Absence of γ-chain in keratinocytes alters cytokine secretion resulting in reduced immune cell recruitment

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Short title (45 characters or fewer): Skin γ-chain influences cytokine secretion

Abbreviations:

γc – common gamma chain

DC – dendritic cell

HPV – human papilloma virus

HSCT - haematopoietic stem cell transplantation

IL – interleukin

KD – γc knock-down cells

LC – Langerhans cell

PBMC – peripheral blood mononuclear cell

(X-)SCID – (X-linked) Severe combined immunodeficiency

scr – scrambled control cells

Abstract

Loss of function mutations in the common gamma (γc) chain cytokine receptor subunit give rise to severe combined immunodeficiency (SCID) characterised by lack of T and natural killer cells and infant death from infection. Haematopoietic stem cell transplantation or gene therapy offer cure but despite successful replacement of lymphoid immune lineages a long-term risk of severe cutaneous human papilloma virus (HPV) infections persists, possibly related to persistent γc-deficiency in other cell types. Here we demonstrate that keratinocytes, the only cell type directly infected by HPV, normally express functional γc and its co-receptors. Following stimulation with the γc-ligand IL-15, γc-deficient keratinocytes demonstrate significantly impaired secretion of specific cytokines including Gro-α, IL-8 and Mip-3α resulting in reduced chemotaxis of dendritic cells and CD4+ T-cells. Furthermore, γc-deficient keratinocytes also exhibit defective induction of T-cell chemotaxis in a model of stable HPV18 infection. These findings suggest that persistent γc-deficiency in keratinocytes alters immune cell recruitment to the skin which may contribute to the development and persistence of warts in this condition and would require novel treatment approaches.

Introduction

The common gamma chain (γc) is the shared signalling subunit, for the interleukin (IL) cytokine receptors IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Inherited deficiency of γc results in X-linked severe combined immunodeficiency (X-SCID) characterised by absence of T- and natural killer (NK) cells and opportunistic infections (Buckley, 2004; Kovanen and Leonard, 2004). The natural history is death at a very young age but haematopoietic stem cell transplantation (HSCT) or gene therapy are effective treatments conferring protection from life threatening infections (Antoine *et al.*, 2003; Gaspar *et al.*, 2004b; Gaspar *et al.*, 2013). However, despite excellent long term survival following curative therapy, a persistent susceptibility to human papillomavirus (HPV) infections is well described that does not appear overall to relate to the conditions of transplant or immune reconstitution (Gaspar *et al.*, 2004a; Laffort *et al.*, 2004).

In three separate cohorts severe cutaneous warts developed in 19-64% of treated children, with warts onset 4 to 19 years after transplant (Gaspar *et al.*, 2004a; Kamili *et al.*, 2014; Laffort *et al.*, 2004). Original genotype is the main risk factor suggesting that γc cytokine-signalling is important for host defence against HPV. In support of this, similar HPV infections are seen in patients with SCID caused by deficiency of Janus kinase 3 (JAK3), the immediate downstream signalling partner for γc and to a lesser extent in patients with a defect of IL-7Rα, which selectively abrogates γc signalling following IL-7R ligation (Gaspar *et al.*, 2004a; Horev *et al.*, 2015; Neven *et al.*, 2009). The observation that warts are milder in IL-7Rα deficiency suggests that other γc cytokines in addition to IL-7 are likely to play a protective role against HPV.

The main HPV types found in lesions from affected patients were from the β1 (e.g. HPV5, HPV14 and HPV36) and α4 (e.g. HPV2 and HPV57) clades which usually only produce lesions in immunodeficient patients (Laffort *et al.*, 2004). Similar susceptibility to papillomavirus infection has been described in a canine model of γc-deficiency with severe chronic cutaneous

lesions observed in the majority of X-SCID dogs post-HSCT despite good immune reconstitution. Importantly, a high percentage (67%) of dogs with persistent canine PV infections developed invasive squamous cell carcinoma 3½ years after transplantation (Goldschmidt *et al.*, 2006), highlighting a potential long term cancer risk for affected X-SCID patients and the need for improved therapy that depends on better understanding of the pathogenesis of HPV infections in γc-deficiency.

