected into mice retain their antigen specificity [31]. This might explanation why we did not observe any signs of an X-GVHR in our mice as the polyclonality of the injected T cell cultures were found to be low.

In conclusion, we found that human AD skin-derived T cells can induce a CSR in NOG mice upon s.c. injection. The Th2 associated cytokines IL-5 and IL-13 may be involved in the induction of the CSR, but additional skin-associated expression of CCR10 facilitating migration of the injected T cells from subcutis to papillary dermis seemed to be central for induction of a pronounced CSR. The CSR was associated with a transient induced proliferation of the keratinocytes, which disappeared as the T cells vanished from the skin. We suspect that the disappearance of the skin-derived T cells from the mice within two weeks is due to their culture time *in vitro* down-regulating expression of molecules enabling prominent engraftment. Although, a proliferative response was induced the mice

This article is protected by copyright. All rights reserved.

keratinocytes, no scratching behavior was noticed, and expression of S100A8 was not detected in the mouse epidermis, thus, the induced reaction in the mice only shared peripheral resemblance with human lesional AD skin.

Acknowledgements

Britta C Martel is supported by LEO Pharma A/S.

The authors wish to thank the patients for their participation in this study, and the laboratory animal staff and laboratory technicians at LEO Pharma for taking great care of the laboratory animals and for assistance and guidance with the experimental work. Also Kristian Moller and Andreas Hald are thanked for their help with analyses of epidermal thickness and Ki-67, and Hanne Norsgaard and Paola Lovato for valuable input during preparation of this manuscript. Department of Dermato-Allergology, Gentofte Hospital, University of Copenhagen, would like to thank Toyota Fonden (Denmark) for financial support to their Flow cytometer.

All authors have approved the submitted versions of this manuscript. Britta C Martel has drafted the paper and all the other authors have revised it critically. In addition, the authors have made substantial contribution to the following:

Britta C Martel: research design, acquisition, analysis, and interpretation of data; Lars Blom: research design, acquisition, analysis, and interpretation of data; Beatrice Dyring-Andersen: research acquisition and interpretation of data; Lone Skov: research design, acquisition and interpretation of data; Kristian Thestrup-Pedersen: research acquisition and interpretation of

This article is protected by copyright. All rights reserved.

data; Søren Skov: interpretation of data; Kresten Skak: research design and interpretation of data; Lars K. Poulsen: research design and interpretation of data.

References

- 1 Homey B, Steinhoff M, Ruzicka T, Leung DY. Cytokines and chemokines orchestrate atopic skin inflammation. *J Allergy Clin Immunol* 2006; 118:178-89.
- 2 Kawakami T, Ando T, Kimura M, Wilson BS, Kawakami Y. Mast cells in atopic dermatitis. *Curr Opin Immunol* 2009; 21:666-78.
- 3 Werfel T. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol* 2009; 129:1878-91.
- 4 Matsuda H, Watanabe N, Geba GP *et al.* Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997; 9:461-6.
- 5 Andrade D, Redecha PB, Vukelic M *et al.* Engraftment of peripheral blood mononuclear cells from systemic lupus erythematosus and antiphospholipid syndrome patient donors into BALB-RAG-2^{-/-} IL-2Rgamma^{-/-} mice: a promising model for studying human disease. *Arthritis Rheum* 2011; 63:2764-73.
- 6 Davis LS, Sackler M, Brezinschek RI *et al.* Inflammation, immune reactivity, and angiogenesis in a severe combined immunodeficiency model of rheumatoid arthritis. *Am J Pathol* 2002; 160:357-67.
- 7 Nolte T, Zadeh-Khorasani M, Safarov O *et al.* Induction of oxazolone-mediated features of atopic dermatitis in NOD-scid IL2Rgamma(null) mice engrafted with human peripheral blood mononuclear cells. *Dis Model Mech* 2013; 6:125-34.

