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CD40-mediated amplification of local immunity by epithelial cells is impaired by HPV

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ABSTRACT

The interaction between the transmembrane glycoprotein surface receptor CD40 expressed by skin epithelial cells (ECs) and its T cell-expressed ligand CD154 was suggested to exacerbate inflammatory skin diseases. However, the full spectrum of CD40-mediated effects by ECs underlying this observation is unknown. Therefore, changes in gene expression after CD40 ligation of ECs were studied by microarrays. CD40-mediated activation for 2 hours stimulated the expression of a coordinated network of immune-involved genes strongly interconnected by *IL8* and *TNF*, while after 24 hours anti-proliferative and anti-apoptotic genes were upregulated. CD40 ligation was associated with the production of chemokines and the attraction of lymphocytes and myeloid cells from peripheral blood mononuclear cells (PBMCs). Thus, CD40-mediated activation of ECs resulted in a highly coordinated response of genes required for the local development and sustainment of adaptive immune responses. The importance of this process was confirmed by a study on the effects of human papilloma virus (HPV) infection to the EC's response to CD40 ligation. HPV infection clearly attenuated the magnitude of the response to CD40 ligation and the EC's capacity to attract PBMCs. The fact that HPV attenuates CD40 signalling in ECs indicates the importance of the CD40-CD154 immune pathway in boosting cellular immunity within epithelia.

INTRODUCTION

CD40 is a 48 kDa transmembrane glycoprotein surface receptor also known as the tumour necrosis factor receptor superfamily member 5 (TNFRSF5). It is expressed at the cell surface of antigen presenting cells of the hematopoietic lineage, including B cells, dendritic cells (DCs), Langerhans cells and macrophages, and is also expressed by non-hematopoietic cells, such as endothelial cells (Hollenbaugh *et al.*, 1995), fibroblasts (Fries *et al.*, 1995; Yellin *et al.*, 1995), smooth muscle cells and epithelial cells (Galy and Spits, 1992). The ligand for CD40 is the type II membrane protein CD40L (CD154), which is primarily expressed on activated $CD4^+$ T helper cells. The CD40–CD154 interaction plays a role in both cellular and humoral immune responses. Upon CD40 ligation, DCs mature and become activated to produce high levels of pro-inflammatory cytokines and chemokines, and upregulate MHC class II and co-stimulatory molecules such as CD80 and CD86. Together, these up-regulated molecules facilitate effective priming of $CD8^+$ T cells and stimulate activated $CD8^+$ T cells to become cytotoxic effector cells (Ma and Clark, 2009). In B cells, CD40 ligation induces immunoglobulin isotype switching and differentiation as well as inhibits apoptosis by upregulating anti-apoptotic genes like cIAPs, members of the BCL2 family and MYC (Kehry, 1996; Laman *et al.*, 1996). Deregulation of CD40–CD154 interaction can lead to various clinical conditions (Peters *et al.*, 2009), such as autoimmune diseases, multiple sclerosis, allograft rejections, intraepithelial pre-malignancies and inflammatory skin diseases such as psoriasis and subacute cutaneous lupus erythematosus (Caproni *et al.*, 2007).

In the epidermis, CD40 is expressed at low levels by basal and para-basal layer epithelial cells (ECs). ECs upregulate CD40 expression when stimulated with IFNγ (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Peguet-Navarro *et al.*, 1997), that normally is produced by effector cells of the innate immune system and by activated type 1 polarized (IFNγ-producing) CD40L-expressing $CD4^+$ T-helper (Th1) cells that enter the skin (Swamy *et al.*, 2010; van den Bogaard *et al.*, 2013). Indeed, CD40 is highly expressed by ECs in T-cell infiltrated psoriatic lesions (Denfeld *et al.*, 1996). A limited number of *in vitro* studies on CD40 ligation of human primary IFNγ-stimulated ECs showed that these cells

express ICAM-1 and secrete RANTES (CCL5), TNFα, IL-6, IL-8 and MCP-1 (CCL2) (Companjen *et al.*, 2002; Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Pasch *et al.*, 2004; Peguet-Navarro *et al.*, 1997). In addition, there is evidence that CD40-activated ECs stop proliferating and start differentiating (Concha *et al.*, 2003; Grousson *et al.*, 2000; Peguet-Navarro *et al.*, 1997; Villarroel Dorrego *et al.*, 2006). However, the full spectrum of effects mediated by CD40 ligation on the response of ECs is still unknown.

The basal and parabasal layers ECs of squamous epithelia are a well-known target for different viruses (Andrei *et al.*, 2010), including high-risk human papilloma virus (hrHPV). Chronic infections with hrHPV can last for many years, probably as a result of several sophisticated mechanisms employed by hrHPV to evade the hosts' innate immune response (Karim *et al.*, 2011; Karim *et al.*, 2013; Reiser *et al.*, 2011). Interestingly, an *in vivo* model for EC-specific human-CD40 expression and activation showed that CD40 ligation on ECs enhanced DC migration and T cell priming in a mouse model (Fuller *et al.*, 2002), suggesting that ECs boost the activity of cells from the adaptive immune system. HPV-specific cellular immunity, however, develops quite late and slowly during persistent HPV infections (van der Burg and Melief, 2011), posing the question if HPV may also impair pathways typically associated with activation of the adaptive immune response.

To obtain a better understanding of the outcome between the interaction of epithelial cells and CD40 ligand-expressing $CD4^+$ Th1 cells, we analysed the genome-wide expression profiles of CD40-stimulated undifferentiated primary ECs. We observed that ECs react in a very coordinated fashion to CD40 ligation with the induction of mainly immune-related genes and the attraction of immune cells. The parallel analysis of hrHPV-infected primary ECs revealed that hrHPV did not grossly change the gene expression pattern but attenuated the magnitude of the CD40-stimulated immune response, resulting in an impaired immune cell attraction. These data strengthen the notion that the CD40-CD154 pathway plays an important role in protective epithelial immune responses.

RESULTS

CD40 upregulation and functionality on epithelial cells

To study how ECs respond to CD40 ligation on a genome-wide scale, we mimicked the CD40 – CD154 interaction between ECs and IFNγ-secreting $CD4^+$ T cells. Basal CD40 levels on cultured ECs are too low for efficient *in vitro* ligation with CD154, however ECs upregulate the expression of CD40 when stimulated with IFNγ (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Peguet-Navarro *et al.*, 1997). Therefore, we measured by flow cytometry the CD40 expression on primary undifferentiated ECs stimulated with increasing concentrations of IFNγ for 72 hours. In line with previous reports, CD40 expression was enhanced by IFNγ at all concentrations but became optimal at a concentration equal or more than 50 IU/ml IFNγ for the primary ECs obtained from 4 different healthy donors (Figure 1a and b). Therefore, this dose was used in our subsequent studies.