Very persistent warts are uncommon in immunocompetent hosts, where most cutaneous HPV infections spontaneously regress within 1-5 years (Bruggink *et al.*, 2013; Williams *et al.*, 1993). Although the precise mechanisms of skin wart clearance remain to be clarified, lesion regression is associated with the presence of antigen presenting Langerhans cells (LC) in the epidermis, expression of the chemoattractant Mip-3α and recruitment of CD4+ and CD8+T-cells to the dermis (Iwatsuki *et al.*, 1986; Nakayama *et al.*, 2011). In contrast, reduced epidermal LC associated with regulatory T-cells in the dermis are found in non-regressing cutaneous lesions, suggesting that persistent warts are favoured by an immune suppressed local environment (Leong *et al.*, 2010; Sperling *et al.*, 2012). Studies of human and animal mucosal HPV infections further support an important role for T-cells for control of HPV infection (reviewed in (Hibma, 2012) and (Stanley, 2012)). In various disease models, mucosal lesion regression is associated with an influx of both CD4+ and CD8+ T cells, with a prominence of CD4+ T cells (Monnier-Benoit *et al.*, 2006; Peng *et al.*, 2007; Tong *et al.*, 2015) .

Despite apparently full T-cell correction, patients following HSCT for γc-deficiency present with severe cutaneous infections, mainly located on hands and feet, that are difficult to treat and lead to substantially reduced quality of life. While it remains feasible that HPV susceptibility in γc-deficient patients is caused by specific defects of haematopoietic immune reconstitution, for example in myeloid lineage dermal dendritic cells (DC) and LCs, it is also possible that an intrinsic defect in keratinocytes that are not replaced in HSCT is responsible.

This is an attractive hypothesis as keratinocytes are the only cell type directly infected with HPV and have an important role in skin immunity through secretion of a variety of chemokines and cytokines, such as IP-10, RANTES and Mip-3α, that recruit haematopoietic immune cells (Grone, 2002; Tokura *et al.*, 2008; Uchi *et al.*, 2000).

Here, we show that keratinocytes express functional γc and its co-receptors. We also show that secretion of chemokines by keratinocytes, following cytokine stimulation or when harbouring HPV genomes, is reduced in γc-deficient keratinocytes and that this leads to changes in lymphoid and myeloid migration. Our data suggests that altered immune cell recruitment as a result of intrinsic keratinocyte dysfunction may contribute to the retained susceptibility of X-SCID patients to HPV-associated disease following HSCT.

Results and Discussion

Keratinocytes express functional γc and co-receptors

Keratinocyte expression of γc and some of its co-receptors have been previously reported (Distler *et al.*, 2005; Hong *et al.*, 2015; Kagami *et al.*, 2005; Raingeaud and Pierre, 2005; Zhang *et al.*, 2008). To confirm and extend published findings, we measured expression of γc and all co-receptors in the NIKS keratinocyte cell line and primary keratinocytes. Both NIKS and primary keratinocytes expressed γc mRNA (Fig. 1a) and expressed protein at a comparable level to an EDR7 T-cell line engineered to overexpress γc and to primary CD8+ T-cells (Fig. S1a). These data suggest that keratinocytes express γc in physiologically relevant quantities.

In addition, mRNA and protein were detected for the co-receptors IL-2Rβ, IL-4R, IL-7Rα, IL-9R, IL-15Rα and IL-21R (Fig. 1a, S1b, S1c), while mRNA for IL-2Rα was absent in both NIKS and primary keratinocytes using two sets of primers, despite detectable expression of IL-2Rβ (Fig. 1a, S1c). This indicates that keratinocytes express a specific subset of γc-expressing cytokine receptors that does not include the heterotrimeric high affinity IL-2R critical for T-cell survival and proliferation (Sadlack *et al.*, 1995; Wang *et al.*, 2005) but is likely to include the low affinity IL-2R comprising IL-2Rβ and γc. Presence of a range of γc-containing receptors suggests that their cytokine ligands play an important role in keratinocyte biology. To test whether keratinocyte γc and co-receptors are functional, we measured phosphorylation of the downstream signalling molecules Signal transducer and activator of transcription 5 (STAT5) and Akt, following cytokine stimulation. Stimulation with IL-7, IL-9, IL-15 and IL-21 resulted in increased phosphorylation of STAT5 and pAkt, respectively, (Fig. 1b, S2a and data not shown), confirming intact signalling of multiple γc receptors in keratinocytes.

To further test the role of γc for keratinocyte function, we generated a cell line using shRNA technology in which γc mRNA was reduced by more than 70% (Fig. S2b) and γc expression was completely abrogated at the cell surface (Fig. 1c). Functional knock-down, resulting in a

failure to upregulate Akt phosphorylation following IL-15 stimulation, was confirmed (Fig. 1d).

