Crosstalk between Keratinocytes and T Cells in a 3D Microenvironment: A Model to Study Inflammatory Skin Diseases

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The interaction between keratinocytes and immune cells plays a major role in the development of inflammatory skin diseases like psoriasis and atopic dermatitis. Pharmacological intervention to inhibit T cell–derived proinflammatory mediators is an effective therapy in the treatment of psoriasis. Here, we present a model to study the interaction between keratinocytes and T cells in a three-dimensional (3D) microenvironment, based on human skin equivalents populated with CD4+ T cells. T cell migration into the dermis initiated keratinocyte activation within 2 days, with hallmarks of a psoriasiform inflammation after 4 days. Expression of epidermal psoriasis marker genes was upregulated, and proinflammatory cytokines and chemokines were highly expressed. Disturbed epidermal differentiation was shown by downregulated filaggrin expression and involucrin expression in the spinous layer. These effects were mediated via soluble factors produced by the T cells. The psoriasiform inflammation was also observed using T helper type 1 (Th1)- and Th17-polarized CD4+ T cells. We validated our model by treatment with anti-inflammatory drugs that reduced the expression of proinflammatory cytokines and chemokines and suppressed the psoriasiform inflammation. We propose that our T cell–driven inflammatory skin equivalent model has potential to study the pathogenesis of inflammatory skin diseases and may serve as a preclinical screening tool for anti-inflammatory drugs.

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INTRODUCTION

Inflammatory skin diseases such as psoriasis and atopic dermatitis are highly prevalent and pose a significant burden on patients. Our understanding of these diseases is greatly advanced, but many aspects of their etiology and pathogenesis are still unknown. Recent genetic studies now lead to an emerging consensus that psoriasis and atopic dermatitis result from gene polymorphisms related to immune and skin barrier function (Palmer *et al.*, 2006; Roberson and Bowcock, 2010; Bergboer *et al.*, 2012). The translation of genetic characteristics to (immuno)biological function in these diseases is clearly a challenge as suitable models will require the presence of both keratinocytes and immune cells to study the crucial interaction between these cellular components.

At present, crosstalk between keratinocytes and lymphocytes is studied in submerged co-cultures (Muhr *et al.*, 2010; Renne *et al.*, 2010; Martin *et al.*, 2012), but these models are limited by the lack of a three-dimensional (3D) microenvironment such as found in the skin. The generation of a 3D human inflammatory skin model comprising both keratinocytes and lymphocytes has many requirements such as the correct geometry and spatial distribution of the cells, a matrix that allows lymphocyte trafficking, and a culture medium that allows adequate growth and survival of all cell types involved. In the absence of clinical parameters (erythema, itch), the readout relies on the quantification of molecular markers of disease. In psoriasis, the changes in the keratinocyte transcriptome are well documented with many molecular markers described, including Ki67, cytokeratin 16 (*KRT16*), elafin (*PI3*), psoriasin (*S100A7*), MRP8 (*S100A8*), and β-defensin-2 (*DEFB4*) (Suarez-Farinas *et al.*, 2012; Tian *et al.*, 2012); for gene and protein nomenclature of molecular markers used in this study, see Supplementary Table S1 online). In addition, proinflammatory cytokines and chemokines such as IL-6, IL-8, CXCL10, and CCL2 are highly

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Abbreviations: 3D, three dimensional; ATRA, all-trans-retinoic acid; CsA, cyclosporine A; Th, T helper; TNF-α, tumor necrosis factor-α

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upregulated in psoriatic keratinocytes (reviewed in Nickoloff *et al.*, 2007). Disturbed terminal differentiation in psoriatic plaques is witnessed by low levels of filaggrin, upregulated involucrin expression (Bernard *et al.*, 1988), and induction of the LCE3 family members, e.g., *LCE3A* (Bergboer *et al.*, 2011). For atopic dermatitis, only few studies describe molecular epidermal markers that discriminate between atopic dermatitis and psoriasis, such as *NELL2*, *TARC*, and *CA2* (Nomura *et al.*, 2003; Kamsteeg *et al.*, 2010). Atopic dermatitis has been associated with T helper type 2 (Th2) cytokines that are well documented for their effects on epidermal differentiation. The Th2-mediated inflammation reduces expression of epidermal differentiation genes, filaggrin, involucrin, and loricrin (Kim *et al.*, 2008; Howell *et al.*, 2009; van den Bogaard *et al.*, 2013) and induces eosinophil chemoattractants (e.g., CCL26) (Bao *et al.*, 2012).

Recently, *in vitro* cultured skin equivalents were successfully introduced in humanized mouse models and injection of psoriatic immune cells induced inflammation in these skin equivalents (Guerrero-Aspizua *et al.*, 2010; Carretero *et al.*, 2013), recapitulating features of psoriasis. However, the low-throughput, high costs, and ethical considerations of animal experimentation limits the use of these models for drug development. Inflammatory skin equivalents using disease-associated cytokines have been successfully developed by our group (Tjabringa *et al.*, 2008; Kamsteeg *et al.*, 2011), but these models lack the presence of immune cells. Attempts to generate *in vitro* human skin equivalents with immune cells resulted in human skin equivalents populated with Langerhans cells or dermal dendritic cells to study skin sensitization (Rees *et al.*, 2011).

In our current work, we report on the successful population of 3D skin equivalents with activated CD4+ T cells and Th1- and Th17-polarized T cells. These T-cell subsets and their cytokines are strongly associated with psoriasis pathology. We studied the crosstalk between the T cells and keratinocytes at the mRNA and protein levels upon direct and indirect contact between both cellular compartments. The keratinocyte transcriptome was indicative of a psoriasiform inflammation, and anti-inflammatory drugs reduced the inflammatory phenotype. This T cell–driven inflammatory skin equivalent model may aid to study pathogenesis of inflammatory skin diseases and may serve as a preclinical screening tool for anti-inflammatory drugs.