ECs were reported to secrete the pro-inflammatory chemokines IL8 (CXCL8) and RANTES (CCL5) upon CD40 ligation (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Pasch *et al.*, 2004; Peguet-Navarro *et al.*, 1997). Indeed, this was also observed for CD40 expressing ECs stimulated with CD154-expressing L-cells (CD40L) as compared to ECs cultured with control L-cells (Figure 1c and d), showing that our ECs expressed functionally active CD40. To determine the optimal time points for measuring the response of CD40-ligated ECs on a genome-wide scale, ECs were stimulated for up to 24 hours with CD40L and the peak gene expression of *IL8* and *RANTES* was determined. The highest expression of *IL8* was detected after 2 hours (Figure 1e), *RANTES* peaked after 24 hours of CD40 ligation (Figure 1f). We concluded that these two time-points were most suited for studying early and late responses of ECs to CD40 ligation.

Epithelial cells upregulate genes involved in immune signalling and proliferation after CD40 ligation

The effects of CD40 ligation on four freshly isolated uninfected primary EC cultures from healthy donors of foreskin, vaginal or cervical origin were studied by genome-wide expression profiling. These ECs are the natural target for hrHPV, which is most commonly transmitted by sexual contact. We

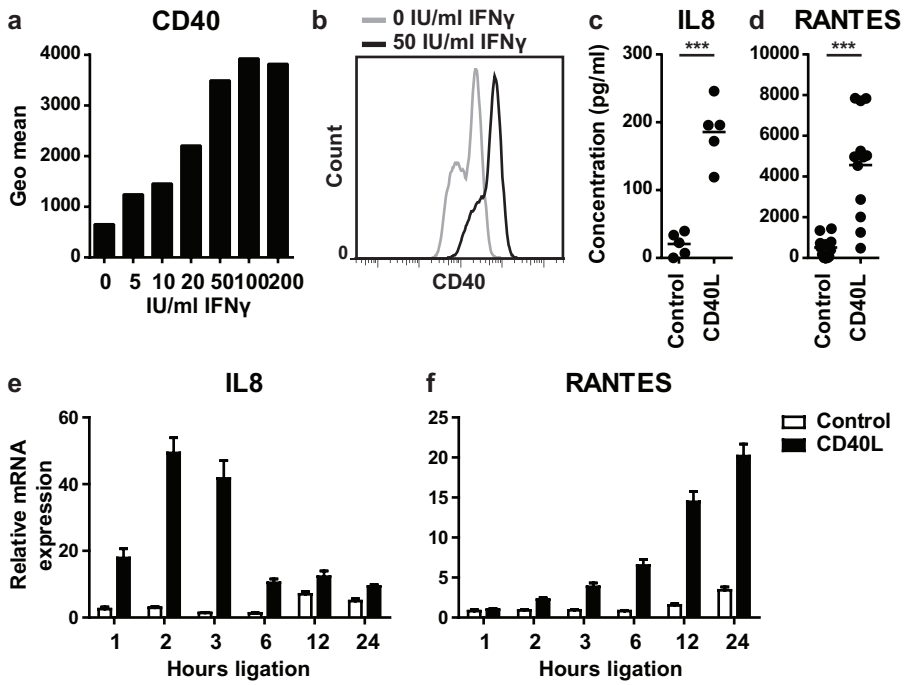

Figure 1. Epithelial cells produce cytokines and chemokines upon CD40 ligation
*(a) CD40 upregulation on vaginal ECs upon stimulation with 0, 5, 10, 20, 50, 100 or 200 IU/ml IFNγ for 3 days. The height of the bars represent the CD40 mean fluorescence intensity as determined by flow cytometry. (b) Histogram of CD40 expression on vaginal ECs stimulated 3 days with 0 and 50 IU/ml IFNγ. ELISA for IL8 (c) and RANTES (d) in cleared supernatants from IFNγ-pre-stimulated foreskin, vaginal and cervical EC cultures (n=5-12) co-cultured for 24 hours with Control or CD40L-expressing L cells in presence of IFNγ. *** indicates p<0.0005. RT-qPCR of IL8 (e) and RANTES (f) expression by IFNγ-pre-stimulated vaginal ECs co-cultured with L-Control or L-CD40L cells in presence of IFNγ for 0, 1, 2, 3, 6, 12 or 24 hours. Gene expression was normalized using GAPDH as the calibrator gene. Fold changes over 0 hours co-culture were calculated and depicted. These data are representative for two to three independent experiments.*

verified that the cells were activated via CD40 by confirming the increased expression of *IL8* (2 hours) and *RANTES* (24 hours) (Supplemental figure S1) and subsequently subjected the samples to microarray analysis. Plots with microarray log2 intensities confirmed that *IL8* and *RANTES* were upregulated after 2 and 24 hours, respectively (Supplemental figure S1) and confirmed the results obtained by quantitative PCR.

Using a False Discovery Rate (FDR) ≤0.05 the response to CD40 ligation in the four primary EC cultures was analyzed for genes that were at least two-fold up- or down-regulated (log2-fold change filter (LogFC) ≥1) after 2 or 24 hours of stimulation. The response obtained in EC cultures with control cells was used to correct the results obtained with CD40-ligated ECs for both the time of co-culture with L-cells and total cell density. In total 60 probes showed differential expression, representing 49 differentially expressed genes. Twenty-four genes were upregulated after 2 hours and twenty-nine genes after 24 hours, five genes were upregulated at both time points. One gene (*MMP3*) was significantly downregulated after 24 hours (Figure 2a, Supplemental table S1).

By Ingenuity Pathways Analysis (IPA), we explored if these 49 differentially expressed genes were enriched for biological pathways and how they were connected. IPA enrichment analysis showed that the 24 genes differentially expressed after 2 hours CD40 ligation were mainly involved in 'Cellular movement', especially 'Leukocyte migration', 'Cell-to-cell signalling and interaction', and 'Cell death and survival'. The highest upregulated gene was *IL8*, followed by *CCL20*, *TNFAIP3*, *TNF*, *CXCL1*, *EFNA1* (*TNFAIP4*), *IL36G* and *UBD*, all having a LogFC≥2. At 24 hours post-stimulation the highest upregulated genes were *CCL5* (*RANTES*), *UBD*, *MMP9*, *C15orf48*, *SOD2*, *SerpinA3* and *BIRC3* (*cIAP2*). The 30 genes differentially expressed at this time-point are involved in 'Cellular movement', 'Cell death and survival pathways', 'Post-translational modification', and 'Protein degradation'.

According to the IPA knowledge database 37 of these 49 differentially expressed genes formed a network (117 connections) including 23 out of the 24 genes differentially expressed after 2 hours, and 19 out of the 30 genes differentially expressed after 24 hours (Figure 2b and c). The most interconnected genes within the center of the network were *TNF* and *IL8*, both upregulated only after 2 hours of CD40 ligation. These data indicated that CD40 stimulation of epithelial cells results in a very coordinated reaction; first highly connected immune-involved genes that are able to recruit leukocytes or regulate cytokine expression are upregulated, and subsequently genes involved in the regulation of cell death and survival are upregulated.