γc deficient keratinocytes display impaired chemokine secretion

In hematopoietic lineages, ligation of γc-containing receptors is required for numerous cell functions including secretion of cytokines and chemokines that can act in an autocrine or paracrine manner (Chenoweth *et al.*, 2012; Kotlarz *et al.*, 2013). In the skin, chemokine secretion by both hematopoietic derived cells and keratinocytes plays a key role in cutaneous immune surveillance, recruiting a variety of different immune cell subsets to sites of infection or inflammation (Tan *et al.*, 2015). Although the early immune events in cutaneous HPV infection are poorly understood, micro-wounding of the skin is thought to be a pre-requisite for HPV entry and access to basal layers of the skin. Keratinocyte damage caused by micro-wounding induces keratinocyte release of IL-15 (Kennedy-Crispin *et al.*, 2012) which is distinct among γc receptor ligands in its ability to be cross presented to neighbouring cells through direct cell:cell interaction (Dubois *et al.*, 2002; Olsen *et al.*, 2007). Thus keratinocyte release of IL-15 is likely to impact neighbouring keratinocytes to activate specific cell functions. Therefore, we tested whether IL-15 stimulation was sufficient to induce keratinocyte production of chemokines that may be important for recruitment of other immune cells to the site of HPV infection.

Using a cytokine array to measure a broad range of chemokines, we observed that control keratinocytes produced a range of chemokines which was enhanced following IL-15 stimulation (Table 1). To specifically test whether keratinocyte γc-deficiency altered cytokine release in response to IL-15, we utilised a quantitative Luminex bead assay to analyse eight chemokines identified by the array to be strongly expressed or upregulated by IL-15 in control keratinocytes. Additionally, we included Mip-3α (CCL20/LARC) which was not part of the

array screen but which is known to be released by keratinocytes, modified in HPV infection and has been implied to be γc-dependent in other cell types (Kotlarz *et al.*, 2013; Sperling *et al.*, 2012; Tokura *et al.*, 2008). Using this method, significant increases (1.5 – 2-fold) were only observed in the secretion of IL-8 (CXCL8), Mip-3α and Gro-α (CXCL1) from control keratinocytes after stimulation with IL-15 but not RANTES, IP-10, TNF-α, GM-CSF or IL-1α (Fig. 2 and data not shown). Similar basal levels of these cytokines were produced by γc knock-down keratinocytes but upregulation of IL-8, Mip-3α and Gro-α following IL-15 stimulation was completely abolished, suggesting that γc-deficiency impairs cytokine release from keratinocytes following specific stimulation.

Impaired immune cell recruitment in response to cytokine secretion by γc-deficient cells

To test whether the reduced levels of IL-8, Mip-3α and Gro-α secretion by γc-deficient keratinocytes were sufficient to impact immune cell recruitment we tested migration of primary human cells *in vitro*. As the cytokines identified are best known for their ability to recruit neutrophils, we first analysed neutrophil migration to a cocktail of IL-8, Mip-3α and Gro-α combined at the concentrations detected after IL-15 stimulation of control keratinocytes. A significant increase in both average migration speed and directionality (defined in Materials and Methods) was seen in response to the cytokine cocktail, comparable to the positive control fMLP (Fig. 3a, 3b, S3b) demonstrating that the level of cytokines produced is sufficient to induce immune cell migration. As neutrophils are not thought to be associated with protection against HPV, we tested the response of DC and CD4+ T-cells that are considered to be more relevant during HPV infection (Amador-Molina *et al.*, 2013; Handisurya *et al.*, 2014; Handisurya *et al.*, 2013; Wang *et al.*, 2015). As seen with neutrophils, the average speed of DC migration was significantly increased using the concentration of cytokines released by IL-15-stimulated control keratinocytes (Fig. 3c, S3d). In contrast, lower chemokine concentrations,

equivalent to levels secreted from γc-deficient keratinocytes after IL-15 stimulation, induced no increase in migration speed. Similar effects were seen for CD4+ T cell migration using transwells, where significantly increased migration (comparable to fractalkine which is known to potently attract T cells and monocytes, Fig. S4b (Bazan *et al.*, 1997)) was seen towards the higher concentration of cytokines compared to the lower concentration (Fig. 3d). Together these findings suggest that reduced chemokine secretion following wounding of γc-deficient skin may impair immune cell recruitment, leading to enhanced susceptibility to basal layer keratinocyte infection after HPV entry.

γc-deficient keratinocytes stably harbouring HPV18 fail to induce CD4+ T cell migration