RESULTS

Migration of T cells in the dermis of human skin equivalents

To introduce T cells into the skin equivalents, fully developed skin equivalents were lifted from the transwell system and CD4+ T cells stimulated with anti-CD3/CD28 mAb-coated beads (further referred to as T cells) were placed between the transwell membrane and dermal side of the skin equivalents (Figure 1a). The activation status of the T cells was confirmed by flow cytometry (Supplementary Figure S1A online). We started off by adding 2×10^6 CD4+ T cells and observed T-cell migration in time, with T cells present in the dermis from day 2 onwards (Figure 1b). However, as the epidermal morphology was negatively affected at these high cell numbers (Figure 1c), we tested a concentration series and observed that with numbers as low as 0.25×10^6, T cells could still be demonstrated in the dermis, whereas epidermal morphology was preserved (Figure 1d). Activated T cells, just before addition to the skin equivalents, were able to produce IFN-γ, tumor necrosis factor-α (TNF-α), IL-17, and IL-22 (Figure 1e), but not IL-4 and IL-5 (data not shown). Nonactivated CD4+ T cells were unable to migrate and populate the dermis (Supplementary Figure S1B online). After 2 and 4 days of

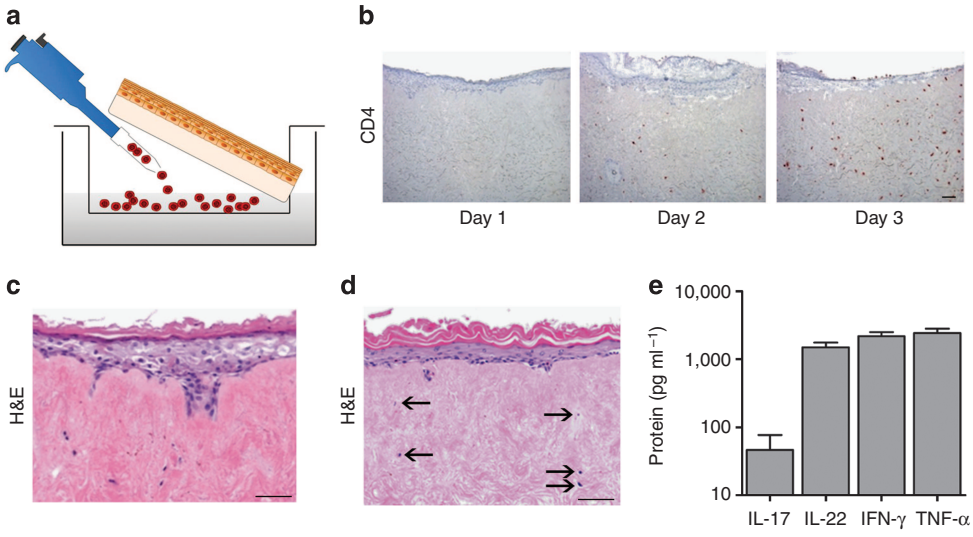

Figure 1. Activated CD4+ T cells migrate into skin equivalents. (**a**) The skin equivalents are lifted from the transwell support and T cells are placed underneath the dermis. (**b**) CD4 immunostaining of migrating T cells. Images are representative of $n = 2$ individual experiments. Bar = 100 μm. (**c**) Epidermal morphology of skin equivalents with 2×10^6 T cells at day 4 after T cell administration. Representative image of $n = 3$ individual experiments. Bar = 50 μm. H&E, hematoxylin and eosin. (**d**) Epidermal morphology of skin equivalents with 0.25×10^6 T cells. Arrows indicate T cells in the dermis. Representative image of $n = 3$ individual experiments. Bar = 50 μm. (**e**) Cytokine levels produced by the T cells just before addition to the skin equivalents, and after stimulation with anti-CD3/CD28 beads ($n = 5$ T-cell donors; mean ± SEM). TNF-α, tumor necrosis factor-α.

migration, we analyzed the gene expression levels of the T cells in the dermis of the skin equivalents and observed that the expression of proinflammatory cytokines was highest at 2 days of migration, and *IFNG* expression was relatively most abundant as compared with *IL17*, *IL22*, and *TNF* (Supplementary Figure S1C online).

Activated CD4+ T cells induce an activated, inflammatory phenotype in epidermal cells

After administration of 0.25×10^6 T cells to the skin equivalents (day 0), molecular markers of epidermal inflammation were determined at days 2 and 4 by quantitative PCR analysis. We found a time-dependent induction of the psoriasis-associated genes *DEFB4*, *PI3*, and *LCE3A*, whereas *KRT16* and *S100A7* reached a plateau at day 2 (Figure 2a and b). Molecular markers of atopic dermatitis, *CA2* and *NELL2*, were not induced (data not shown). Proinflammatory cytokine and chemokine production by the keratinocytes reached a maximum at day 2 (Figure 2c). Immunostaining of the inflammatory skin equivalents confirmed the mRNA expression data, showing induced expression of psoriasis-associated proteins hBD2, KRT16, and elafin (Figure 2d). As psoriatic skin is characterized by increased proliferation and disturbed differentiation, we analyzed these parameters in our model. The terminal differentiation process was clearly disturbed, as seen by reduced filaggrin expression and increased involucrin expression in the stratum spinosum (Figure 2e). However, Ki67 expression at the mRNA (Figure 2f) and protein levels (data not shown) was unaffected and no signs of acanthosis or hyperkeratosis were observed.