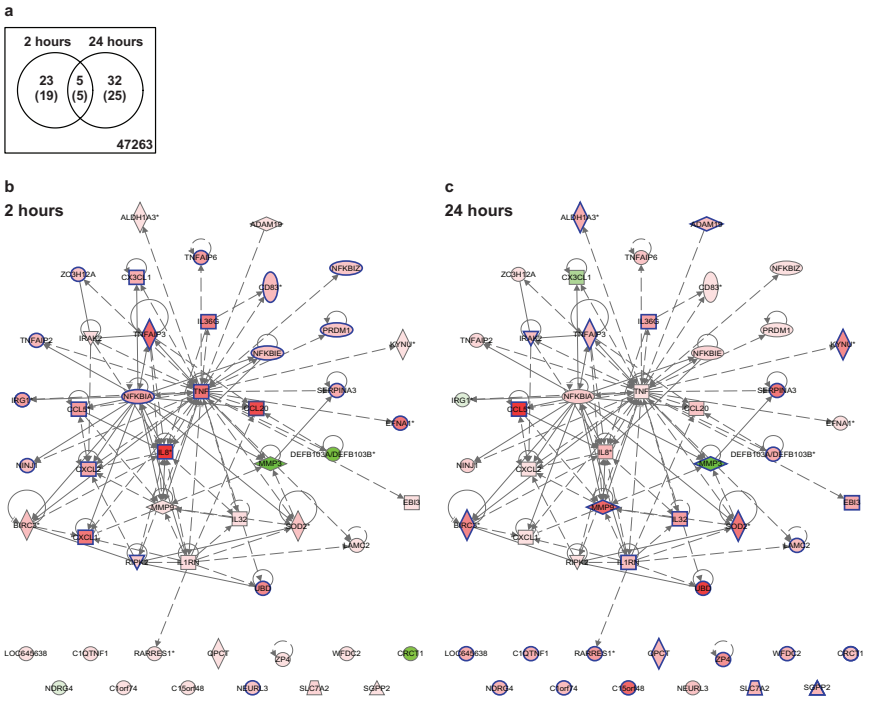

Figure 2. CD40 stimulation stimulates a highly coordinated immune response by ECs
(a) Venn diagram depicting the overlap between 49 signature genes (60 microarray probes) differentially expressed at 2 and/or 24 hours L-CD40L stimulation versus L-Control stimulation with adjusted p-value≤0.05 and absolute log2-fold change≥1. Networks with expression changes at 2 (b) and 24 (c) hours were constructed of 49 connected CD40L signature genes using interaction data curated from literature and high-throughput screens by Ingenuity Pathways Analysis. The colours show the degree of upregulation (red) or downregulation (green) in the L-CD40L condition versus the L-Control condition. The genes meeting the adjusted p-value≤0.05 and absolute log2-fold change≥1 thresholds, shown in the Venn diagram in (a), are indicated by blue borders.

CD40 ligation amplifies immune cell attraction to epithelial cells.

Many of the genes that were expressed by ECs after CD40 stimulation belonged to the 'Leukocyte migration' group, indicating that CD40-CD154 interactions between T cells and ECs may serve primarily to boost the attraction of immune cells. Therefore, as a second functional assay to study the impact of CD40 ligation, we assessed the capacity of ECs to induce immune cell migration after stimulation with CD40L or control cells. The culture

supernatants were isolated and used in a trans-well system with PBMCs seeded in the top wells. To confirm that CD40 ligation is associated with the production of chemokines belonging to the “Leukocyte migration” group, the production of the representative cytokines IL8 and RANTES were measured. Their increased secretions are representative for the production of several chemoattractants following CD40 stimulation (Figure 3a). Indeed, higher numbers of PBMCs migrated towards the supernatants from CD40-ligated ECs when compared to supernatants of control ECs (Figure 3b and c). Analysis of the fraction of lymphocytes and myeloid cells in the migrated PBMCs suggested that the myeloid fraction in the total pool of migrated PBMCs was slightly more increased (Figure 3b). These data indicate that CD40 stimulation of ECs mainly results in the secretion of pro-inflammatory cytokines that aid ECs in the attraction of PBMCs.

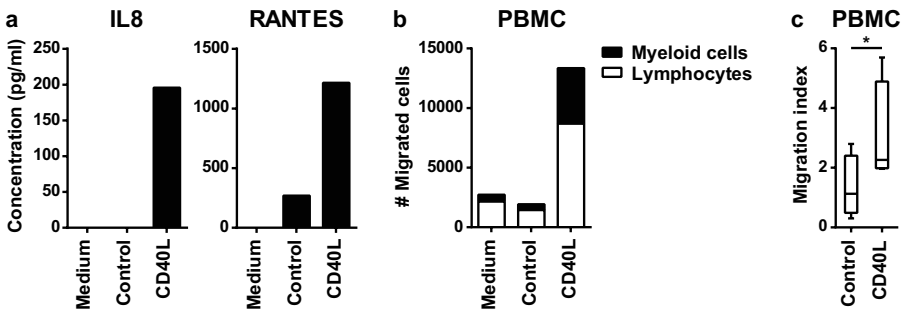

Figure 3. CD40 ligation induces immune cell migration towards epithelial cells
*Example of a representative experiment of the (a) production of IL8 and RANTES in cleared supernatants of vaginal EC donors used for the migration assay by ELISA, and (b) PBMC migration towards these cleared supernatants from vaginal EC donors prepared for the migration assay. PBMC numbers were determined by flow cytometry in presence of FACS counting beads and subsequently gated on lymphocyte or monocyte fractions. Within the total cell numbers (total bar) the fractions of lymphocytes (white) and myeloid cells (black) are depicted. (c) Migration index of total PBMC towards indicated supernatants of ECs of foreskin or vaginal origin of four independent experiments. * indicates p<0.05.*

Persistent infection with hrHPV attenuates the intensity of the CD40-induced gene expression

High-risk HPVs are known to deregulate the response of epithelial cells to TNF (Termini *et al.*, 2008). In view of the cellular mediators shared between the TNF- and the CD40-pathway, we studied if a persistent infection with hrHPV influences the gene expression pattern of CD40-stimulated ECs by genome wide expression analysis. We confirmed the expression of CD40 after IFNγ stimulation at the cell surface of hrHPV-positive ECs as well as the expression of *IL8* after 2 hours and *RANTES* after 24 hours of CD40 ligation (Supplemental figure S1a, b and c) and the secretion of these cytokines in the supernatant of hrHPV-infected ECs (Figure 5a). The gene expression profiles of four hrHPV-positive primary EC cultures, stably harbouring HPV16 or HPV18 episomes, were compared with those of the four uninfected primary EC cultures. The expression of *IL8* and *RANTES* of HPV-infected ECs after CD40-stimulation was verified by qPCR (Supplemental figure S1e). The log2 intensity plots of these genes as measured by microarray (Supplemental figure S1f), showed that the results obtained by both methods were comparable.