- 8 Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 1980; Suppl 92:44-47.
- 9 Blom L, Poulsen LK. In vitro Th1 and Th2 cell polarization is severely influenced by the initial ratio of naive and memory CD4+ T cells. *J Immunol Methods* 2013; 397:55-60.
- 10 Ito M, Hiramatsu H, Kobayashi K *et al.* NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 2002; 100:3175-82.
- 11 Hijnen D, De Bruin-Weller M, Oosting B *et al.* Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. *J Allergy Clin Immunol* 2004; 113:334-40.
- 12 Kakinuma T, Nakamura K, Wakugawa M *et al.* Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001; 107:535-41.
- 13 Niwa Y. Elevated RANTES levels in plasma or skin and decreased plasma IL-10 levels in subsets of patients with severe atopic dermatitis. *Arch Dermatol* 2000; 136:125-6.
- 14 de Jongh GJ, Zeeuwen PL, Kucharekova M *et al.* High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis. *J Invest Dermatol* 2005; 125:1163-73.
- 15 Khattri S, Shemer A, Rozenblit M *et al.* Cyclosporine in patients with atopic dermatitis modulates activated inflammatory pathways and reverses epidermal pathology. *J Allergy Clin Immunol* 2014; 133:1626-34.

- 16 Gittler JK, Shemer A, Suarez-Farinas M *et al.* Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol* 2012; 130:1344-54.
- 17 Kamsteeg M, Bergers M, de Boer R *et al.* Type 2 helper T-cell cytokines induce morphologic and molecular characteristics of atopic dermatitis in human skin equivalent. *Am J Pathol* 2011; 178:2091-9.
- 18 Howell MD, Kim BE, Gao P *et al.* Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2007; 120:150-5.
- 19 Campbell HD, Tucker WQ, Hort Y *et al.* Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5). *Proc Natl Acad Sci U S A* 1987; 84:6629-33.
- 20 de Vries JE. Molecular and biological characteristics of interleukin-13. *Chem Immunol* 1996; 63:204-18.
- 21 McKenzie AN, Culpepper JA, de Waal Malefyt R *et al.* Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci U S A* 1993; 90:3735-9.
- 22 Park LS, Friend D, Sassenfeld HM, Urdal DL. Characterization of the human B cell stimulatory factor 1 receptor. *J Exp Med* 1987; 166:476-88.
- 23 Hennino A, Vocanson M, Toussaint Y *et al.* Skin-infiltrating CD8+ T cells initiate atopic dermatitis lesions. *J Immunol* 2007; 178:5571-7.
- 24 Hennino A, Jean-Decoster C, Giordano-Labadie F *et al.* CD8+ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. *J Allergy Clin Immunol* 2011; 127:1064-7.

- 25 Homey B, Alenius H, Muller A *et al.* CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* 2002; 8:157-65.
- 26 Santulli-Marotto S, Fisher J, Petley T *et al.* Surrogate antibodies that specifically bind and neutralize CCL17 but not CCL22. *Monoclon Antib Immunodiagn Immunother* 2013; 32:162-71.
- 27 Wang W, Soto H, Oldham ER *et al.* Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2). *J Biol Chem* 2000; 275:22313-23.
- 28 Morales J, Homey B, Vicari AP *et al.* CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci U S A* 1999; 96:14470-5.
- 29 Kagami S, Kakinuma T, Saeki H *et al.* Increased serum CCL28 levels in patients with atopic dermatitis, psoriasis vulgaris and bullous pemphigoid. *J Invest Dermatol* 2005; 124:1088-90.
- 30 Pan J, Kunkel EJ, Gossler U *et al.* A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. *J Immunol* 2000; 165:2943-9.
- 31 Petzelbauer P, Groger M, Kunstfeld R, Petzelbauer E, Wolff K. Human delayed-type hypersensitivity reaction in a SCID mouse engrafted with human T cells and autologous skin. *J Invest Dermatol* 1996; 107:576-81.

Tables

Table 1. Characteristics of the six AD T cell donors and the skin-derived T cell cultures.