Direct contact is not absolutely required for crosstalk between keratinocytes and T cells

To address the question of whether the inflammatory phenotype is caused by direct contact between T cells and the skin equivalent or whether soluble factors produced by these cells suffice, we exposed skin equivalents to 0.25×10^6 T cells placed in the lower chamber of a transwell system (indirect) or allowed T cells to migrate into the dermis of the skin equivalents (direct). Only upon direct but not indirect contact,

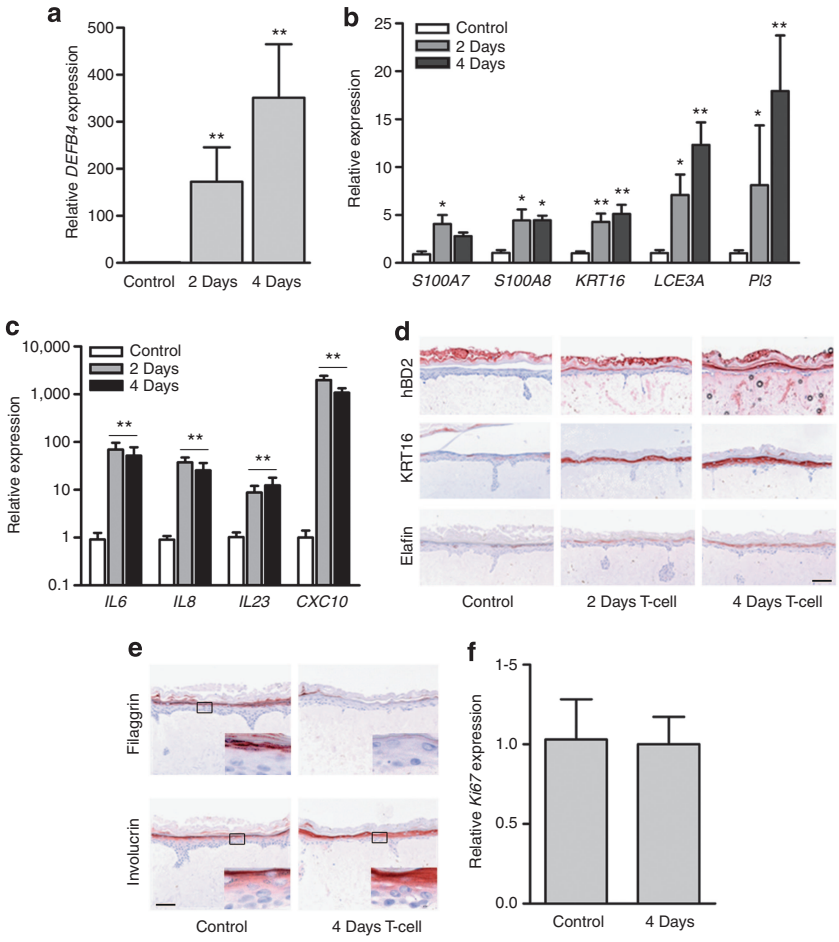

Figure 2. Epidermal response to T cells represents a psoriasiform phenotype. Keratinocyte mRNA expression levels of (**a**) *DEFB4* and (**b**) other psoriasis-associated genes in the epidermis after 2 and 4 days of 0.25×10^6 T-cell migration. (**c**) mRNA expression levels of proinflammatory cytokines and chemokines by keratinocytes after 2 days and 4 days of T-cell migration. (**d**) Immunostaining of psoriasis-associated proteins hBD2, KRT16, and elafin after 2 and 4 days of T-cell migration. Bar = 100 μm. (**e**) Epidermal differentiation proteins filaggrin and involucrin demonstrated by immunostaining. Bar = 100 μm. (**f**) *Ki67* mRNA levels of control and T cell–populated skin equivalents (for all mRNA data: $n = 5$ T-cell donors in 2 independent experiments, $n = 2$ keratinocyte donors; mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$ relative to control skin equivalents).

we observed epidermal inflammation as exemplified by the induced expression of psoriasis-associated marker genes *DEFB4* (Figure 3a), *LCE3A*, *PI3*, *KRT16*, and others (Figure 3b) and the expression of proinflammatory cytokines *IL6*, *IL8*, and *IL23* (Figure 3c). Of interest, upon indirect contact with the T cells, *CCL2* and *CXCL10* expression was highly upregulated in keratinocytes (Figure 3d). This would indicate that the T cells, through soluble factors, can activate keratinocytes to produce chemokines. We then wondered whether the relatively low cell numbers, and the associated concentration gradient of the proinflammatory mediators produced by the T cells, might have been a limiting factor with regard to the lack of disease marker expression. Therefore, we increased the number of T cells to 2×10^6 in the lower chamber. Although the epidermal morphology was still not affected, epidermal inflammation was instigated with upregulation of all investigated psoriasis-associated markers (Figure 3e and f) and proinflammatory cytokine expression (Figure 3g). As soluble factors produced by the T cells appear to be the main factor responsible for the inflammatory phenotype in our model, we measured the cytokine levels in the culture medium after 4 days of co-culture with skin equivalents. Indeed, higher levels of IFN-γ, IL-6, IL-22, IL-17, and TNF-α were associated with higher T-cell numbers (Figure 3h). The Th2 cytokines IL-4 and IL-5 were undetectable at either T-cell concentration, which could well clarify the lack of an atopic dermatitis phenotype in our model.