We studied differential gene expression in HPV-positive epithelial cells after CD40 ligation. At 2 hours, HPV-positive ECs differentially expressed 13 genes, 11 of which overlapped with the 24 genes differentially expressed in uninfected ECs (Figure 4a). At 24 hours, HPV-positive ECs differentially expressed 19 genes, 10 of which overlapped with the 30 genes differentially expressed in uninfected ECs (Figure 4b). This was a first indication that HPV does not grossly alter the reaction to CD40. All differentially expressed genes, 65 in total, were analyzed by IPA and the resulting network (159 connections) was highly similar to the network of genes expressed by CD40-stimulated non-infected ECs (Supplemental figure S3, Supplemental table S1). There were no specific clusters of genes that were either up- or down-regulated in HPV-positive ECs but not in uninfected ECs (Supplemental figure S2), rather the expression intensities of the differentially expressed genes were attenuated in HPV-positive ECs. Focusing on the immune-related genes (Figure 4c), revealed that the presence of hrHPV in ECs impaired the expression of twelve immune-related genes after 2 hours of CD40 stimulation, whereas one gene (*BDKRB1*) was enhanced. After 24 hours of stimulation, hrHPV impaired the expression of eight genes and upregulated seven immune-related genes in ECs. A closer

look into the seven upregulated genes was carried out. Three genes, *IL7R*, *LTB* and *SAA1*, showed similar upregulation in the uninfected ECs but did not reach our significance and fold change thresholds (Supplemental figure S4). The remaining four genes, *CXCL9*, *CXCL10*, *CXCL11* and *RSAD2*, were already strongly upregulated in uninfected ECs compared with HPV-positive ECs in response to the IFNγ pre-stimulation, and were not further increased by additional CD40 ligation (Supplemental figure S4). In HPV-positive ECs, CD40 ligation resulted in the upregulation of these genes to levels similar as in uninfected ECs (Supplemental figure S4).

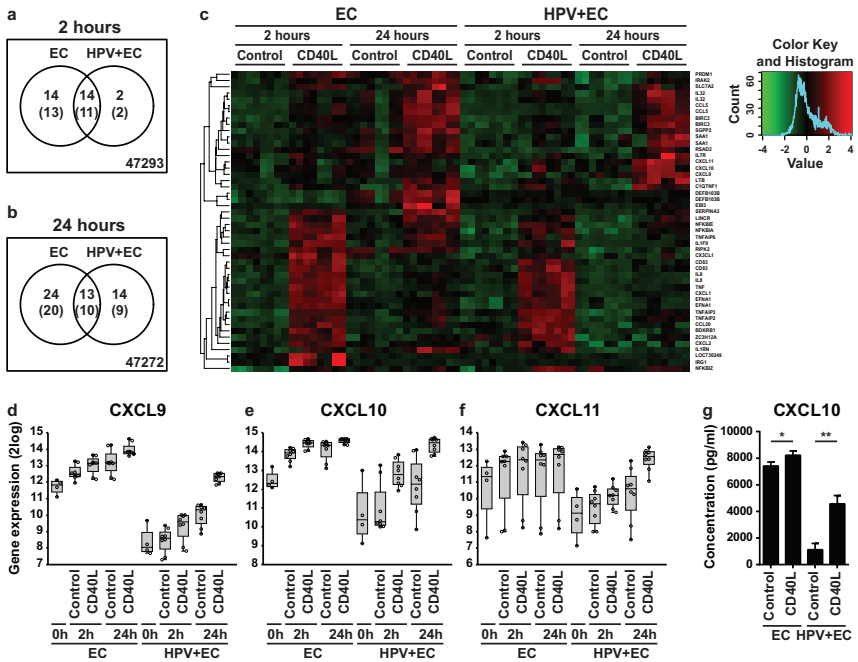

Figure 4. HPV infection results in an attenuated response of ECs to CD40 ligation
*Venn diagrams showing the overlapping genes between ECs and HPV-positive ECs in their response to L-CD40L versus L-Control stimulation for 2 (a) and 24 (b) hours; significance thresholds as in Figure 2a, numbers in brackets represent unique genes. (c) Heat-map of differentially expressed immune-involved genes as determined by IPA. Expression ratios for each condition compared to the 0h time point per cell line were mean-centered and scaled over all conditions. The genes were hierarchically clustered using cosine similarity and average linking. Microarray intensities for CXCL9 (d), 10 (e) and 11 (f) represented in a box plot. (g) CXCL10 concentration as measured by ELISA in supernatants of 24 hours IFNγ-stimulated and L-Control or L-CD40L co-cultured foreskin ECs and HPV-positive foreskin ECs (n=3). * indicates p<0.05 and ** p<0.005.*

hrHPV impairs CD40-ligation mediated immune cell attraction to epithelial cells.

The T cell-attracting chemokines CXCL9, 10 and 11 are known to be induced by IFNγ in various cell types, including ECs (Kanda *et al.*, 2007; Kanda and Watanabe, 2007; Kawaguchi *et al.*, 2009; Ohta *et al.*, 2008; Sauty *et al.*, 1999). Although CD40 stimulation salvaged the expression levels of *CXCL9*, *CXCL10* and *CXCL11* in HPV-positive ECs to similar levels found in non-infected ECs (Figure 4d, e and f), ELISA assays showed that hrHPV-positive ECs still secreted lower levels of CXCL9 and CXCL10 than non-infected ECs (Figure 4g and not shown). On average the CD40-ligated HPV-positive ECs also produced lower amounts of IL8 and RANTES (Figure 5a) albeit that in some experiments the levels approached that of non-infected ECs. To obtain a broader view on the impact of HPV to CD40L-induced immune activation, also their capacity to attract PBMCs was tested. Notwithstanding the production of the earlier tested cytokines, no increased attraction of PBMCs to the supernatants of CD40L-stimulated HPV-positive ECs was observed (Figure 5b and c). This indicates that also the production of other chemokines within the 'Leukocyte migration' group, those that are key in the attraction of PBMCs, must have been impaired in HPV-positive ECs. In independent experiments, the absolute numbers of migrated PBMCs differed per primary EC culture and PBMC donor used, however, the increase in PBMC attraction following CD40 ligation was consistently and significantly higher in uninfected ECs (Figure 3c), but not in hrHPV+ ECs (Figure 5c). Together these data show that hrHPV does not grossly alter, but rather attenuates the intracellular response of epithelial cells to CD40 ligation, resulting in a hampered ability of the HPV-positive ECs to attract immune cells.

