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Allele-specific cytokine responses at the *HLA-C* locus, implications for psoriasis

Christian Hundhausen[1,5], **Anna Bertoni**[1,5], **Rose K Mak**[1], **Elisabetta Botti**[1,2], **Paola Di Meglio**[1], **Alex Clop**[1], **Ute Laggner**[1], **Sergio Chimenti**[2], **Adrian C Hayday**[3,4], **Jonathan N Barker**[1], **Richard C Trembath**[1], **Francesca Capon**[1], and **Frank O Nestle**[1]

[1]Division of Genetics and Molecular Medicine, King's College London, London, UK

[2]Department of Dermatology, "Tor Vergata" University of Rome, Rome, Italy

[3]Division of Immunology, Infection and Inflammatory Disease, King's College London, London, UK

[4]London Research Institute, Cancer Research UK.

Abstract

Psoriasis is an inflammatory skin disorder that is inherited as a complex trait. Genetic studies have repeatedly highlighted *HLA-C* as the major determinant for psoriasis susceptibility, with the *Cw*0602* allele conferring significant disease risk in a wide-range of populations. Despite the potential importance of *HLA-C* variation in psoriasis, either *via* an effect on peptide presentation or immuno-inhibitory activity, allele-specific expression patterns have not been investigated. Here, we used reporter assays to characterize two regulatory variants, which virtually abolished the response to TNF-α (rs2524094) and IFN-γ (rs10657191) in *HLA-Cw*0602* and a cluster of related alleles. We validated these findings through the analysis of *HLA-Cw*0602* expression in primary keratinocytes treated with TNF-α and IFN-γ. Finally, we showed that *HLA-Cw*0602* transcripts are not increased in psoriatic skin lesions, despite highly elevated TNF-α levels. Thus, our findings demonstrate the presence of allele-specific differences in *HLA-C* expression and indicate that *HLA-Cw*0602* is unresponsive to up-regulation by key pro-inflammatory cytokines in psoriasis. These data pave the way for functional studies into the pathogenic role of the major psoriasis susceptibility allele.

Introduction

Psoriasis is a chronic, inflammatory skin disorder, affecting approximately 2% of the Caucasian population. The disease is characterized by keratinocyte hyperproliferation and altered differentiation, in the presence of an inflammatory skin infiltrate, typically consisting of dendritic cells, macrophages, T cells and neutrophils (Nestle *et al.*, 2009). Psoriasis has a strong genetic component and is widely regarded as a multifactorial disorder, resulting from gene-gene and gene-environment interactions (Griffiths and Barker, 2007; Liu *et al.*, 2007).

A highly significant association between psoriasis and the HLA-Cw6 antigen has long been established (Henseler and Christophers, 1985; Mallon *et al.*, 1999). In agreement with these

Correspondence: Prof. Frank O Nestle, St. John's Institute of Dermatology, Division of Genetics and Molecular Medicine, King's College London, London, UK; Dr Francesca Capon, Division of Genetics and Molecular Medicine, King's College London, London, UK. frank.nestle@kcl.ac.uk; francesca.capon@kcl.ac.uk.
[5]These authors contributed equally to this work

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historical observations, genome-wide linkage scans have repeatedly identified a primary disease susceptibility locus (*PSORS1*), lying within the class I region of the Major Histocompatibility Complex (MHC), on chromosome 6p21.3 (Nair *et al.*, 1997; Trembath *et al.*, 1997). Refinement studies, deep sequencing experiments and recent genome-wide association data all point to *HLA-C* as the most likely *PSORS1* gene (Liu *et al.*, 2008; Nair *et al.*, 2006; Strange *et al.*, 2010; Veal *et al.*, 2002). However, the extended Linkage Disequilibrium (LD) that characterizes the MHC region has complicated the analysis of the *PSORS1* locus and has so far prevented the identification of the casual susceptibility variant. At the same time, the analysis of *PSORS1* sequences associated with different *HLA-C* alleles has demonstrated that the Single Nucleotide Polymorphisms (SNPs) that are unique to *HLA-Cw*0602* haplotypes lie exclusively in non-coding regions (Nair et al., 2006).