Donors	AD1	AD2	AD3	AD4	AD5	AD6
Sex	K	M	M	K	K	K
Age	22	22	56	47	27	25
EASI Score	3.7	2	31.8	7.5	0.9	8.2
Total IgE (kU/L)	8.41	23.4	256	549	22.3	60.6
T cell cultures	AD1	AD2	AD3	AD4	AD5	AD6
Days of culture**	27	21	46	35	34	27
% Living cells*	97.1	96.5	95.8	82.9	90.3	82.5
CD4**	7.9	99.8	100	34.2	99.0	68.7
CD8**	91.4	0.1	0	1.7	0.0	30.4
CD4⁺CD8⁺	0	0	0	64.1	1.0	0.9
Supernatant (pg/ml)						
IL-4**	1326	1252	1328	1560	2222	1437
IL-5**	1.43	11.23	36.91	32.64	843.07	32.48
IL-13**	219	1767	2928	1112	3116	829
IFN-γ**	nd	nd	nd	nd	nd	7.04

* Analyzed day 0

** Analyzed prior to freezing

Table 2. Expression of CCR4 and CCR10 on human T cells from all 8 donors (AD1-AD6 and BD1-BD2).

Donors	AD1	AD2	AD3	AD4	AD5	AD6	BD1	BD2
CCR4	55%	86%	88%	71%	64%	72%	<0.5%	<0.5%
CCR10	13%	16%	1%	1%	<1%	2%	0%	0%

Figure legends

Figure 1. Experimental set up for transfer of human skin-derived T cells into NOG mice. Skin-derived T cell cultures were thawed one week prior to injection, and the mice were tattooed with 4 small dots marking the site of injection. Human skin-derived T cells were injected s.c. or i.v. at day 0 and mice received s.c. injections on day 2, 4, 6, and 8 with either IL-2+IL-4 or saline. Mice were euthanized at day 1, 3, 6, and 10, where blood and spleen was taken for flow cytometry analysis and skin biopsies for immunohistochemistry. * mice injected i.v. with human skin-derived T cells did receive s.c. injection with a chemokine cocktail consisting of human recombinant CCL5/RANTES, CCL17/TARC, and CCL27/CTACK.

Figure 2. Immunohistochemical staining of human CD4 and CD8 T cells.

Representative pictures from mice showing that s.c. injected human T cells has migrated from subcutis to papillary dermis. Negative controls seen in column 1, skin from mice not injected with human T cells stained with anti-human CD8 (upper row) and CD4 (lower row). Upper row and lower row, column 2-5, mice injected s.c. with T cells from donor AD1 and AD2 respectively and receiving regular IL-2 + IL-4 s.c. injections, stained with anti-human CD8 and CD4 respectively. Scale bars = 500µm.

Figure 3. Clinical skin reaction and presence of papillary dermal human T cells in mice injected with human T cells.