Th1- and Th17-polarized CD4+ T cells elicit psoriasis-like epidermal inflammation

Our skin equivalents populated with T cells express many molecular markers associated with psoriasis. As it is commonly accepted that psoriasis is a Th1/Th17-mediated disease, we used *in vitro* polarized Th1 and Th17 cells to populate the skin equivalents. To this end, we cultured naive CD4+ T cells under Th1- or Th17-polarizing conditions during 5 days using well-established cytokine and blocking antibody cocktails (Koenen *et al.*, 2008). Following T-cell polarization, the activation status, which is vital for migration, as well as cytokine expression of the polarized cells, was analyzed. All cultured T cells were proven activated based on CD69 expression (Supplementary Figure S2A online). As compared with activated T cells cultured for 5 days in the absence of polarizing cytokines/antibodies (referred to as nonpolarized T cells), the Th1-polarized cells showed clear induction of *IFNG* with a loss of *IL4* and *IL17A* (Figure 4a). In the Th17-polarized cells, *IL17A/F* and *IL22* was induced, whereas *IL4* expression was repressed. The *IL17A* expression in Th17-polarized cells was confirmed at the protein level (Figure 4b). We also attempted to include polarized Th2 cells. Although these cells showed downregulated expression of *IFNG*, *IL17A/F*, and *IL22* (Figure 4b), no expression of the Th2 cytokine *IL4* was induced. At 4 days after T-cell administration, the skin equivalents exposed to nonpolarized and Th1-, Th17-, or Th2-polarized cells were analyzed for signs of inflammation at the mRNA and protein levels. The Th1- and Th17-polarized T cells, but not nonpolarized or Th2 cells (Supplementary Figure S2C online), significantly induced *DEFB4* and *LCE3A* (Figure 4c) and *CXCL10* chemokine (Figure 4d) expression. In addition, a trend for upregulated *PI3* and *KRT16* was observed, but this was not statistically significant (Supplementary Figure S2B online). Induction of hBD2 and KRT16 was confirmed at the protein level by immunostaining (Figure 4e). Reduced filaggrin expression and spinous cell expression of involucrin indicated disturbed

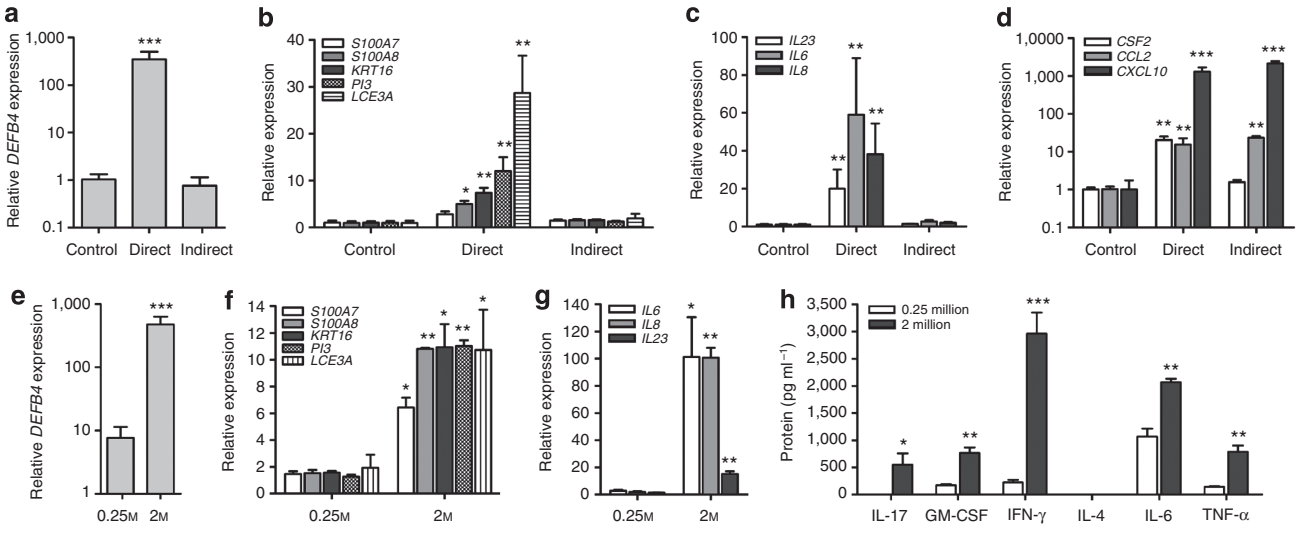

Figure 3. Crosstalk between T cells and keratinocytes upon indirect contact. Activated 0.25×10^6 T cells were allowed to migrate into the skin equivalents (direct contact) or placed in the lower chamber of the transwell system (indirect contact) for 4 days. Keratinocyte mRNA expression levels of (**a**) *DEFB4*, (**b**) other psoriasis-associated genes, (**c**) pro-inflammatory cytokines, and (**d**) chemokines upon indirect and direct contact. Keratinocyte mRNA levels of (**e**, **f**) psoriasis-associated genes and (**g**) proinflammatory cytokine expression when 2×10^6 T cells are used in the indirect model as compared with 0.25×10^6 T cells. (**h**) Proinflammatory cytokine levels measured in the culture medium of the indirect model after 4 days of co-culture with 2×10^6 T cells by Luminex (for all figures, $n = 3$ T-cell donors; mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). TNF-α, tumor necrosis factor-α.