In this context, the hypothesis driving this study is that the *PSORS1* disease susceptibility allele may lie within a regulatory region influencing *HLA-C* expression. To explore this pathogenic model, we investigated the differential regulation of three *HLA-C* alleles, using reporter assays, in combination with patient-based expression studies. This integrated approach demonstrated the previously unreported existence of allele-specific *HLA-C* expression patterns and specifically identified two regulatory variants affecting the response to IFN-γ and TNF-α.

Results

Two sequence variants in the *HLA-C* promoter cause non-responsiveness to TNF-α and interferons

To assess the presence of *HLA-Cw*0602* specific gene expression patterns, we measured the reporter activity driven by the *HLA-Cw*0602*, *-Cw*0702* and *-Cw*0304* promoters. We examined a 426 bp fragment corresponding to the gene minimal promoter (Johnson, 2003) and found that the *HLA-Cw*0602* reporter construct was significantly less active than its *HLA-Cw*0702* counterpart (Figure 1a). Importantly, the *HLA-C* promoter contains an experimentally defined enhancer κB element (Johnson and Pober, 1994), which displays a G to A substitution (SNP rs2524094, see Figure S1) in *HLA-Cw*0602* and in a cluster of related alleles. To investigate whether this polymorphism was responsible for the differential activity of the *HLA-Cw*0602* and *-Cw*0702* promoters, we repeated the reporter assays following site-directed mutagenesis of the *HLA-Cw*0702* enhancer κB. These experiments revealed a significant decrease in reporter activity for the mutagenized construct (Figure 1b), thus confirming the functional impact of the rs2524094 polymorphism.

To investigate whether SNP rs2524094 also affects inducible promoter activity, we next carried out reporter assays in cells that had been stimulated with TNF-α, IL-17A and IL-22, three inflammatory cytokines which signal through the NF-κB pathway and play a key role in the pathogenesis of psoriasis (Nickoloff *et al.*, 2007). Surprisingly, none of the examined *HLA-C* alleles responded to IL-17A or IL-22 treatment (Figure 1f). Conversely, the *HLA-Cw*0702* promoter displayed significant TNF-α induced luciferase activity, in keeping with the role of this cytokine as an inducer of MHC gene expression (Johnson, 2003). At the same time, *HLA-Cw*0602* showed a weak and non-significant TNF-α response (Figure 1c). The analysis of mutagenized constructs demonstrated that the introduction of a wild-type rs2524094 allele in the -Cw*0602 promoter restored TNF-α responsiveness (Figure 1e). Conversely, disruption of the HLA-Cw*0702 enhancer κB resulted in a more modest TNF-α response (Figure 1d). Thus, the TNF-α induced activity of *HLA-Cw*0702* is dependent on the integrity of the enhancer κB element.

The *HLA-C* minimal promoter also contains an Interferon Response Stimulated Element (ISRE), similar to the one mediating IFN induction of the *HLA-A* and *HLA-B* genes

(Johnson, 2003). Of note, the *HLA-C* ISRE has a different base composition in *HLA-Cw*0602* and -*Cw*0702,* due to the occurrence of a 3bp deletion in the latter allele (−166_−163delTCT, rs10657191; Figure S1). Reporter assays showed that *HLA-Cw*0702,* but not *HLA-Cw*0602* could respond to IFN stimulation. The analysis of a mutagenized construct confirmed that the ISRE deletion polymorphism was responsible for this difference (Figures 1g and 1h).

Harbouring the same κB and ISRE elements as *HLA-Cw*0602*, -*Cw*0304* served as control and showed similar reporter activity to -*Cw*0602* throughout all experiments (Figures 1a, 1c, 1f, 1g and 1h).

Taken together, these results demonstrate that the rs2524094 and the rs10657191 promoter variants affect *HLA-C* cytokine responses, with the A and insertion alleles determining non-responsiveness to TNF-α and IFNs.