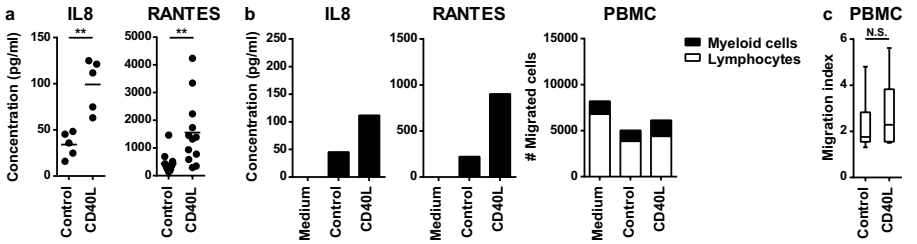

Figure 5. HPV infection hampers the enhanced attraction of immune cells by CD40-stimulated epithelial cells

*(a) ELISA for IL8 and RANTES in cleared supernatants from IFNγ-pre-stimulated HPV-positive foreskin, vaginal and cervical EC cultures (n=5-12) co-cultured for 24 hours with Control or CD40L-expressing L cells in presence of IFNγ. ** indicates p<0.005 using unpaired Welch corrected t-test. (b) Example of a representative experiment of the production of IL8 and RANTES in cleared supernatants of vaginal EC donors used for the migration assay by ELISA, and PBMC migration towards these cleared supernatants from vaginal EC donors prepared for the migration assay. (c) Migration index of total PBMC towards indicated HPV-positive foreskin, vaginal and cervical EC supernatants of four independent experiments. N.S. indicates p=not significant.*

DISCUSSION

We studied the response of epithelial cells to CD40 ligation, a major immune trigger of B and T cell immunity, and a major cue for leukocyte migration towards the skin. Stimulation of ECs via CD40 resulted in a highly coordinated regulation of predominantly immune-related genes involved in the attraction, sustainment and amplification of adaptive immune responses as well as resulted in the attraction of immune cells. Interestingly, hrHPV infection did not qualitatively alter the gene expression profile of CD40-stimulated EC, instead the extent of the response was attenuated. The fact that HPV attenuates CD40 signalling in ECs indicates the importance of the CD40-CD154 immune pathway in boosting immunity in epithelia.

Microarray expression studies showed that CD40 ligation of non-hematopoietic cells, such as endothelial cells (Pluvinet *et al.*, 2008), pancreatic cells (Klein *et al.*, 2008), renal proximal tubule epithelial cells (Li and Nord, 2005), smooth muscle cells (Stojakovic *et al.*, 2007), microglia (Ait-Ghezala *et al.*, 2005) and epithelial cells (this report), generally results in the upregulation of genes involved in immunity and inflammatory responses, cell fate and cell adhesion. The response of ECs to CD40 stimulation is alike that of muscle cells and pancreatic cells. Endothelial cells seem to have a broader response as they also upregulate genes involved in the viral immune surveillance system, e.g. the 2'-5'-oligoadenylate/RNase L system and guanylate-binding proteins (GBP1-4), potentially to keep the vasculature from harmful consequences and prevent the spread of systemic viral infection in the host (Pluvinet *et al.*, 2008). Epithelial cells are well equipped with viral sensors which can launch an antiviral response upon infection (Karim et al., 2011), and the CD40 pathway may help to establish efficient adaptive B and T cell immunity to expand the precision of protection after the initial innate immune cell response.

Interestingly, we found that late CD40-mediated responses in ECs involved the upregulation of the anti-apoptosis genes *cIAP2* and *BCL3* as well as the negative regulator of proliferation *RARRES1*. These observations may explain earlier findings that epithelial cells do not go into apoptosis but rather stop proliferating after CD40 ligation (Peguet-Navarro *et al.*, 1997). We are currently exploring this further. The response of ECs to CD40 stimulation is paralleled by

B cells, which respond to CD40 ligation by preventing apoptosis through the upregulation of several anti-apoptotic genes, including *cIAPs*, *MYC* and *BCL2* members (Kehry, 1996; Laman *et al.*, 1996).

CD40 stimulation of dendritic cells (DCs) has been thoroughly studied as it plays a key role in the activation, maturation and T-cell priming capacity of DC. Upon CD40 stimulation DCs produce pro-inflammatory cytokines and chemokines, upregulate HLA class I and II as well as the co-stimulatory molecules CD86 and CD80 (Ma and Clark, 2009). This allows DC to convey the appropriate signals to T cells required for them to become effector cells. Candidate gene studies showed that ECs can express CD40, HLA class I and II, CD86, but not CD80 (Black *et al.*, 2007; Ortiz-Sanchez *et al.*, 2007; Romero-Tlalolini *et al.*, 2013) as well as the co-stimulatory molecules CD83 and ICAM-1 and a number of cytokines after being exposed to IFNγ and CD40 activation (Companjen *et al.*, 2002; Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Pasch *et al.*, 2004; Peguet-Navarro *et al.*, 1997). This may allow CD40-stimulated ECs to process and present antigen to effector/memory $CD4^+$ and $CD8^+$ T cells (Black *et al.*, 2007) as well as to amplify immune responses. However, it is not likely that such activated ECs function as professional antigen-processing cells (APCs) as it was shown that CD40L-activated ECs fail to prime allogeneic T-cell reactions, underlining the difference of CD40 ligation on professional and non-professional APCs (Grousson *et al.*, 2000).

The pathogenesis of skin diseases such as psoriasis is based on an influx of immune cells into psoriatic lesions where cytokine levels are elevated. Our results sustain the notion that tissue-infiltrating T cells may exacerbate the disease via the production of IFNγ and the interaction with CD40 on ECs. The resulting cytokines may amplify the immune response via the attraction of more immune cells thereby forming a loop in EC stimulation and cytokine production. The involvement of ECs in the exacerbation of disease has been questioned as CD40 expression on epithelial cells *in vivo* can be weak (Ohta and Hamada, 2004). However, we and others have shown that CD40 expression is rapidly upregulated (at least temporarily) under the influence of physiological doses of IFNγ and thus weak steady state expression does not preclude robust action under conditions of immune activation.

HPV attenuates the extent of the epithelial cells' response to CD40 ligation, suggesting that HPV interferes with CD40 ligation-induced signal transduction and subsequent canonical and non-canonical NFκB activation (Gommerman and Summers deLuca, 2011; Hostager and Bishop, 2013; Ma and Clark, 2009). Several research groups have reported that hrHPV deregulates NFκB activation following the activation of pattern recognition receptors (PRRs) (Karim *et al.*, 2011; Reiser *et al.*, 2011) or the TNF receptor (Termini *et al.*, 2008). We and others have previously shown that hrHPV attenuates the PRR-induced (Karim *et al.*, 2013) and TNFR-induced (Takami *et al.*, 2007) NFκB pathway activation by upregulating UCHL1, a cellular deubiquitinase/E3 ligase. Therefore, the expression of UCHL1, or other non-identified modulators, may explain how HPV mediates the attenuation of CD40 ligation-induced gene expression.