Long-term persistence of HPV papillomata in X-SCID patients suggests not only increased susceptibility to initial infection but also failure to eradicate established HPV infection. Our initial results suggest that changes in host immunity could increase susceptibility to HPV infection of γc-deficient basal layer keratinocytes but this does not explain why wart lesions, once established, are difficult to treat and do not regress in γc-deficiency despite restoration of T-cell immunity. Immunological control of HPV following established infection is thought to depend largely on T-cells, and a role for CD4+ and CD8+ T-cells is supported by the observation that in humans and in multiple animal models (murine, canine, rabbit and bovine), wart regression is associated with an influx of both subsets (Coleman *et al.*, 1994; Handisurya *et al.*, 2014; Knowles *et al.*, 1996; Nicholls *et al.*, 2001; Uberoi *et al.*, 2016; Wilgenburg *et al.*, 2005). Therefore, we set out to test whether defects in T-cell recruitment are seen with γc-deficient keratinocytes stably transfected with HPV18 to mimic established infection (Fig. 4a, Fig. S5). HPV18 was chosen as it enables stable transfection of keratinocytes whereas as low-risk α-types and β-types do not persist in cultured keratinocytes (Doorbar, 2016). In transwell assays, supernatants from control HPV18-transfected NIKS induced significantly greater

CD4+ T cell migration than supernatants from untransfected cells. This effect was completely abolished in γc-deficient keratinocytes (Fig. 4b) implying that CD4+ T cell recruitment to sites of established HPV infection could be impaired in γc-deficient patients. In contrast, an increase in CD8+ T cell migration was observed only with supernatants from γc-deficient cells (Fig. 4c), in keeping with dysregulated cytokine secretion by γc-deficient keratinocytes. Our data suggests that, in γc-deficient patients, altered balance between CD4+ and CD8+ T cell recruitment to skin sites could impact the efficacy of the induced immune response.

Together, our results demonstrate a role for γc-containing receptors in keratinocyte biology, in particular for cytokine secretion and induction of immune cell recruitment. Our *in vitro* experiments indicate that γc-deficient keratinocytes are unable to efficiently recruit lymphoid and myeloid immune cells in response to specific stimulation. Of particular relevance for HPV infection, γc-deficient keratinocytes were defective in recruiting CD4+ T cells and DC under conditions that mimicked early infection, which could favour viral persistence and subsequent formation of wart lesions. In addition, the ability to induce CD4+ T-cell recruitment, known to be important for wart regression in different animal models and humans (Coleman *et al.*, 1994; Handisurya *et al.*, 2014; Nicholls *et al.*, 2001; Peng *et al.*, 2007; Wilgenburg *et al.*, 2005), was impaired in response to established infection in γc-deficient keratinocytes which could be a factor favouring persistence of warts. We propose a model in which γc-deficiency limited to keratinocytes alters the host response both at the onset and in the persistence of HPV infection (Fig. 5). Further work is required to demonstrate the importance of our findings for the susceptibility to HPV infection in a γc-deficient context *in vivo* and to investigate the contribution of other cell types that also express receptors for IL-8 and Gro-α (Inngjerdingen *et al.*, 2001). In particular, analysing the role of NK cells would be of interest as poor NK cell engraftment was associated with HPV infection in a small cohort of X-SCID patients (Kamili *et al.*, 2014).

Understanding the role of keratinocytes in persistent HPV in X-SCID is an important goal to inform the design of improved treatment for this intractable complication, including novel vaccinations that could be administered prophylactically to provide protection against cutaneous HPV types.

Materials and Methods

Information about the isolation of primary keratinocytes, immunoblot, flow cytometry, reverse transcription PCR, analysis of phopho-proteins and transfection of NIKS with HPV18 vector can be found in the supplements.

Cell lines

ED-7R cells are a human T cell line derived from a patient with adult T cell leukaemia. They express IL-2Rα, IL-2Rβ but they lack γc (Arima *et al.*, 1992). The ED-7R + γc line was genetically modified to express the wild-type γc (Ishii *et al.*, 1994).

Normal Immortalised KeratinocyteS (NIKS) are a spontaneously immortalized keratinocyte cell line derived from neonatal human foreskin (Allen-Hoffmann *et al.*, 2000). This cell line was kindly provided by Dr. John Doorbar, Department of Pathology, University of Cambridge.

Lentiviral preparation and transductions of NIKS

pGIPZ vectors carrying the shRNA against γc (TCAGTAACAAGATCCTCTA) and the scrambled control (TGAACTCATTTTTCTGCTC) as well as a puromycin resistance cassette and turbo-GFP fluorescent marker were obtained from UCL Openbiosystems. The lentiviruses containing the shRNAs were produced, stored and concentration quantified as described previously using the VSVG packing plasmid (Metelo *et al.*, 2011). NIKS were transfected at a multiplicity of infection of 10. 72 hours post-transduction, selection with 2 µg/ml puromycin was carried out for a week and confirmed by ensuring >95% GFP expression by flow cytometry.

Cytokine Array and Luminex assays

NIKS were serum-depleted for 6 hours and then stimulated for 24 hours with 100 ng/ml IL-15 in serum-free E-medium at 37 °C or left unstimulated. Supernatants were collected and used for Proteome Prolifer™ Assay or Luminex Bead Assay (R&D Systems). All assays were carried out according to the manufacturers' instructions.