TNF-α treatment of primary keratinocytes fails to up-regulate *HLA-Cw*0602*

Having established that the *HLA-Cw*0602* promoter displays reduced cytokine responses in reporter assays, we proceeded to investigate *HLA-C* expression in cultured primary keratinocytes. As the presence of inflammatory cytokines in psoriatic skin would have confounded the interpretation of our results, we carried out these experiments in cells isolated from healthy donor skin samples. In keeping with the results obtained in reporter assays neither IL-17A nor IL-22 had a significant effect on *HLA-C* expression, regardless of the *HLA-C* genotype (Figures 2a, 2b and 2d). In both *HLA-Cw*0602* positive and negative keratinocytes, IFN-γ was the only single cytokine that profoundly upregulated *HLA-C* (Figures 2a and 2b). The strong synergistic effect of TNF-α in combination with IFN-γ as observed for donor N1 (Figure 2a) was significantly reduced in *HLA-Cw*0602* positive keratinocytes (donor N2, Figure 2a). Following the validation of antibody specificity (Figure S2a), Western blotting for HLA-C was performed confirming the mRNA data at protein level (Figure 2b). To analyze cytokine induced HLA-C cell surface expression, immunofluorescence staining was also performed on keratinocytes from donor N1. The results confirmed IFN-γ as the strongest modulator of HLA-C expression (Figure 2c).

To specifically investigate the regulation of *HLA-Cw*0602* transcripts, we next designed allele-specific, exon-spanning primers (Figure S2b), which we used in real-time PCR measurements of gene expression. The results of these experiments were again consistent with those obtained in reporter assays. Total *HLA-C* and *HLA-Cw*0602* mRNA levels were both significantly up-regulated in response to IFN-γ, however *HLA-Cw*0602* expression was markedly less enhanced (Figure 2d). Moreover, TNF-α treatment had no effect on *HLA-Cw*0602* transcript levels, and total *HLA-C* expression increased only in the cells derived from donor N3 (Figure 2d,), whose non-risk allele (Cw*17) carries a canonical enhancer κB sequence (Figure S1).

Thus, the analysis of endogenous *HLA-Cw*0602* expression in human keratinocytes confirmed the non-responsiveness to TNF-α, which we had observed in our reporter assays.

*HLA-Cw*0602* expression levels do not differ significantly in normal, peri-lesional and lesional skin

In the final phase of this study, we investigated *HLA-C* expression in whole tissue, by analyzing total RNA isolated from normal skin (N) (n= 31) and from matched pairs of peri-lesional (PL) and lesional (L) psoriasis skin biopsies (n= 26). We found that there was variation in non-risk *HLA-C* transcript levels (Figures 3a), potentially reflecting the presence of various combinations of regulatory elements in the different non-risk alleles (Table S1, Figure S1, Figure S3). In the genetically homogenous *HLA-Cw*0602*

chromosome population, no significant up-regulation of *HLA-Cw*0602* gene expression was detected between normal, peri-lesional and lesional samples (Figure 3b), despite the presence of elevated TNF-α and IFN-γ levels in psoriatic skin (Figure S4). The amount of total HLA-C protein found in the epidermis was variable, but no evidence of up-regulation was observed in lesional (highlighted with an L in Figure 3c) vs. non-lesional (PL) samples, obtained from *HLA-Cw*0602* heterozygous individuals (Figure 3c).

Our data also show that there is no correlation between *HLA-C* expression and PASI score (Figure S5) and that transcript levels are not affected by the use of topical or systemic therapeutic agents (Figure S6). Thus, our results are unlikely to be confounded by individual variation in disease severity or treatment.

Taken together, these findings are in agreement with the notion of *HLA-Cw*0602* non-responsiveness to TNF-α, as they show that *HLA-Cw*0602* gene expression remains largely unchanged in psoriatic skin, despite the presence of elevated cytokine levels.

Discussion

The present study explores allele-specific patterns of *HLA-C* regulation, using a combination of reductionist promoter assays and studies in human tissue samples.