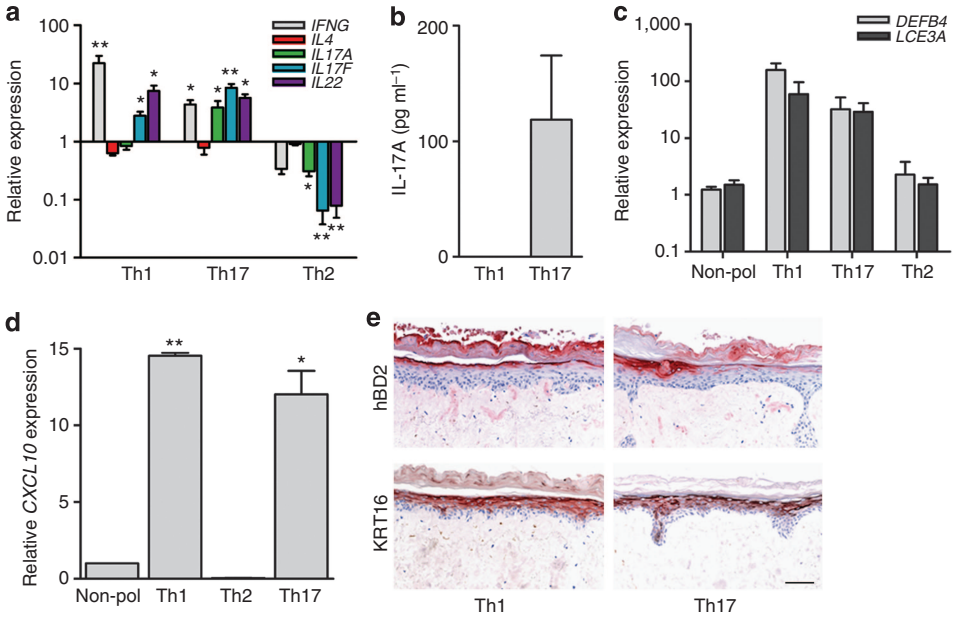

Figure 4. T helper type 1 (Th1)- and Th17-polarized CD4+ T cells induce inflammation. Naive CD4+ T cells were polarized for 5 days using cytokines and blocking antibodies or left untreated (nonpolarized (Non-pol)). After 5 days, the T cells were stimulated for 6 hours using anti-CD3/anti-CD28 mAb-coated beads in a T-cell/bead ratio of 5:1 to ensure T-cell activation. (**a**) mRNA expression profiles of the Th1-, Th17-, and Th2-polarized T cells just before addition to the skin equivalents ($n=3$ T-cell donors; mean ± SEM; $^*P<0.05$, $^{**}P<0.01$, relative to nonpolarized T cells). (**b**) IL-17A secreted in the culture medium by the different T-cell subsets just before addition to the skin equivalents ($n=3$ T-cell donors; mean ± SEM). (**c**) Keratinocyte mRNA expression levels of *DEFB4* and *LCE3A* after 4 days of T-cell migration by Th1, Th2, and Th17 cells ($n=3$ T-cell donors; mean ± SEM; $^*P<0.05$, $^{**}P<0.01$, relative to nonpolarized T cells). (**d**) *CXCL10* chemokine expression by keratinocytes in response to polarized T cells ($n=3$ T-cell donors; mean ± SEM; $^*P<0.05$, $^{**}P<0.01$, relative to nonpolarized T cells). (**e**) Immunostaining of hBD2 and KRT16 protein in skin equivalent populated with Th1- and Th17-polarized T cells (representative image of $n=3$ T-cell donors). Bar = 50 μm.

epidermal differentiation in the Th1- and Th17-polarized inflammatory skin equivalents (Supplementary Figure S2D online).

Anti-inflammatory drugs counteract the epidermal inflammatory phenotype

To further validate our T cell–based inflammatory 3D skin model, we studied the effect of targeting either the epidermal compartment by all-*trans*-retinoic acid (ATRA) or the T-cell compartment by cyclosporin A (CsA). Based on the initial experiments where disease markers were found to be induced after 2 days of T cell migration, we allowed T cells (as used in Figure 2) to migrate for 2 days. Subsequently, the culture medium was supplemented with ATRA or CsA and the culture was continued for another 2 days. Psoriasis-associated genes *DEFB4*, *PI3*, and *S100A8* were found to be downregulated upon treatment with either ATRA or CsA (Figure 5a). We also observed the same trend for *LCE3A* and to a lesser extent *KRT16*, but this decrease was not statistically significant (Supplementary Figure S2E online). Protein expression analysis of hBD2 and elafin confirmed the effect of ATRA or CsA on the inflamed skin equivalents, showing reduced expression of both proteins upon treatment. The expression of filaggrin was partially restored upon CsA treatment (Figure 5b). To determine the treatment effects on the proinflammatory mediators present in our model, we measured cytokine levels in the conditioned medium from the inflammatory skin equivalents. IL-17A and IL-22 levels were reduced upon treatment with ATRA and CsA, whereas IFN-γ was only affected by CsA treatment (Figure 5c). Again, Th2 cytokines IL-4 and IL-5 were undetectable in all experimental conditions (data not shown). *CXLC10* and *CCL2* chemokine expression by the keratinocytes was only reduced upon CsA treatment (Figure 5d). In addition, we used the antipsoriatic drug Infliximab (TNF-α antibody) to target the TNF-α produced by either the T cells or keratinocytes in our model, and found significantly reduced expression levels of *TNF*, *DEFB4*, and *CXCL8* and a similar trend to *S100A8*, *CXCL10*, and *CCL2* (Supplementary Figure S2F online).