Isolating immune cells from whole blood

Blood was obtained from healthy volunteers with consent. Neutrophils were isolated directly from peripheral blood using the Neutrophil Isolation Kit/ MACSexpress. Peripheral blood mononuclear cells (PBMC) were collected following centrifugation of whole blood (diluted 1:1 in PBS) over ficoll. CD14+ cells and CD4+ and CD8+ T cells were isolated from PBMC using CD14 Microbeads, CD4+ and CD8+ T cell isolation kits, respectively (Miltenyi Biotec) following the manufacturer's instructions. Purity of CD8+ and CD4+ T cells was analysed by flow cytometry and was generally over 95%. DCs were generated using our established laboratory protocols. Briefly, CD14+ cells were cultured for 6 days in RPMI supplemented with 100 ng/ml GM-CSF and 25 ng/ml IL-4 which typically yields >95% CD11c+ cells with an immature phenotype (Burns *et al.*, 2004; Metelo *et al.*, 2011) and which have a characteristic dendritic appearance following adhesion to substrate (Fig. S3c).

Migration experiments using neutrophils

For migration experiments with neutrophils, Dunn Chambers (Hawksley, Medical & Laboratory Equipment) were used. Migration was carried out as previously described (Record *et al.*, 2015). In brief, $1 * 10^5$ neutrophils were seeded onto fibrinogen coated coverslips. These coverslips were placed on a Dunn Chamber (Zicha *et al.*, 1997) where the inner ring was filled with medium only and the outer ring was filled with chemoattractant-containing agarose so that a gradient formed across the bridge. The bridge was imaged by time-lapse microscopy for

1 h at 37 °C taking a photo every minute. Analysis was carried out tracking individual cells using the software Icy (de Chaumont *et al.*, 2012), which enables automatic tracking. For each condition in each experiment, 25 to 40 cells were analysed for their migratory speed and the directionality of their movement which is defined as the ratio of the direct distance between the start and end position of a cell divided by the total distance of the cell path.

Migration experiments using DCs

Migration experiments with DCs were carried out using the µ-Slide Chemotaxis 3D / Collagen IV coated slides (Ibidi) according to the manufacturer's instructions. In brief, 6 µl of a $3 * 10^6$ cells/ml cell suspension was seeded per slide and cultured at 37 °C, 5% CO_2 for 4 hours to allow cells to adhere to the collagen substrate. Plating of immature DCs to substrate induces their maturation (Burns *et al.*, 2004). The chemoattractant was filled into one of the outer chambers so that a gradient formed along the bridge of the migration chamber where the cells adhered and then the chamber was sealed to ensure CO_2 buffering. The bridge of the slide was imaged for 4 hours at 37 °C taking pictures every 5 minutes. Migration of cells over the bridge was analysed using the Manual Tracking plugin from ImageJ from around 10 cells per condition using the same method as previously described (Record *et al.*, 2015).

Migration experiments using T cells

Migration experiments using T cells were carried out using transwells with a 5.0 µm polycarbonate membrane in a 24 well plate (Costar) with T cells freshly isolated from donor blood. Cytokines diluted in culture medium at concentrations indicated in the figure legends or keratinocyte supernatants were added to the 24 well plate chamber. $1 – 2 * 10^5$ T cells were placed into the overlying transwell. After culturing for 37 °C for 2 hours, transwells were removed, the bottom of the transwell incubated in trypsin to remove cells that were attached to

the bottom of the well and these cells were pooled with the cells that had migrated into chamber of the 24 well plate. The total number of cells collected after migration was determined using a live cell dye (CyQUANT® NF cell proliferation assay kit, Thermo Fisher Scientific) following the manufacturer's instructions to stain the T cells at the end of the migration assay. Fluorescence was read using an excitation wave length of 490 nm and emission detection at 540 nm. Fold-migration was calculated using relative fluorescence levels.

Statistical Analysis

All graphs were made and statistical analysis carried out using GraphPad Prism. Data was analyzed using statistical tests according to data-sets: Kruskal-Willis test for multiple groups and Mann-Whitney test to compare two groups.

Conflict of Interest

The authors state no conflict of interest.

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Table 1: Cytokines and Chemokines with differential expression after IL-15 stimulation using the Cytokine Array

Cytokines printed in bold showed strong expression, changes in expression were determined using densitometry.