Our work was driven by the results of serologic studies, which have long established an unequivocal association between HLA-Cw6 and psoriasis (Henseler and Christophers, 1985; Mallon *et al.*, 1999) and those of recent genome-wide association scans, which have highlighted *HLA-C* as the most likely *PSORS1* candidate gene (Ellinghaus *et al.*, 2010; Strange *et al.*, 2010; Stuart *et al.*, 2010; Sun *et al.*, 2010). Due to its function as innate and adaptive immunoregulator, an involvement of HLA-C in psoriasis pathogenesis is conceivable. HLA-Cw6 in particular has peptide-presenting capacity (Dionne *et al.*, 2004; Falk *et al.*, 1993) and can trigger $CD8^+$ T cell specific responses (Dionne *et al.*, 2004; Johnston *et al.*, 2004).

Although total *HLA-C* expression has previously been investigated in psoriasis (Carlen *et al.*, 2007; Carlen *et al.*, 2005; Nair *et al.*, 2009; Zhou *et al.*, 2003), allele-specific patterns have never been examined. In this context, our study provides important insights into the regulation and expression of the risk allele *HLA-Cw*0602,* in normal, peri-lesional and lesional psoriatic skin.

We have used a combination of reporter and real-time PCR assays to characterize a promoter variant (rs2524094) mediating reduced responsiveness to TNF-α. We also investigated an insertion/deletion polymorphism (rs10657191) and found that it determined reduced sensitivity to IFNs, in reporter assays. However, this effect was not replicated in real-time PCR experiments, where differential IFN responses were observed in alleles sharing the same ISRE element (Figure 2d). Thus, we suggest that other regulatory elements outside of the *HLA-C* minimal promoter might be of importance for the IFN response. We further argue that the Th17 association with psoriasis (Di Cesare *et al.*, 2009) is not related to an effect on *HLA-C* transcript levels, as both IL-17A and IL-22 failed to alter *HLA-C* promoter activity or endogenous expression. Our observation that *HLA-Cw*0602* transcript levels do not differ significantly between normal and psoriatic skin is in keeping with the above findings and further support the notion of *HLA-Cw*0602* reduced responsiveness to pro-inflammatory cytokines.

It is worth pointing out that the rs2524094 variant is not unique to *HLA-Cw*0602*, so that other *HLA-C* alleles are likely to show reduced responsiveness to TNF-α. In fact our reporter assays show that this is the case for *HLA-Cw*0304*, which harbours the same

enhancer κB element as -*Cw*0602*. At the same time, reduced responsiveness to TNF-α is likely to have a different impact on risk and non-risk *HLA-C* alleles, as they are likely to have differential affinities for self-peptides and to interact with different binding partners. For instance the HLA-Cw6 (but not the HLA-Cw3) protein has been shown to interact with KIR2DL1 inhibitory receptor, which regulates the activity of Natural Killer and T cell subsets (Parham, 2005). Of note, associations between psoriasis and KIR receptor polymorphisms have been reported in the past (Luszczek *et al.*, 2004), with a number of studies suggesting pathogenic and protective roles for specific HLA-C/KIR genotype combinations (Holm *et al.*, 2005; Nelson *et al.*, 2004). Further functional experiments will now be required to explore the impact of TNF-α non-responsiveness on HLA-C KIR interactions and self-peptide presentation. Such studies hold the promise to elucidate the molecular mechanisms underlying the contribution of *HLA-Cw*0602* to psoriasis susceptibility.

Material and Methods

Study population

Blood samples and skin biopsies were obtained from 26 patients with plaque psoriasis (Table S1). Control skin tissue was obtained from 31 healthy volunteers undergoing breast or abdominal reduction surgery. Our study was conducted in accordance with the Helsinki Declaration, with informed consent obtained from each volunteer and ethical approval granted by the institutional review board of Guy's Hospital.

Cell culture and cytokine stimulation assays

HeLa and HEK293T cell lines were cultured in DMEM medium (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% Penicillin/Streptomycin (PAA, Yeovil, Somerset, UK). LCL721.221-Cw*0602, LCL721.221-mock and HLA class I K562 transfectants were kindly provided by Dr Matthias Marget (Institute of Immunology, University Medical Center Schleswig-Holstein Germany), and Dr Eric Champagne (INSERM Toulouse, France), respectively; both LCL721.221 and K562 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin.