DISCUSSION

Recent studies combining transcriptomics data from psoriasis and healthy skin resulted in a consensus molecular profile, giving us more insight in the disease pathogenesis (Suarez-Farinas *et al.*, 2012; Tian *et al.*, 2012). These molecular markers of disease are highly valuable for validating *in vitro* disease models as presented here. Many of the top 50 of upregulated genes in psoriatic lesions are also upregulated in our model such as *DEFB4*, *PI3*, *IL8*, *S100A7*, and more. In addition, the protein expression was similar to psoriatic skin with increased levels of the aforementioned molecular markers and induced keratin 16 protein expression, which is typical for hyperproliferative disorders such as psoriasis (Leigh *et al.*, 1995). The key feature of hyperproliferative skin

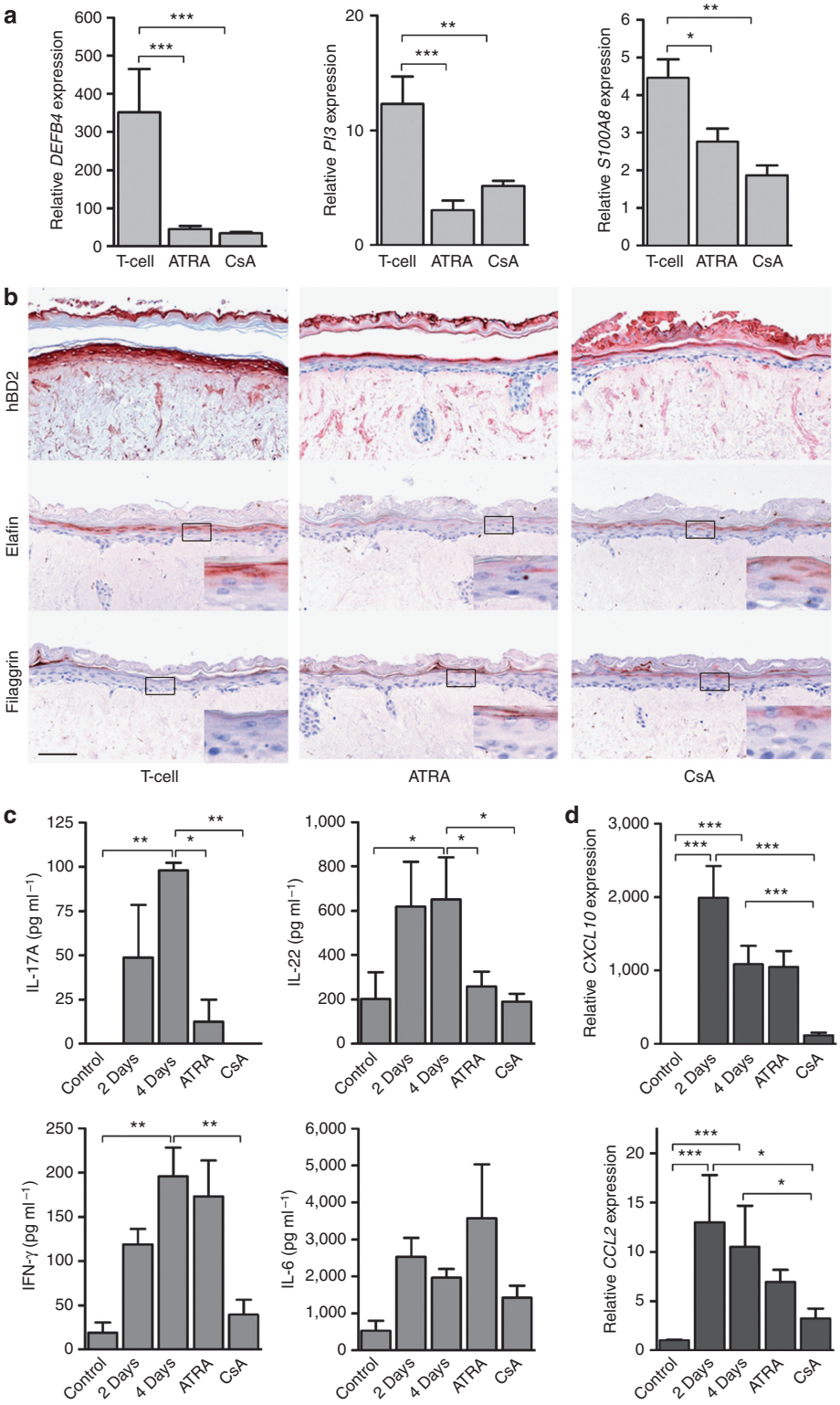

Figure 5. Anti-inflammatory drugs target crosstalk and reduce the inflammatory phenotype. CD4 + CD25 − T cells were activated by anti-CD/CD28 bead stimulation for 5 hours and allowed to migrate into the skin equivalents for 2 days to initiate inflammation. Thereafter, the culture medium was supplemented with cyclosporine A (CsA) or all-*trans*-retinoic acid (ATRA) for another 2 days. (**a**) Keratinocyte mRNA expression levels of the psoriasis-associated genes *DEFB4*, *PI3*, and *S100A8* in untreated (T cell) and ATRA-, and CsA-treated inflammatory skin equivalents ($n = 3$ T-cell donors; mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (**b**) Immunostaining of hBD2 and elafin and filaggrin protein before (T cell) and after ATRA or CsA treatment. Bar = 100 μm. (**c**) Cytokine levels of IL-17A, IL-22, IFN-γ, and IL-6 in the culture medium of the skin equivalents ($n = 3$ T-cell donors; mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**d**) Keratinocyte mRNA levels of *CXCL10* and *CCL2* ($n = 3$ T-cell donors; mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