Surprisingly, PBMCs were more attracted to supernatants of non-CD40-ligated HPV-positive ECs than to uninfected ECs, implying that supernatants of HPV-positive ECs contain higher cytokine levels than supernatants of uninfected ECs. However, not only in this study, but also in previous studies (Karim *et al.*, 2011; Karim *et al.*, 2013), we observed that hrHPV generally downregulates the basal expression and secretion of many pro-inflammatory cytokines. Recent literature has shown that metabolism intermediates can act as inflammatory signals (Tannahill *et al.*, 2013), implying that a simple difference in cell density can affect basal immune cell attraction. Although both the HPV-positive and uninfected ECs have been treated exactly the same throughout the experiments, HPV-positive ECs proliferate faster than uninfected ECs, and as such the supernatants may contain higher metabolite levels to mediate CD40-independent PBMC attraction towards HPV-positive cells. In hrHPV+ ECs, despite the higher basal numbers of attracted PBMC, CD40 stimulation does not result in an increased number of PBMC attracted whereas in uninfected ECs this is the case.

In conclusion, epithelial cells show a coordinated response to CD40 ligation, mainly inducing the expression of genes involved in leukocyte migration, cell-to-cell signalling and interaction, as well as cell death and survival. HPV attenuates the extent of CD40-signalling, resulting in lower amounts of chemoattractants produced and a failure to enhance immune cell migration.

These data suggest that progression of inflammatory skin diseases may be driven by highly programmed immune activation scenario's in epithelial cells, that have their evolutionary basis in the epithelial cells' response to infections.

MATERIALS & METHODS

Ethics Statement

The use of discarded human foreskin, cervical and vaginal keratinocyte tissues to develop cell lines for these studies was approved by the Institutional Review Board at the Pennsylvania State University College of Medicine and by the Institutional Review Board at Pinnacle Health Hospitals. The Medical Ethical Committee of the Leiden University Medical Center approved the human tissue sections (healthy foreskin, healthy cervix, HPV16- or 18-positive cervical neoplasias) used for staining. All sections and cell lines were derived from discarded tissues and de-identified, therefore no informed consent was necessary.

Cell culture

Primary cultures of human epithelial cells (ECs) were established from foreskin, vaginal and cervical tissues as previously described (Karim *et al.*, 2011) and grown in keratinocyte serum-free medium (K-SFM; Medium 154 supplemented with HKGS kit, Invitrogen, Breda, The Netherlands). The cells morphologically and biochemically resembled ECs in both monolayer and organotypic raft cultures, as indicated by keratin expression, hemidesmosome and desmosome structures, and ability to differentiate to full thickness epithelium (McLaughlin-Drubin *et al.*, 2004; Meyers *et al.*, 1997). Using the microarray data, the cells were verified to express high levels of keratin (KRT) 10, 14, 17, and 19, and low levels of KRT18 (Supplemental figure S5), a signature specific for keratinocytes (Bononi *et al.*, 2012; Moll *et al.*, 2008). Epithelial cell lines stably maintaining the full episomal HPV genome following electroporation (HPV-positive ECs) were grown in monolayer culture using E medium in the presence of mitomycin C treated J2 3T3 feeder cells (McLaughlin-Drubin *et al.*, 2004; Meyers *et al.*, 1997) for two passages and were then adapted to K-SFM for one passage before experimentation. Since primary ECs have a limited life span and do not survive long enough to undergo a mock electroporation procedure similar to that used to obtain HPV-positive ECs, normal undifferentiated primary epithelial cells were used as control. J2 3T3 mouse fibroblasts and L-cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 2mM l-glutamine and 1%

penicillin-streptomycin (complete DMEM medium) (Gibco-BRL, Invitrogen, Breda, The Netherlands).

CD40 ligation on epithelial cells

Uninfected ECs or HPV-positive ECs were seeded at $1.5x10^5$ cells/well in 6-wells plates in K-SFM and allowed to attach for 24 hours, after which the cells received fresh K-SFM containing 50 IU/ml IFNγ (Immunotools, Friesoythe, Germany) for 72 hours. Control or CD40L-expressing L-cells were harvested, irradiated (4800 – 5200 rad) and resuspended in K-SFM containing 50 IU/ml IFNγ. L-cells were co-cultured with ECs in a 1:1 ratio for indicated time points, after which the supernatant was collected, the L-cells were removed and the RNA of the ECs was harvested. CD40L expression and functionality of the L-cells were validated as was the percentage of residual L-cells after co-culture (<1%; data not shown).

RNA expression analyses and ELISA

Total RNA was isolated using the NucleoSpin RNA II kit (Machery-Nagel, Leiden, The Netherlands) according to the manufacturer's instructions. Total RNA (0.5 – 1.0 µg) was reverse transcribed using the SuperScript III First Strand synthesis system from Invitrogen. TaqMan PCR was performed using TaqMan Universal PCR Master Mix and pre-designed, pre-optimized primers and probe mix for *RANTES* (*CCL5*), *IL8*, and *GAPDH* (Applied Biosystems, Foster City, USA). Threshold cycle numbers (Ct) were determined using the CFX PCR System (BioRad, Veenendaal, The Netherlands) and the relative quantities of cDNA per sample were calculated using the ΔΔCt method using *GAPDH* as the calibrator gene. ELISA's for CCL2, RANTES, IL8 and CXCL10 were performed according to the manufacturer's instruction (PeproTech, London, United Kingdom). Statistical differences in cytokine production were evaluated using a Welch-corrected t-test, correcting for possible unequal variances between the groups.

Gene expression profiling

Four primary EC cultures were used, HVK (vaginal), HCK (cervical), HFK_1 and HFK_2 (both foreskin), as well as four EC cell lines stably maintaining episomal HPV16 or 18, HVK16 (vaginal), HVK18 (vaginal), HCK18 (cervical),

and HPV16 (foreskin). Cells were harvested at five conditions: 0 hrs, 2 hrs and 24 hrs of 50 IU/ml IFNγ in combination with either L-Control or L-CD40L cells. Stimulated 2 hrs and 24 hrs samples were generated in duplo. Total RNA for these 72 samples was isolated as stated above. The microarray experiment was performed by ServiceXS according to their protocols (ServiceXS, Leiden, The Netherlands). Briefly, total RNA was analyzed by Lab-on-a-Chip. All RNA showed a RIN score of >9.5. Total RNA was reverse-transcribed, amplified and biotin labeled. cRNA was hybridized to Illumina Human HT-12 v4 BeadChips in a randomized fashion and scanned with the Illumina iScan. Samples passed quality control as assessed by Illumina GenomeStudio software. Values for missing bead types on the HumanHT-12 BeadChip were estimated using the k-Nearest Neighbor (k-NN) algorithm (Troyanskaya *et al., 2001) in Illumina's BeadStudio Gene Expression Module (v3.3+).*

Microarray data preprocessing

The expression array data was analyzed using R2.14.1 and Bioconductor (R Development Core Team, 2008). The data were normalized using the Bioconductor package lumi version 2.6.0 (Du *et al.*, 2008; Lin *et al.*, 2008), resulting in log2-transformed normalized intensities. Quality control plots were generated using limma version 3.10.2 (Smyth, 2005) and mpm version 1.0-22 (Wouters, 2011; Wouters *et al.*, 2003). Uninfected and HPV-positive ECs correlated in separate blocks, and within these blocks the next level similarity was at the cell line level, and within cell line at the exposure level, indicating that the data behaved as expected (data not shown). All microarray data is MIAME compliant and the raw data has been deposited in the MIAME compliant database Gene Expression Omnibus with accession number GSE54181, as detailed on the MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html.