Primary keratinocyte cell cultures were established using the procedure described by (Mee *et al.*, 2007). Keratinocytes were cultured for 2-4 passages in KGM-2 medium, in the presence of growth supplements (KGM-2 Bullet Kit CC-3107, Lonza, Slough, UK). For cytokine treatment, cells were stimulated for 24 hours with either 10 ng/ml TNF-α, 500 U/ml IFN-γ (PeproTech, London, UK), 10 ng/ml IL-17A or IL-22 (R&D Systems, Abingdon, UK).

Plasmids

Three BAC clones carrying *HLA-C*, but not *HLA-A* or *HLA-B*, were used as templates for the specific amplification of the *HLA-C* promoter. In particular, the BACs containing the *Cw*0602* and *Cw*0702* promoters were selected from a library, which had been previously generated from the leukocytes of a heterozygous psoriatic patient, using the procedure described by Osoegawa *et al.* (2001). A third BAC, which carries the *HLA-Cw*0304* promoter, was kindly provided by Dr Lucy Matthews (Wellcome Trust Sanger Institute, Cambridge, UK). Allele specific oligonucleotides tagged with XhoI and HindIII (New England Biolabs, Hitchin, UK) restriction sites, were used to clone the minimal *HLA-C* promoter (Johnson, 2003) into the pGL3-basic vector (Promega, Southampton, UK). Primer sequences were HLA-C.F 5′-AATCGCTCGAGAGGGACGGGGATTCCAGGAG-3′,

HLA-Cw*0602/*0304.R 5′-AGGCAAGCT TCTCGGCGTCTGGGGAGA-3′, HLA-Cw*0702.R 5′-AGGCAAGCTTCTCGGCCTCTGGGGAGA-3′.

Mutagenized constructs were generated using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to manufacturer instructions. The presence of the correct insert was verified by direct sequencing of all wild-type and mutant constructs.

Transient transfection and dual-light reporter assay

HeLa or HEK293T cells were transfected with 1 μg pGL3-basic vector and 0.45 μg pJ7lacZ vector (kindly provided by Dr Talat Nasim, King's College London, UK), using the FuGene HD transfection reagent (Roche, Mannheim, Germany). Cells were harvested 24 hours post-transfection and reporter gene expression was measured with the Dual-Light® luminescent reporter gene assay (Applied Biosystems, Bedford, UK), according to the manufacturer's instructions.

RNA isolation and real-time PCR

Total RNA was isolated from cell lines and primary human keratinocytes using the RNeasy Plus Mini Kit (Qiagen) or TRI REAGENT (Sigma-Aldrich), according to the manufacturer's protocol. 500-1000 ng total RNA was reverse transcribed into cDNA, using SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions.

Total *HLA-C* and *HLA-Cw*0602* mRNA levels were assessed by quantitative PCR (gene expression assay Hs03044135_m1) and SYBR Green based PCR, respectively. Primers for specific detection of the *HLA-Cw*0602* transcript were: Forward 5′ TACTACAACCAGAGCGAGGA 3′ and Reverse 5′ GGTCGCAGCCATACATCCA 3′. *PPIA*, *GAPDH* beta-actin transcripts were used as endogenous controls. Samples were amplified on a Applied Biosystems 7900HT Sequence Detection System, and real-time data were presented either as individual data points normalized to the internal control ($2^{-\Delta Ct}$) or expressed as fold change using the comparative C_T ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008).

HLA-C locus typing

Genomic DNA was extracted from peripheral blood or skin, using the DNeasy Blood & Tissue Kit (Qiagen). Medium resolution *HLA-C* typing was performed using polymerase chain reaction with sequence specific primers (PCR-SSP) (Bunce *et al.*, 1995).

Western blotting

Cell and tissue lysates were analysed by Western blotting, using 1 μg/ml anti HLA-C goat polyclonal antibody Q-18 (Santa Cruz Biotechnology, CA, USA) together with 1 μg/ml anti HSP90 rabbit monoclonal antibody H-114 (Santa Cruz Biotechnology) or anti β-actin mouse monoclonal antibody AC-15 (1:2000, Sigma-Aldrich) as endogenous controls.