disorders such as psoriasis is a thickened epidermis with high numbers of Ki67-positive cells in the basal and suprabasal layers. We did not observe this feature in our model. IL-22 has been shown to be critical for epidermal hyperproliferation in mouse models (Zheng *et al.*, 2007; Wang *et al.*, 2013), submerged keratinocytes cultures (Mitra *et al.*, 2012), and skin equivalents stimulated with IL-22 showed acanthosis (Boniface *et al.*, 2005; Sa *et al.*, 2007). The concentration of IL-22 used to stimulate the skin equivalents was, however, 40-fold higher than we measured in our model. In addition, the previously reported acanthosis was a result from epidermal hyperplasia and not from increased proliferation (Boniface *et al.*, 2005). Apparently, the crucial mediator(s) that cause keratinocyte proliferation are lacking in our model and peripheral blood–derived CD4+ T cells and the soluble mediators they produce (e.g., IFN-γ, TNF-α, IL-22, or IL-17) are probably not the main instigators of epidermal hyperproliferation. Alternatively, an excess of growth-inhibitory molecules may be present (e.g.. transforming growth factor-β). In addition, *Ki67* expression levels of our skin equivalent model when compared with normal skin *in vivo* indicate that the skin equivalents still have a high proliferation rate after a culture period of 14 days (data not shown). Perhaps this could explain why we were not able to induce proliferation as the proliferation rate was already high. It could be expected that upon a longer culture period of our normal skin equivalents (e.g., 3 to 4 weeks), the proliferation rate will lower, and then the T cells may be capable of inducing keratinocyte hyperproliferation. Obviously, several other potentially relevant cell types such as fibroblasts, dendritic cells, endothelial cells, and mast cells are absent here, some of which may be pivotal in promoting keratinocyte proliferation.

The main goal of our study was to develop a model for the crosstalk between T cells and keratinocytes in a 3D microenvironment. This type of crosstalk is an essential feature in disease pathogenesis, as immune cells produce mediators to activate keratinocytes that in turn modulate the expression of skin-homing markers on T cells (Gunther *et al.*, 2005; McCully *et al.*, 2012) and produce chemokines to attract immune cells (Giustizieri *et al.*, 2001; Peters *et al.*, 2013), thereby completing the vicious circle of chronic inflammation. Here, we have demonstrated such crosstalk by the induction of keratinocyte-derived chemokines (Figure 3d) in response to soluble factors produced by peripheral blood–derived CD4+ T cells. In our indirect model using low number of T cells, this chemokine production appeared to be the initial step in epidermal activation as proinflammatory cytokine levels and epidermal disease markers (e.g., *DEFB4*, *KRT16*) were undetectable. Comparing the levels of soluble factors produced by the T cells in our skin equivalent model with the concentrations used in previous studies (Tjabringa *et al.*, 2008) indicates that the T cells produce a wider variety of cytokines but at lower concentrations (data not shown). This suggests that our T cell–driven inflammatory skin model is probably more physiologically relevant than using an overload of cytokines to stimulate skin equivalents.

Using polarized T cells, we aimed to generate inflammatory skin equivalents with a disease phenotype specific for the subset of helper T cells introduced in the skin equivalents. For Th1- and Th17-polarized cells, this was successful as shown by the induction of molecular markers for psoriasis, even though we did not observe hyperproliferation or acanthosis. The epidermal inflammation observed by addition of activated T cells was similar to that of Th1- or Th17-skewed T cells that indicates that our T cell–populated skin equivalent model represents a psoriasiform inflammation. Despite activation, the 5-day cultured, nonpolarized, or Th2-skewed T cells did not induce any signs of epidermal inflammation and we did not detect any of these cells in the dermis of our skin equivalents (data not shown). In addition, the keratinocytes did not respond to these cells as shown by the lack of chemokine production (Figure 4d), which appeared to be the initial step in the inflammatory process in our model, as discussed above. The lack of chemokine production could be an explanation for the absence of T cells in the dermis of the skin equivalents, and also demonstrates the importance of crosstalk between the cellular compartments.

The inflammatory phenotype of our skin equivalents rather resembled psoriasis than atopic dermatitis. Molecular markers of atopic dermatitis (*CA2* and *NELL2*) (Kamsteeg *et al.*, 2010) and morphological features such as spongiosis and/or apoptosis (Trautmann *et al.*, 2000) were absent, likely because of lack of IL-4 and IL-5 production by the T cells. To generate a more atopic dermatitis-like phenotype, we cultured naive CD4+ T cells under Th2-polarizing conditions and added these to our skin equivalents. The Th2 polarization protocol led to downregulation of Th1- and Th17-associated genes, but the cells failed to demonstrate IL-4 upregulation (Figure 4a). Thus, so far, to study atopic dermatitis pathogenesis and therapeutic intervention *in vitro*, we are still limited to cytokine-stimulated skin equivalents (van den Bogaard *et al.*, 2013).

The use of patient-derived keratinocytes and T cells or skin-resident T cells would be a valuable application of our model. Humanized mouse models based on the initial studies using the severe combined immunodeficient mouse xenograft model (Nickoloff *et al.*, 1995) have been crucial for our current knowledge of psoriasis pathogenesis. Many pathophysiological pathways and therapeutic strategies have been elucidated by xenografting human skin and immune cells from healthy volunteers or psoriasis patients onto immunodeficient mice or mice deficient in type I and type II IFN receptors (Gilhar *et al.*, 2002; Boyman *et al.*, 2004; Ma *et al.*, 2008). It would be very interesting to study if our model has the potential to partially replace these models by using patient-derived keratinocytes versus healthy immune cells and vice versa. As the *in vivo* models all show hyperproliferation and acanthosis, this might tackle the problem in our model where these features are absent.