Analysis of differentially gene expression

Differentially expressed genes were identified using maanova version 1.24.0 (Wu; Wu *et al.*, 2003). We modelled the cell line effect as a random effect and indicated the technical replicates in the model. We calculated test statistics for testing the null hypotheses of no difference in expression between L-CD40L-stimulated and L-Control stimulated cells at 2 and 24

hours for uninfected epithelial cells as well as HPV-positive epithelial cells for each gene. We applied the Fs statistic, which uses a shrinkage estimator for gene-specific variance components based on the James-Stein estimator. To correct for multiple testing, false discovery rates (FDR) were calculated using the q-value method (Dabney; Storey, 2002). The ranking and selection of the genes is based on these adjusted p-values.

Functional genomics analyses

The networks were constructed using Ingenuity Pathways Analysis (IPA version 17199142; Ingenuity systems, Inc., www.ingenuity.com). The list of differentially expressed genes was used to generate the network. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathway Knowledge Base.

Boxplot representations

Boxplots are drawn as a box, containing the 1st quartile up to the 3rd quartile of the data values. The median is represented as a line within the box. Whiskers represent the values of the outer 2 quartiles. These whiskers are however maximized at 1.5 times the size of the box (a.k.a. inter quartile distance). If 1 or more values outside of the whiskers are present, then this is indicated with a single mark ‘o’ next to the implicated whisker. Plots were generated using the webtool R2: microarray analysis and visualization platform (http://r2.amc.nl).

Migration assays

IFNγ pre-stimulated (HPV-positive) ECs were co-cultured with L-cells for 3 hours after which the L-cells were removed. The ECs were cultured a subsequent 24 hours with fresh K-SFM. Cleared (HPV-positive) EC supernatants were added to the lower compartment of a transwell plate (Corning). The upper compartment was filled with PBMCs, which were allowed to migrate for 16 hours, after which the cells in the lower compartment were counted by flow cytometry in the presence of counting beads (Invitrogen) according to the manufacturer’s protocol. Myeloid cells and lymphocytes were differentiated by their respective size in the FSC/SCC plot (data not shown). To normalize for biological differences between PBMC donors and EC cultures, a migration

index was calculated of the total number of PBMCs migrated towards the indicated stimulation over the medium control. The statistical significance of differences in migration towards supernatants of EC cultures stimulated with CD40L or control L-cells was assessed using a paired t-test.

Flow cytometry

Expression of CD40 on epithelial cells was analyzed by flow cytometry using FITC-coupled Mouse-anti-human CD40 (BD Biosciences, Breda, The Netherlands). 50.000 cells/live gate were recorded using the BD FACS Calibur with Cellquest software (BD Bioscience) and data were analyzed using Flowjo (Treestar, Olten, Switzerland).

Conflict of interest

CM has received speaker honoraria from Merck, Quest Diagnostics, GSK, and Bristol-Myers. CM has performed research funded by Merck, The Phillip Morris External Research Program, NexMed, GSK, OriGenix, and Interferon Sciences Inc.

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REFERENCES

Ait-Ghezala G, Mathura VS, Laporte V, et al. (2005) Genomic regulation after CD40 stimulation in microglia: relevance to Alzheimer's disease. Brain research Molecular brain research 140:73-85.

Andrei G, Duraffour S, Van den Oord J, et al. (2010) Epithelial raft cultures for investigations of virus growth, pathogenesis and efficacy of antiviral agents. Antiviral research 85:431-49.

Black AP, Ardern-Jones MR, Kasprowicz V, et al. (2007) Human keratinocyte induction of rapid effector function in antigen-specific memory CD4+ and CD8+ T cells. European journal of immunology 37:1485-93.

Bononi I, Bosi S, Bonaccorsi G, et al. (2012) Establishment of keratinocyte colonies from small-sized cervical intraepithelial neoplasia specimens. Journal of cellular physiology 227:3787-95.

Caproni M, Torchia D, Antiga E, et al. (2007) The CD40/CD40 ligand system in the skin of patients with subacute cutaneous lupus erythematosus. The Journal of rheumatology 34:2412-6.

Companjen AR, van der Wel LI, Boon L, et al. (2002) CD40 ligation-induced cytokine production in human skin explants is partly mediated via IL-1. International immunology 14:669-76.

Concha M, Vidal MA, Moreno I, et al. (2003) Evidence for modulation of human epidermal differentiation and remodelling by CD40. The British journal of dermatology 148:1105-14.

Dabney A qvalue: Q-value estimation for false discovery rate control. (R package version 1.28.0).

Denfeld RW, Hollenbaugh D, Fehrenbach A, et al. (1996) CD40 is functionally expressed on human keratinocytes. European journal of immunology 26:2329-34.

Du P, Kibbe WA, Lin SM (2008) lumi: a pipeline for processing Illumina microarray. Bioinformatics 24:1547-8.

Fries KM, Sempowski GD, Gaspari AA, et al. (1995) CD40 expression by human fibroblasts. Clinical immunology and immunopathology 77:42-51.

Fuller BW, Nishimura T, Noelle RJ (2002) The selective triggering of CD40 on keratinocytes in vivo enhances cell-mediated immunity. European journal of immunology 32:895-902.

Galy AH, Spits H (1992) CD40 is functionally expressed on human thymic epithelial cells. Journal of immunology 149:775-82.

Gaspari AA, Sempowski GD, Chess P, et al. (1996) Human epidermal keratinocytes are induced to secrete interleukin-6 and co-stimulate T lymphocyte proliferation by a CD40-dependent mechanism. European journal of immunology 26:1371-7.

Gommerman JL, Summers deLuca L (2011) LTbetaR and CD40: working together in dendritic cells to optimize immune responses. Immunological reviews 244:85-98.

Grousson J, Ffrench M, Concha M, et al. (2000) CD40 ligation alters the cell cycle of differentiating keratinocytes. The Journal of investigative dermatology 114:581-6.

Hollenbaugh D, Mischel-Petty N, Edwards CP, et al. (1995) Expression of functional CD40 by vascular endothelial cells. The Journal of experimental medicine 182:33-40.

Hostager BS, Bishop GA (2013) CD40-Mediated Activation of the NF-kappaB2 Pathway. Frontiers in immunology 4:376.

Kanda N, Shimizu T, Tada Y, et al. (2007) IL-18 enhances IFN-gamma-induced production of CXCL9, CXCL10, and CXCL11 in human keratinocytes. European journal of immunology 37:338-50.

Kanda N, Watanabe S (2007) Prolactin enhances interferon-gamma-induced production of CXC ligand 9 (CXCL9), CXCL10, and CXCL11 in human keratinocytes. Endocrinology 148:2317-25.