Coordinate	**Target**	**Unstimulated**	**+ IL-15**
A9/10	GM-CSF	Undetectable	Expressed
A11/12	**Gro-α**	**Strongly expressed**	**Upregulated**
B3/4	IL-1α	Expressed	Upregulated
B17/18	**IL-8**	**Strongly expressed**	**Upregulated**
D5/6	IP-10	Expressed	Upregulated
E3/4	RANTES	Undetectable	Slightly expressed
E7/8	TNF-α	Undetectable	Slightly expressed

Figure legends:

Fig. 1: Keratinocytes express functional γc and generation of a knock-down cell line:

a) RNA of ED-7R cell lines, NIKS and primary keratinocytes (KC) was isolated and RT-PCR was performed with primers specific for γc, IL-2Rα, IL-2Rβ, IL-4R, IL-7Rα, IL-9R, IL-15Rα and IL-21R and GAPDH as control.

b) NIKS were cultured in serum-free medium overnight at 37 °C, the following day they were stimulated with 100 ng/ml of the indicated cytokines for 10 min at 37 °C. After stimulation, cells were analysed by immunoblot for the expression of pAKT and GAPDH as control.

c) NIKS were transduced with shRNA against γc or scrambled control (scr) shRNA; γc expression was analysed by flow cytometry and compared to an isotype control.

d) NIKS cell lines (scrambled control = scr and γc-knock-down = KD) were cultured in serum-free medium overnight at 37°C, the following day they were stimulated with 10 ng/ml IL-15 for 10 min at 37 °C, lysed and analysed for the expression of pAKT and GADPH as control. Bar chart showing the compared intensities of the western blot bands obtained using ImageJ, mean ± SEM, *$p < 0.05$, $n = 5$.

Fig. 2: Secretion of chemokines after IL-15 stimulation:

Scrambled control (scr) and γc-knock-down (KD) NIKS were serum-depleted for 6 hours and then stimulated for 24 hours with 100 ng/ml IL-15 at 37°C, supernatants were collected and analysed for secretion of RANTES (a), IL-8 (b), Mip-3α (c) and Gro-α (d) using Luminex bead assays, mean ± SEM, $n = 6$, * $p < 0.05$.

Fig. 3: Migration of neutrophils, dendritic cells and CD4+ T cells.

a and b) Neutrophils were isolated from whole blood and used for migration experiments using Dunn chambers; shown is quantification for migration where each dot represents the mean value for all cells analysed from one donor; "none" contains no chemoattract, "cytokines" represents a cocktail of 10 μg/ml Gro-α, 3 μg/ml IL-8 and 1 μ/ml Mip-3α. fMLP at 100 nM was used as a positive control, mean ± SEM, *p<0.05, n = 4, shown are migratory speed (a) and directionality (b).

c) CD14+ derived dendritic cells were cultured on μ-Slides Chemotaxis 3D (Ibidi, Collagen IV coated) for migration assays. Shown is quantification where each dot represents the mean value for all cells analysed from one donor, "none" contains no chemoattractant, "scr + IL-15" is a cocktail of 10 μg/ml Gro-α, 3 μg/ml IL-8 and 1 μ/ml Mip-3α and "KD + IL-15" the same chemokines at half the concentration, mean ± SEM *p<0.05, n = 4.

d) CD4+ T cells were isolated from whole blood and used for transwell migration assays; values were normalized to the sample containing no chemoattractant; "scr + IL15" is a cocktail of 10 μg/ml Gro-α, 3 μg/ml IL-8 and 1 μ/ml Mip-3α and "KD + IL-15" the same chemokines at half the concentration, mean ± SEM, *p<0.05, n = 4, run in triplicate.

Fig. 4: Chemokine Secretion from HPV18 positive cells

a) Scrambled control (scr) and γc-knock-down (KD) NIKS were transfected with HPV18wt plasmid. DNA was isolated and used for PCR using HPV18 specific primers.

b) CD4+ T cells were isolated from whole blood and used for transwell migration assays as before; migration was measured towards supernatants harvested from scr control and KD NIKS with and without HPV18. Values shown represent the number of migrating cells normalised to experiments using supernatant from HPV18 negative samples.

c) CD8+ T cells were isolated from whole blood and used for transwell migration assays towards supernatant as described for CD4+ T cells. Values shown represent the number of

migrating cells normalised to experiments using supernatant from HPV18 negative samples.; b) and c) shown mean ± SEM, *p < 0.05, measured with four different sets of supernatants and two different blood donors, everything run in duplicate.

Fig. 5: Model for the effect of γc-deficiency on HPV infection

The absence of the common γc has an impact on the immune response to HPV infection in early and late stages. In the early stage of infection, IL-15 is released after wounding which is thought to be necessary for entry of HPV. Due to the lack of γc dependent signalling, chemokines such as Gro-α, IL-8 and Mip-3α are not induced and immune cells such as DCs and CD4+ T cells are not recruited. Therefore, the infection is not cleared resulting in wart lesion formation. In the later stages, when the infection is already established, alterations in chemokine secretion lead to changes in CD4+ and CD8+ T cell migration. Due to the resulting imbalances in the T cell subsets, warts do not regress.

