Human papillomavirus 16-encoded E7 protein inhibits IFN-γ-mediated MHC class I antigen presentation and CTL-induced lysis by blocking IRF-1 expression in mouse keratinocytes

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Human papillomavirus 16 (HPV16) infection causes 50% or more of cervical cancers in women. The HPV16 E7 oncogene is continuously expressed in infected epithelium with its oncogenicity linked to cervical cancer. The E7 protein is an ideal target in control of HPV infection through T-cell-mediated immunity. Using HPV16 E7-transgenic mouse keratinocytes (KCs–E7) to investigate T-cell-mediated immune responses, we have shown previously that HPV16-encoded E7 protein inhibits IFN-γ-mediated enhancement of MHC class I antigen processing and T-cell-induced target cell lysis. In this study, we found that HPV16 E7 suppresses IFN-γ-induced phosphorylation of STAT1 (Tyr701), leading to the blockade of interferon regulatory factor-1 (IRF-1) and transporter associated antigen processing subunit 1 (TAP-1) expression in KCs–E7. The results of a 51 Cr release assay demonstrated that IFN-γ-treated KCs–E7 escaped from CTL recognition because HPV16 E7 downregulated MHC class I antigen presentation on KCs. Restoration of IRF-1 expression in KCs–E7 overcame the inhibitory effect of E7 protein on IFN-γ-mediated CTL lysis and MHC class I antigen presentation on KCs. Our results suggest that HPV16 E7 interferes with the IFN-γ-mediated JAK1/JAK2/STAT1/IRF-1 signal transduction pathway and reduces the efficiency of peptide loading and MHC class I antigen presentation on KCs–E7. These results may reveal a new mechanism whereby HPV16 escapes from immune surveillance in vivo.

INTRODUCTION

Human papillomavirus (HPV) infection causes cervical carcinoma, one of the leading causes of cancer death in the world (Guan et al., 2012; Sasagawa et al., 2012; Stanley et al., 2007). Among the over 120 HPV types identified, HPV16 is the most common high-risk type, accounting for approximately 50% of cervical cancers worldwide, while HPV 18 causes approximately 20% of cervical cancers (Barnabas et al., 2006). Currently, two virus-like-particle-based vaccines against HPVs are commercially available: a bivalent (HPV16/18) and a quadrivalent (HPV6/11/16/18) vaccine, which may provide sufficient immunogenicity to confer long-term protection (Frazer, 2007; Leggatt & Frazer, 2007). However, there is still a great need worldwide for effective therapeutic HPV vaccines (Bhat et al., 2011; Frazer et al., 2011).

The genome of HPV16 includes an early region (E), which encodes six ORFs (E1, E2, E4, E5, E6 and E7), and a late region (L), which encodes a major capsid protein L1 and a minor capsid protein L2. All HPV16-encoded proteins are encoded by one DNA strand (Münger & Howley, 2002). Both E6 and E7 proteins, called oncoproteins, play very important roles in HPV-induced cancers (Van Doorslaer & Burk, 2010). Expression of E6 and E7 proteins blocks p53 and retinoblastoma (Rb) protein and alters the biological functions of virus-infected epithelial cells (keratinocytes, the major host cells of HPV infection), immortalizing them to be cancerous by modulating the cell cycle (Dürst et al., 1987; Romanczuk et al., 1991). Furthermore, constitutive expression of the two oncoproteins disrupts the immune function of host cells (Münger & Howley, 2002). For example, CTLs play an important role in cellular immunity, particularly in the control of tumours and viral diseases. However, vaccine-induced immune cells fail to eliminate the HPV-infected keratinocytes (KCs) (Frazer,
KCs using anti-mouse SIINFEKL/H-2Kb antibody and thus processing and presentation of OVA on mouse primary KCs to observe the effect of HPV16 E7 on MHC class I antigen transgenic KCs in our studies. For instance, we can directly found that there are several advantages to using the mouse endogenous antigen in KCs and CTL ability to kill KCs. HPV16 E7 protein inhibits CTL epitope presentation from induction pathway (Zhou, 2009a). Recently, we reported that HPV16 E7 transgenic mice (K14–E7 transgenic mice) have been previously developed to stably express the oncogene E7 of HPV16 under control of the keratin-14 promoter in keratinocytes (Lambert et al., 1993). This transgenic animal model provides a basis for studying the mechanisms of action of E7 in eliciting the observed pathology and the genetic alterations required for HPV-16 E7 associated tumour progression (Lambert et al., 1993). In this animal model, skin in which HPV16 E7 antigen is actively expressed only in epithelial cells was isolated for producing HPV16 E7 transgenic keratinocytes (KCs–E7) (Frazer, 2004). Transgenic KCs–E7 have been used to investigate the mechanism of CTL-mediated cytotoxicity (Frazer et al., 2001; Leggatt et al., 2002).