In lesional psoriatic skin, CD4+ T cells (as we studied in our model) are mainly localized to the dermis, whereas CD8+ T cells are present in the epidermis. The role of CD8+ T cells in the epidermis and in psoriasis pathogenesis is of current interest (Di Meglio and Duarte, 2013; Hijnen *et al.*, 2013). Therefore, we are in the process of introducing CD8+ T cells to our model system. Entrance of these cells in

the epidermal compartment would be biologically relevant and would enable studying the role of these cells in the pathogenesis of psoriasis. In addition, the implementation of other types of immune cells in our model like natural killer cells and dendritic cells would also be of interest as these cell types play an important role in the development of psoriasis (reviewed in Guttman-Yassky *et al.*, 2011). Initial experiments incorporating both T cells and dendritic cells in our skin equivalents show promising results and this is feed for future studies.

In summary, the here presented T cell–driven *in vitro* model of skin inflammation faithfully mimics many characteristics of epidermal psoriasiform inflammation. In this model, clinically used drugs that target the crosstalk between keratinocytes and T cells successfully reduced the inflammatory phenotype, thereby supporting the notion that our model may serve as a highly suitable platform for drug screening purposes in a preclinical setting.

MATERIALS AND METHODS

Generation of human inflammatory skin equivalents

Human skin equivalents were generated using decellularized de-epidermized dermis as described previously and seeded with 10^5 human primary keratinocytes (Tjabringa *et al.*, 2008). After 7 days of culture at the air–liquid interface, activated T cells obtained from donors allogeneic to the skin were introduced in the skin equivalents (Figure 1a). T-cell viability in the skin equivalents was assessed by morphology and TUNEL staining. To study the effect of CsA (400 ng ml^{-1}; Novartis Pharmaceuticals, Arnhem, The Netherlands), and ATRA (10^{-6} M; Sigma-Aldrich, Zwijndrecht, The Netherlands), the drugs were administered 2 days after T-cell migration. Infliximab was administered at 10 μg ml^{-1} at the time of T-cell introduction.

T-cell isolation and stimulation

CD4+ T cells were purified from peripheral blood mononuclear cells obtained from healthy blood donors by negative selection as described previously (Koenen *et al.*, 2008) and depleted from CD25^{high} regulatory T cells by CD25 magnetic-activated cell sorting beads (MACS; Miltenyi-Biotec, Bergisch-Gladbach, Germany). Sorted naive CD4+ T-cell populations were typically >98% pure. To activate the T cells, they were subsequently stimulated with anti-CD3/CD28-beads (Life Technologies, Breda, The Netherlands) at a T-cell/bead ratio of 5:1 for 5 hours. Thereafter, the activated cells were washed and taken in skin equivalent medium and added to the skin equivalent.

T-cell polarization

For Th1, Th2, and Th17 polarization of T cells, isolated naive CD4+ T cells were activated with anti-CD3/CD28 beads (T-cell/bead ratio of 5:1) and cultured for 5 days under Th1-, Th2-, and Th17-polarizing conditions. The following human recombinant cytokines and anti-human mAbs were used to polarize naive T cells: for Th1 polarization: IFN-γ (Endogen, Etten-Leur, The Netherlands, 200 U ml^{-1}), IL-12 (Life Technologies, 50 ng ml^{-1}), and anti-IL-4 (R&D Biosciences, Abingdon, UK 5 μg ml^{-1}); for Th2 polarization: IL-4 (R&D Biosciences, 250 U ml^{-1}) and anti-IFN-γ (R&D Biosciences, 5 μg ml^{-1}); for Th17 polarization: IL-1β (Invitrogen, 50 ng ml^{-1}), IL-6 (R&D Biosciences,, Etten-Leur, The Netherlands 20 ng ml^{-1}), and anti-IL-4 plus anti-IFN-γ. T-cell culture conditions were conducted as reported previously (Koenen *et al.*, 2008).

Transcriptional analysis

Epidermis was separated from the skin equivalents by dispase (Roche Diagnostics, Almere, The Netherlands) treatment for 2 hours at 4 °C. The mRNA isolation, subsequent complementary DNA generation, and quantitative PCR was performed as previously described (de Jongh *et al.*, 2005). Using the comparative $\Delta\Delta C_t$ method and *RPLP0* as reference gene, relative mRNA expression levels were calculated (Livak and Schmittgen, 2001). Transcriptome analysis of polarized T cells was performed using TaqMan quantitative PCR analysis (TaqMan, Life Technologies) according to the manufacturer's instructions using *HPRT* as a reference gene.

Histology and immunohistochemistry

Formalin-fixed human skin equivalents were processed for routine histology. Sections were stained with hematoxylin–eosin or processed for immunohistochemical staining using an indirect immunoperoxidase technique with avidin biotin complex enhancement (Vector Laboratories, Peterborough, UK). Antibodies used are depicted in Supplementary Table S2 online).

Flow cytometry and antibodies

T cells were analyzed by five-color flow cytometry as described previously (Koenen, *et al.*, 2008) using antibodies depicted in Supplementary Table S2 online.

Cytokine analysis by ELISA and Luminex

Human cytokines were determined in culture supernatants using the Bio-Plex systems (Bio-Rad, Veenendaal, The Netherlands) and ELISA (IL-22, R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Statistics

Data are given as mean ± SEM of $n = 3$–5 biological replicates. Statistical analysis of quantitative PCR data was performed on ΔCt values using commercially available software (PASW Statistics 18, Chicago, IL). One-way analysis of variance, followed by Bonferroni *post hoc* testing, and two-sided paired *t*-tests were performed. $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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