Karim R, Meyers C, Backendorf C, et al. (2011) Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. PloS one 6:e17848.

Karim R, Tummers B, Meyers C, et al. (2013) Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. PLoS pathogens 9:e1003384.

Kawaguchi S, Ishiguro Y, Imaizumi T, et al. (2009) Retinoic acid-inducible gene-I is constitutively expressed and involved in IFN-gamma-stimulated CXCL9-11 production in intestinal epithelial cells. Immunology letters 123:9-13.

Kehry MR (1996) CD40-mediated signaling in B cells. Balancing cell survival, growth, and death. Journal of immunology 156:2345-8.

Klein D, Timoneri F, Ichii H, et al. (2008) CD40 activation in human pancreatic islets and ductal cells. Diabetologia 51:1853-61.

Laman JD, Claassen E, Noelle RJ (1996) Functions of CD40 and its ligand, gp39 (CD40L). Critical reviews in immunology 16:59-108.

Li H, Nord EP (2005) CD40/CD154 ligation induces mononuclear cell adhesion to human renal proximal tubule cells via increased ICAM-1 expression. American journal of physiology Renal physiology 289:F145-53.

Lin SM, Du P, Huber W, et al. (2008) Model-based variance-stabilizing transformation for Illumina microarray data. Nucleic acids research 36:e11.

Ma DY, Clark EA (2009) The role of CD40 and CD154/CD40L in dendritic cells. Seminars in immunology 21:265-72.

McLaughlin-Drubin ME, Christensen ND, Meyers C (2004) Propagation, infection, and neutralization of authentic HPV16 virus. Virology 322:213-9.

Meyers C, Mayer TJ, Ozbun MA (1997) Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. Journal of virology 71:7381-6.

Moll R, Divo M, Langbein L (2008) The human keratins: biology and pathology. Histochemistry and cell biology 129:705-33.

Ohta K, Shigeishi H, Taki M, et al. (2008) Regulation of CXCL9/10/11 in oral keratinocytes and fibroblasts. Journal of dental research 87:1160-5.

Ohta Y, Hamada Y (2004) In situ Expression of CD40 and CD40 ligand in psoriasis. Dermatology 209:21-8.

Ortiz-Sanchez E, Chavez-Olmos P, Pina-Sanchez P, et al. (2007) Expression of the costimulatory molecule CD86, but not CD80, in keratinocytes of normal cervical epithelium and human papillomavirus-16 positive low squamous intraepithelial lesions. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 17:571-80.

Pasch MC, Timar KK, van Meurs M, et al. (2004) In situ demonstration of CD40- and CD154-positive cells in psoriatic lesions and keratinocyte production of chemokines by CD40 ligation in vitro. The Journal of pathology 203:839-48.

Peguet-Navarro J, Dalbiez-Gauthier C, Moulon C, et al. (1997) CD40 ligation of human keratinocytes inhibits their proliferation and induces their differentiation. Journal of immunology 158:144-52.

Peters AL, Stunz LL, Bishop GA (2009) CD40 and autoimmunity: the dark side of a great activator. Seminars in immunology 21:293-300.

Pluvinet R, Olivar R, Krupinski J, et al. (2008) CD40: an upstream master switch for endothelial cell activation uncovered by RNAi-coupled transcriptional profiling. Blood 112:3624-37.

R Development Core Team (2008) R: a language and enviroment for statistical computing. R Foundation for Statistical

Computing, Vienna, Austria. ISBN 3-900051-07-0. URL http://wwwR-projectorg.

Reiser J, Hurst J, Voges M, et al. (2011) High-risk human papillomaviruses repress constitutive kappa interferon transcription via E6 to prevent pathogen recognition receptor and antiviral-gene expression. Journal of virology 85:11372-80.

Romero-Tlalolini MA, Chavez Olmos P, Garrido E (2013) Differential DNA methylation patterns in the CD86 gene controls its constitutive expression in keratinocytes. Biochemical and biophysical research communications 438:54-60.

Sauty A, Dziejman M, Taha RA, et al. (1999) The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. Journal of immunology 162:3549-58.

Smyth GK (2005) Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor (Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, eds), New York: Springer.

Stojakovic M, Krzesz R, Wagner AH, et al. (2007) CD154-stimulated GM-CSF release by vascular smooth muscle cells elicits monocyte activation--role in atherogenesis. Journal of molecular medicine 85:1229-38.

Storey J (2002) A direct approach to false discovery rates. Journals of the Royal Statistical Society Series B:479-98.

Swamy M, Jamora C, Havran W, et al. (2010) Epithelial decision makers: in search of the 'epimmunome'. Nature immunology 11:656-65.

Takami Y, Nakagami H, Morishita R, et al. (2007) Ubiquitin carboxyl-terminal hydrolase L1, a novel deubiquitinating enzyme in the vasculature, attenuates NF-kappaB activation. Arteriosclerosis, thrombosis, and vascular biology 27:2184-90.

Tannahill GM, Curtis AM, Adamik J, et al. (2013) Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature 496:238-42.

Termini L, Boccardo E, Esteves GH, et al. (2008) Characterization of global transcription profile of normal and HPV-immortalized keratinocytes and their response to TNF treatment. BMC medical genomics 1:29.

Troyanskaya O, Cantor M, Sherlock G, et al. (2001) Missing value estimation methods for DNA microarrays. Bioinformatics 17:520-5.

van den Bogaard EH, Tjabringa GS, Joosten I, et al. (2013) Crosstalk between Keratinocytes and T Cells in a 3D Microenvironment: A Model to Study Inflammatory Skin Diseases. The Journal of investigative dermatology.

van der Burg SH, Melief CJ (2011) Therapeutic vaccination against human papilloma virus induced malignancies. Current opinion in immunology 23:252-7.

Villarroel Dorrego M, Whawell SA, Speight PM, et al. (2006) Transfection and ligation of CD40 in human oral keratinocytes affect proliferation, adhesion and migration but not apoptosis in vitro. Clinical and experimental dermatology 31:266-71.

Wouters L (2011) Exploratory graphical analysis of multivariate data, specifically gene expression data with different projection methods: principal component analysis, correspondence analysis, spectral map analysis (http://CRAN.R-project.org/package=mpm).

Wouters L, Gohlmann HW, Bijnens L, et al. (2003) Graphical exploration of gene expression data: a comparative study of three multivariate methods. Biometrics 59:1131-9.

Wu H maanova: Tools for analyzing Micro Array experiments. (http://research.jax.org/faculty/churchill).

Wu H, Kerr MK, Cui XQ, et al. (2003) MAANOVA: A Software Package for the Analysis of Spotted cDNA Microarray Experiments. In: The Analysis of Gene Expression Data: An Overview of Methods and Software (Parmigiani G, Garret ES, Irizarry RA, Zeger SL, eds), New York: Springer, 313–41.

Yellin MJ, Winikoff S, Fortune SM, et al. (1995) Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. Journal of leukocyte biology 58:209-16.