Supplemental Methods

Isolation of primary keratinocytes

Patient skin biopsies were obtained with consent from procedures carried out at Great Ormond Street Hospital. Excess dermis and connective tissue underneath the epidermis were removed and the epidermis digested with 0.25% trypsin-EDTA for 3 – 4 hours at 37 °C. Cells were plated together with irradiated 3T3 feeder cells. Experiments with primary keratinocytes were carried out at passage 1.

Primary keratinocytes as well as NIKS were cultured in E-medium **(**3 parts DMEM, 1 part F-12) supplemented with 10% Fetal Calf Serum, 100 u/ml Penicillin-Streptomycin, 0.4 µg/ml hydrocortisone, 10-10 M choleratoxin, 1.8 x 10-4 M adenine, 5 µg/ml insulin and 10ng/ml EGF.

Reverse transcription PCR (RT-PCR)

Isolation of RNA was carried out using TRIzol reagent (Thermo Fisher Scientific) following the provided protocol. Reverse transcription of RNA samples was carried out using the GeneAmp® RNA PCR Core Kit (Thermo Fisher Scientific) following the provided protocol. PCR was carried out using Taq polymerase (Roche).

The following primers were used:

HPV18 E1^4: forward: TGTGCATCCCAGCAGTAAG, reverse: GGTGCTGGAATACGGTGA

γc: forward: ACAGGCCACACAGATGCTAA, reverse: CTATGCTGGTTGCATGGGGA

IL-2Rα: forward 1: CATTTCGTGGTGGGGCAGAT, reverse 1: CCGTGTCCTGTGATGTGACT; forward 2: AATGCAAAGTCCAATGCAGCC, reverse 2: TGTATCCCTGGACGCACTGA

IL-2Rβ: forward: TATGAGTTTCAGGTGCGGGTC, reverse: GAGCCACGGAATGGTGTCC

IL-4R: forward: AATGGGGTGGCTTTGCTCTG, reverse: GCTCATGTAGTCGGAGACGC

IL-7Rα: forward: CTCTGTCGCTCTGTTGGTCAT, reverse: ATCTGGCAGTCCAGGAAACT

IL-9R: forward: ATGTGGTAGAGGAGGAGCGT, reverse: CGACAGCTTGAACAGGAGGT

IL-15Rα: forward: GTCTCTCCTGGCATGCTACC, reverse: GCTGGTTTCCCCGAGTTTCA

IL-21R: forward: CCCGGTCATCTTTCAGACCC, reverse: TGCACCCACCCATTTCTTGA

GAPDH: forward: CCCATCACCATCTTCCAGGA, reverse: CCAGTGAGCTTCCCGTTCAGC

Flowcytometry

For cell surface stainings, keratinocytes (primary and NIKS) were detached using accutase and resuspended in PBS for staining. For intracellular markers, cells were fixed and permeabilised prior to addition of antibody using Lyse/Fix buffer and Perm Buffer III (BD Biosciences) following the manufacturer's protocol. Antibodies and respective isotype controls were from Biolegend; γc-APC, IL-2Rα-PE, IL-4R-PE, IL-9R-PE, IL-15Rα-PE and IL-21R-PE. Human STAT5 (pY694)-Alexa-647 antibody was from BD Biosciences. All staining was performed for 1 hour at 4 °C, followed by washing to remove excess antibody and immediately analysed by flow cytometry.

Immunoblotting

NIKS were scraped off the plate in cold NP-40 lysis buffer, incubated on ice for 10 minutes and cell debris removed. SDS-PAGE was carried out using SDS-PAGE gels at a Bis-Tris concentration of 4-12% (Thermo Fisher Scientific) following the manufacturer's instructions. Samples were transferred onto PVDF membranes following manufacturer's instructions. Membranes were blocked for 1 h in 5% BSA/TBS-Tween 0.1% and incubated overnight with the primary antibody at 4 °C in TBST. After washing and incubation with secondary antibodies, membranes were developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the images acquired using UVIchemi imaging system.

Akt and p-Akt antibodies were from Cell Signalling Technology. Antibodies against GAPDH, IL-2Rβ and IL-7R were from Santa Cruz Biotechnology. Anti-mouse HRP and anti-rabbit HRP were from GE Healthcare Life Sciences.

Analysis of phospho-proteins

NIKS were grown to approximately 80% confluency (assessed by microscopy) and incubated in serum-free E-medium (3 parts DMEM, 1 part F-12) overnight at 37°C. The following day, different concentrations of the various interleukins were added and the cells were incubated for 10 minutes at 37 °C. The cells were prepared for immunoblot or flow cytometry as described above and stained with either Human STAT5 (pY694)-Alexa 647 (BD Biosciences, flow cytometry) or unconjugated p-Akt antibody (Cell Signalling Technology, immunoblot).