Cytospin and immunofluorescence

Cytospin (Cytospin® 3 Cytocentrifuge) was used to deposit monolayers of 2×10^5 primary human keratinocytes on glass slides. Cells were fixed with acetone at −20°C and incubated with HLA-C (Q18) goat polyclonal IgG antibody (1:50 dilution) for 1h at RT. Following incubation with donkey anti-goat Alexa 555 IgG antibody (1:200 dilution) cells were mounted with Prolong® Gold antifade reagent supplemented with DAPI (Invitrogen, Paisley, UK). Pictures were obtained using a fluorescent microscope (Zeiss Axiophot Microscope) with a camera (Nikon Digital Sight Camera).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LD	linkage disequilibrium
PSORS	psoriasis susceptibility locus
PASI	psoriasis area and severity index

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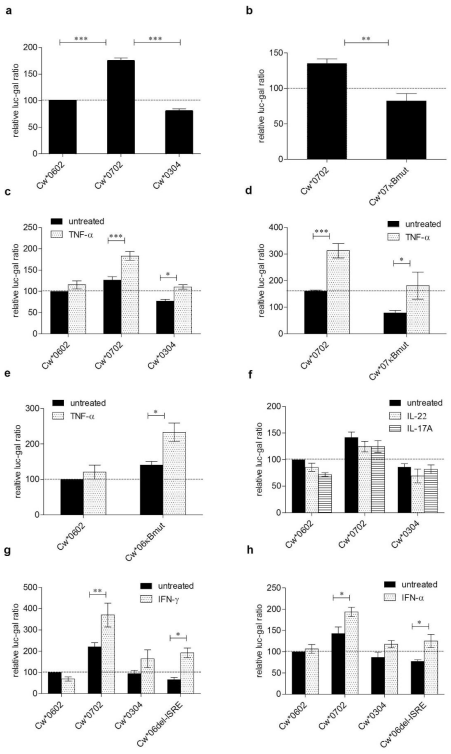

Figure 1. *HLA-C* promoter variants determine non-responsiveness of *HLA-Cw*0602* to TNF-α and interferons

HeLa (a-f) or HEK293T (g-h) cells were transfected with a luciferase gene under the control of wild-type and mutagenized *HLA-C* promoters, along with a control reporter gene encoding for β-galactosidase.

(c-h) Transfected cells were treated with the indicated cytokines for 24 hours and a dual-light reporter assay was performed, in order to measure luciferase and β-galactosidase activities. The results are expressed as the mean +/− SEM of luciferase: β-galactosidase ratios. The Cw*06 luciferase: β-galactosidase ratio was set as baseline to which all other measurements were normalized. Each experiment was performed at least three times in triplicate. Statistical significance was calculated using One-Way ANOVA (a), t-test (b) or Two-Way ANOVA (c-h). $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. κBmut refers to the site-directed mutagenesis performed in the enhancer κB of the -*Cw*0602* and -*Cw*0702* alleles. del-ISRE refers to the introduction of a 3bp deletion in the -Cw*0602 ISRE