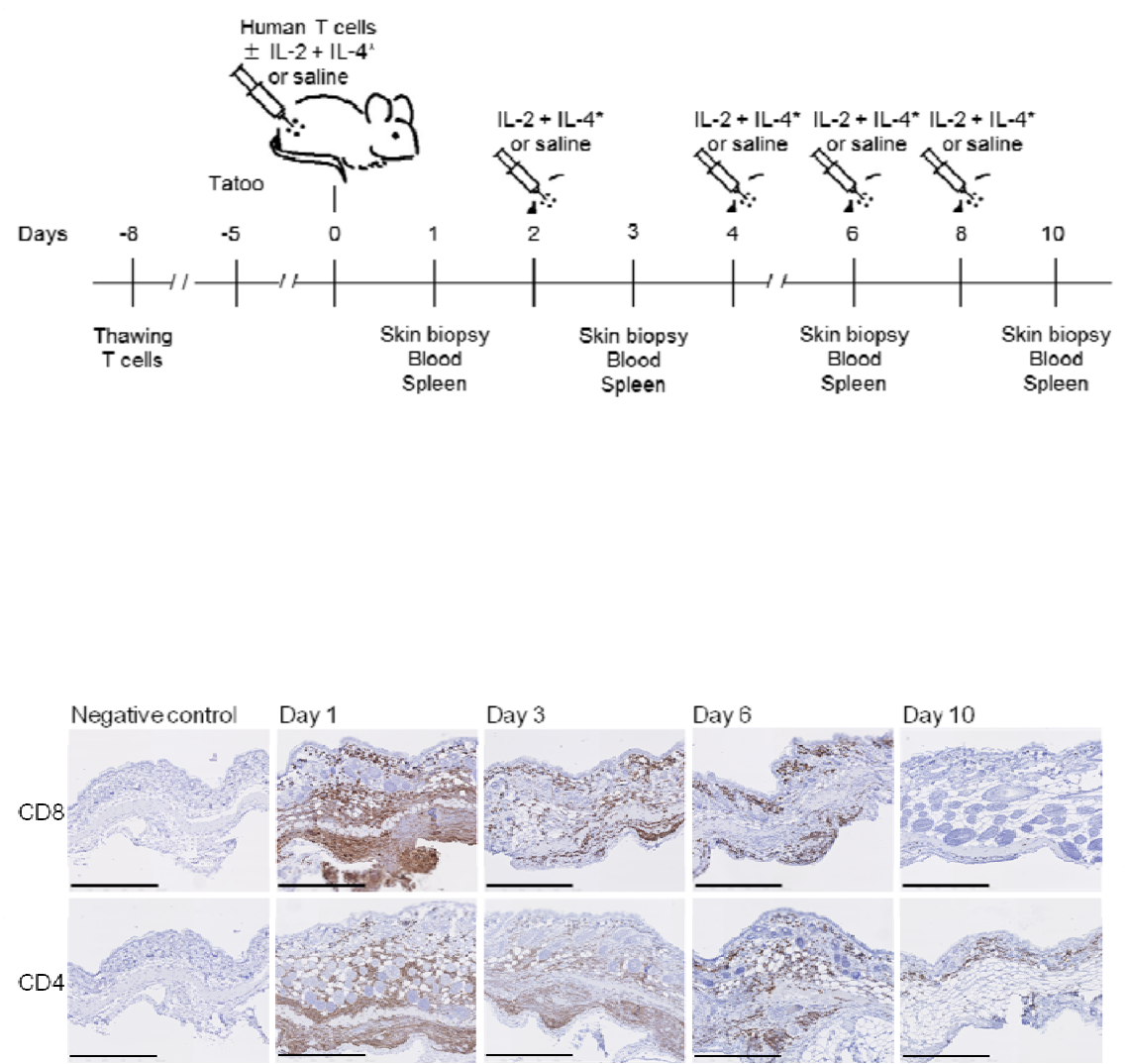
Mice were injected s.c. with human atopic dermatitis (AD) skin-derived T cells from one of six donors (AD1-AD6) or *in vitro* differentiated blood T cells from one of 2 donors (BD1-BD2) with or without IL-2 + IL-4 on day 0. Mice with a clinical skin reaction (CSR) visible as redness and occasional edema at any time point together with presence of papillary dermal human T cells (PDhTc) on the day of termination are represented by black bars. Mice with either CSR or PDhTc are represented by grey bars, and mice not showing any signs of CSR or PDhTc (no phenotype (NP)) are represented by white bars.