Transfection of NIKS with HPV18 vector

To mimic HPV infection we utilised a characterised HPV18 plasmid (Lorenz *et al.*, 2013) which, in contrast with other in HPV plasmids, enables stable transfection of NIK cells (Doorbar, 2016). The HPV18 plasmid and blasticidin resistance plasmid were prepared and NIKS transfected with the two plasmids and selected as previously described (Lorenz *et al.*,

2013). 48 hours after transfection, selection with Blasticidin was started at a concentration of 7 µg/ml. Selection was carried out for 96 hours. Establishment of HPV18 genome in cells was confirmed by either Southern Blot as previously described (Lorenz *et al.*, 2013) or PCR for HPV18 E1^4.

Supplemental figures

Fig. S1: Protein expression of γc and its co-receptors

a) Primary human keratinocytes, the NIKS keratinocyte cell line, CD8+ T cells and ED-7R (negative control) and ED-7R + γc (positive control) cells were stained with anti-γc antibody and isotype control and analysed by flow cytometry.

b) NIKS cells were stained with antibodies for different γc co-receptors and the respective isotype controls and analysed by flow cytometry.

c) NIKS and primary human keratinocytes were lysed and analysed for their expression of IL-7Rα and IL-2Rβ by Western blot. GAPDH was used as internal control.

Fig. S2a: Phosphorylation of STAT5 and knock-down of γc

a) NIKS were cultured in serum-free media overnight at 37°C and then stimulated for 10 minutes with 1,000 ng/ml of IL-7 and IL-15 at 37°C, fixed, permeabilised and stained with a STAT5 (pY694) antibody and analysed by flow cytometry.

b) RNA was isolated from scr control and KD NIKS cell lines and RT-PCR was performed using γc specific primers. The RT-PCR products were separated on a 1% agarose gel; GAPDH RNA was amplified as internal control, bar charts showing the compared intensities of the bands obtained using ImageJ compared to UT, mean ± SEM, * $p < 0.02$, $n = 4$.

Fig. S3: Migration of neutrophils and dendritic cells

a) and b) Neutrophils were isolated from whole blood and used for migration experiments using Dunn chambers, a) Cytopsin of neutrophils before plating them on coverslips for migration, black scale bar equivalent to 25 μm; b) quantification for migration of one representative donor where each dot represents one tracked cell; “control” contains no chemoattract, “cytokines”

represents a cocktail of 10 µg/ml Gro-α, 3 µg/ml IL-8 and 1 µ/ml Mip-3α. fMLP at 100 nM was used as a positive control, mean ± SEM, ***p<0.001, *p<0.05, shown are migratory speed and directionality.

c) and d) CD14+ derived dendritic cells (DCs) were cultured on µ-Slides Chemotaxis 3D (Ibidi, Collagen IV coated) for migration assays, c) shows a microscopic image of DCs directly prior to migration assay; d) Quantification of migration is shown where each dot represents one cell tracked, "none" contains no chemoattractant, "scr + IL-15" is a cocktail of 10 µg/ml Gro-α, 3 µg/ml IL-8 and 1 µ/ml Mip-3α and "KD + IL-15" the same chemokines at half the concentration, mean ± SEM *p<0.05.

Fig. S4: Migration of CD4+ T cells

CD4+ T cells were isolated from whole blood and used for transwell migration assays; the number of migrated cells was determined after migration using the CyQuant NFCell Proliferation Assay.

a) Standard curve for the CyQuant NFCell Proliferation Assay showing correlation between cell number used and fluorescence detected.

b) Migration of CD4+ T cells towards fractalkine used at 40 µg/ml; migration was normalized to wells containing no fractalkine; n = 4.

c) Migration of CD4+ T cells towards cytokine cocktails, "scr" is a cocktail of 10 µg/ml Gro-α, 3 µg/ml IL-8 and 1 µ/ml Mip-3α and "KD" the same chemokines at half the concentration, shown are the four different donors (labelled I-IV) which were run in triplicate, each of the dots represents one of these triplicates.

Fig. S5: Expression of HPV18 in transfected NIKS

Scrambled control (scr) and γc-knock-down (KD) NIKS were transfected with recircularised HPV18 genomes. Extrachromosomal DNA was isolated and digested with NcoI which cuts the HPV18 genome once or HindIII which does not cut the HPV18 genome. Samples were used for Southern blot with a probe against the HPV18 genome. Linearised plasmid DNA was used as standards (labelled with copy number).

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