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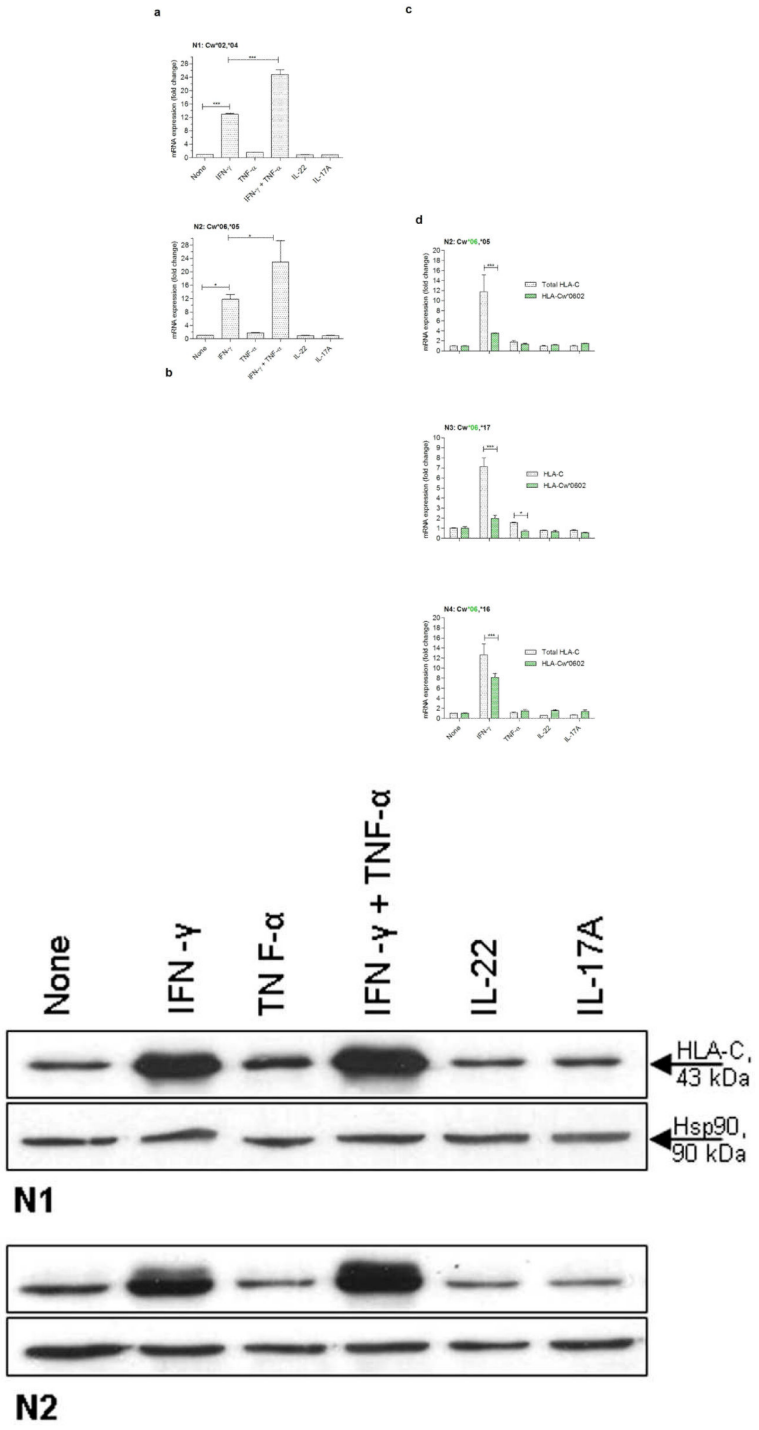

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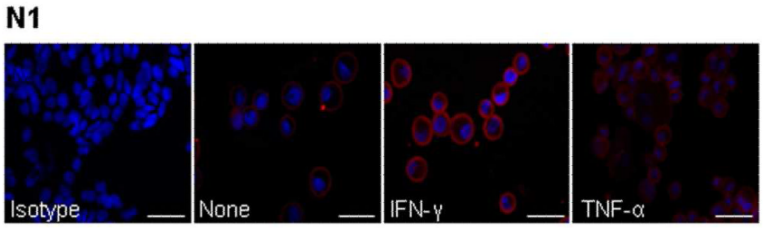