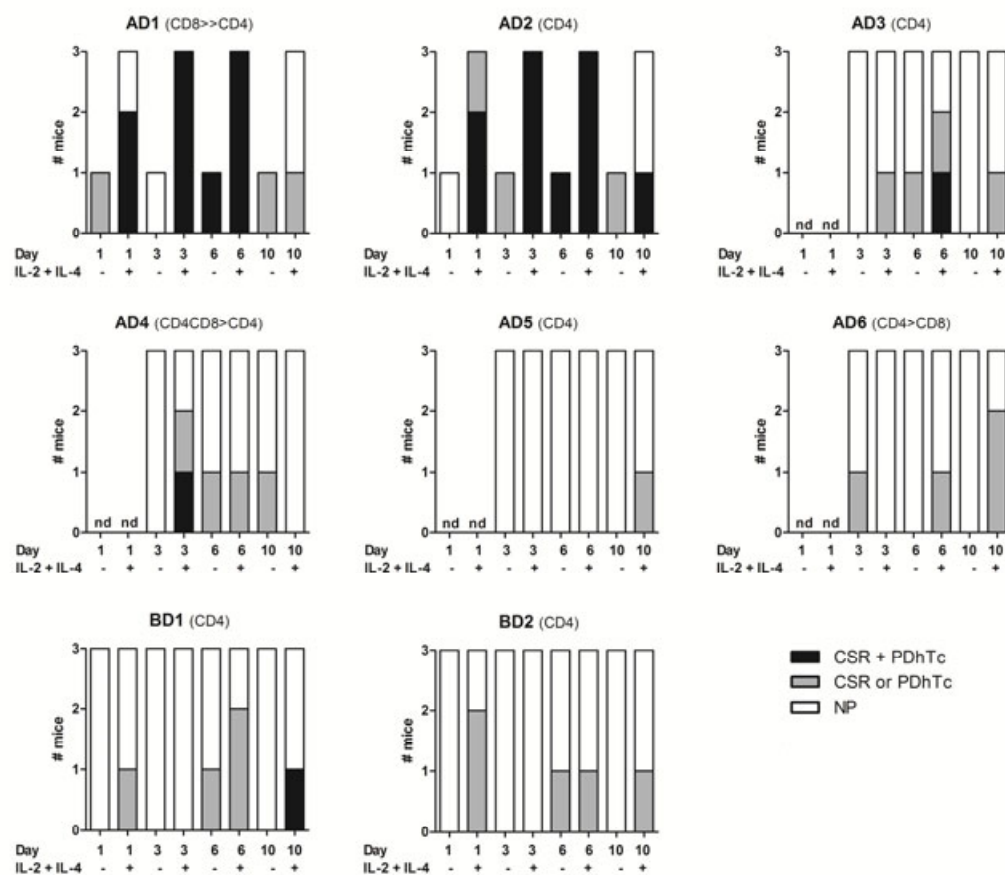
Figure 4. Human skin-derived T cells induced a proliferative response in mouse keratinocytes at day 3. (A) Epidermal thickness score (ETS) of epidermis from mice with and without presence of papillary dermal human T cells (PDhTc) at day 3. **(B)** The number of keratinocytes staining positive for the proliferation marker Ki-67/length of the analyzed epidermis in mice with and without presence of PDhTc at day 3. Horizontal bars: mean with SEM. Compared by Mann Whitney test. * $p < 0.05$; ** $p < 0.01$

Figure 5. Repeated s.c. injections of IL-2 and IL-4 increased the number of human T cells in the circulation. The level of human CD45⁺ T cells in the blood of mice day 6 after injection was analyzed by flow cytometry. **(A)** Gating strategy: gating on singlets, then FSC-SSC followed by living cells, cells positive for human CD45, and finally cells positive for human CD4, CD8, or both. Negative control blood from mice not injected with human T cells was included in all analysis. **(B)** Mice were either injected s.c. or i.v. on day 0 with human AD lesional skin-derived T cells (AD1-AD6), *in vitro* differentiated human Th2 cells (BD1-BD2), or saline in the right flank. Every second day mice received an s.c. injection of recombinant

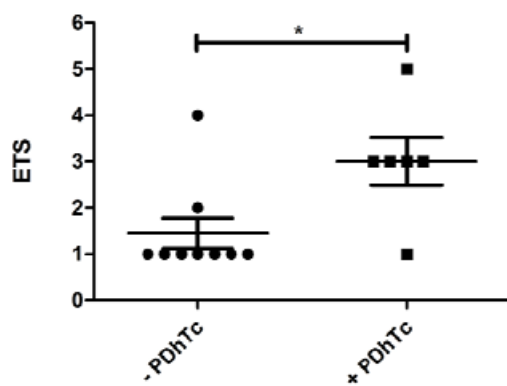
This article is protected by copyright. All rights reserved.

human IL-2 and IL-4 or saline, at the marked injection site. NC: blood from mouse not injected with human T cells. The level of human T cells in the blood of mice injected with T cells from the same donor was compared by unpaired, two-tailed T tests. * $p<0.05$; *** $p<0.001$. Horizontal bars: mean with SEM.





A



B