Figure 2. Cytokine-induced *HLA-C* expression in primary keratinocytes

(a) Primary keratinocytes derived from healthy donors N1 and N2 were treated with TNF-α (10 ng/ml), IFN-γ (500 U/ml), TNF-α (10 ng/ml) + IFN-γ (500 U/ml), IL-17A (10 ng/ml) and IL-22 (10 ng/ml) for 24 h or left untreated. For each condition, cells were split for RNA and protein isolation. *HLA-C* transcript levels were measured by Real-time PCR and normalized to the mean of *cyclophylin A* and *Rplpo* (large ribosomal protein) expression. *HLA-C* expression levels are plotted as fold changes compared to the untreated control. Significance values were calculated using One-Way ANOVA. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. (b) 1% Triton lysates of keratinocytes derived from donors N1 and N2 were analysed for HLA-C protein expression by Western blotting. (c) Immunofluorescence staining of HLA-C. Cytokine-treated keratinocytes from donor N1 were deposited on glass slides by Cytospin and stained for HLA-C protein (red). Nuclei were counterstained with DAPI (blue). Scale bar = 50μm. (d) Total *HLA-C* and *HLA-Cw*0602* mRNA levels were determined in primary keratinocytes derived from three *HLA-Cw*0602* heterozygous donors. Cytokine stimulation, RNA extraction and cDNA synthesis were carried out as described in (a). Relative mRNA levels were expressed as fold change compared to the untreated control. White bars indicate total *HLA-C* and green bars *HLA-Cw*0602* expression. Significance values refer to differences in fold induction.

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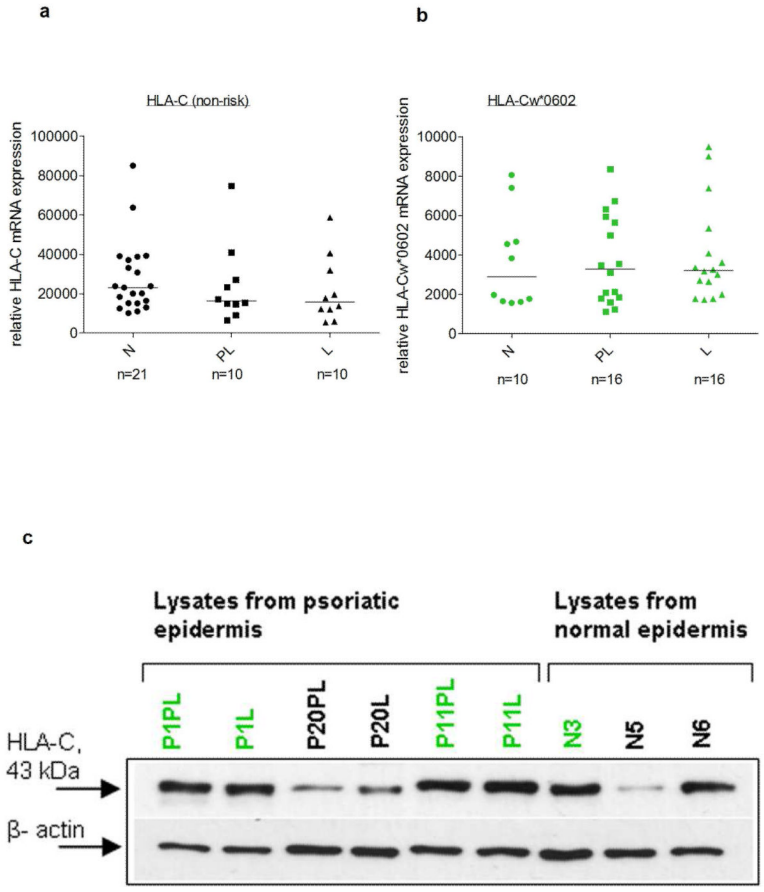

Figure 3. *HLA-C* expression levels in normal and psoriatic skin

(a,b) *HLA-C* and *HLA-Cw*0602* mRNA expression was measured in normal skin (N) and in matched pairs of peri-lesional (PL) and lesional (L) psoriatic skin. (a) *HLA-C* expression in *HLA-Cw*0602* negative samples of N (n=21), PL (n=10) and L (n=10) skin (b) *HLA-Cw*0602* expression in -*Cw*0602* +/− samples of N (n=10), PL (n=16) and L (n=16) skin. Real-time data are presented as individual data points normalized to expression of the internal controls *GAPDH* (a) and beta-actin (b). One-Way Anova statistical test was performed for (a) and (b) yielding p values > 0.05. (c) Western blotting for HLA-C protein in the epidermis of normal and psoriatic skin. Green font indicates samples heterozygous for *HLA-Cw*0602*, black font *HLA-Cw*0602* negative samples.

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