IFN-γ upregulates MHC class I antigen presentation through the Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1)-mediated signal transduction pathway (Zhou, 2009a). Recently, we reported that HPV16 E7 protein inhibits CTL epitope presentation from exogenous antigen in KCs and CTL ability to kill KCs expressing HPV16 E7 (Zhou et al., 2011). We have also found that there are several advantages to using the mouse transgenic KCs in our studies. For instance, we can directly observe the effect of HPV16 E7 on MHC class I antigen processing and presentation of OVA on mouse primary KCs using anti-mouse SIINFEKL/H-2Kb antibody and thus obtain direct evidence of whether or not HPV16 E7 interferes with MHC class I antigen processing and presentation (Zhou et al., 2011). However, we cannot carry out similar experiments using human KCs because no antibody targeting to the human epitope/MHC class I complex is currently available.

Interferon regulatory factor-1 (IRF-1), a tumour suppressor gene, regulates genetic function by modulating activity of telomerase (Lee et al., 2003). However, the immune function of IRF-1 has not been fully elucidated. HPV16 E7 impairs the function of IRF-1 and NFκB to inhibit IFN-γ-mediated MHC class I antigen processing of endogenous antigen (Cordano et al., 2008; Leggatt et al., 2002; Perea et al., 2000). In addition, HPV16 E7 protein can block the activity of transporter associated antigen processing subunit 1 (TAP-1) that is essential for MHC class I antigen processing and presentation (Li et al., 2010; Vambutas et al., 2001). IRF-1, a secondary signal molecule, regulates expression of multiple downstream genes such as tap-1 and low molecular mass protein 2 (lmp-2) that are associated with MHC class I antigen processing and presentation (Saito et al., 2000; Zhou, 2009a). It is unclear, however, whether or not HPV16 E7 can affect expression of IRF-1 protein in HPV-infected host cells via blocking the IFN-γ-mediated JAK1/JAK2/STAT1 signal transduction pathway, and the molecular mechanisms whereby HPV16 E7 inhibits the function of IFN-γ on MHC class I antigen processing and presentation remain obscure. Here, we showed that HPV16 E7 blocks IFN-γ-induced phosphorylation of STAT1 and then inhibits expression of IRF-1 and TAP-1 in KCs–E7. HPV16 E7 in host cells consequently escapes from immune surveillance by inhibiting IFN-γ-mediated MHC class I antigen presentation. Overexpression of IRF-1 protein by transfection of plasmid expressing the irf-1 gene overcomes the inhibitory effect of HPV16 E7 on IFN-γ-mediated MHC class I antigen presentation and CTL-induced KC lysis. The results presented here may help to explain why HPV-infected epithelial cells and E7 transgenic keratinocytes are not effectively eliminated by antigen-specific CD8+ T-cells in vivo and in vitro.

RESULTS

HPV16 E7 inhibits IFN-γ-induced Tyr701 phosphorylation of STAT1 in mouse keratinocytes

Considering that MHC class I antigen presentation is upregulated by the IFN-γ-mediated JAK/STAT signal transduction pathway (Conzelmann et al., 2010; Pamment et al., 2002), we first determined whether HPV16 E7 inhibited transcription and translation of the stat1 gene in KCs and KCs–E7 treated with IFN-γ, or not (Fig. 1). Transcription of stat1 was very low in both KCs and KCs–E7 that were not treated with IFN-γ (Fig. 1b). IFN-γ treatment significantly increased transcriptional levels of stat1. HPV16 E7 did not inhibit stat1 transcription in KCs (Fig. 1b). Expression of the STAT1 protein was also very weak in both KCs and KCs–E7 without IFN-γ treatment (Fig. 1a). IFN-γ treatment dramatically improved protein expression of STAT1 in KCs and KCs–E7 (Fig. 1a, c), consistent with increased transcriptional levels of the stat1 gene (Fig. 1a). In addition, expression of phosphorylated-STAT1(Tyr701) (P-STAT1(Tyr701)) was detected in IFN-γ-treated KCs (Fig. 1a, d), indicating that IFN-γ activates STAT1 by phosphorylation on Tyr701 in KCs. However, P-STAT1(Tyr701) was undetectable in IFN-γ-treated E7-KCs (Fig. 1a, d), suggesting that HPV16 E7 interferes with the IFN-γ-mediated
JAK1/JAK2/STAT1 signal transduction pathway in KCs by inhibiting phosphorylation of STAT1.

We also carried out several time-course experiments from 0.25 to 48 h to examine whether or not HPV16 E7 affected expression of P-STAT1(Tyr701) induced by IFN-γ in KCs. Expression of P-STAT1(Tyr701) was undetectable in IFN-γ-treated KCs-E7 over the time-course, indicating that HPV16 E7 completely inhibits IFN-γ-induced expression of P-STAT1(Tyr701) although it did not inhibit expression of the STAT1 protein in keratinocytes (Fig. S1). In contrast, expression of P-STAT1(Tyr701) could be detected in WT KCs treated with IFN-γ at 0.25 h (Fig. S1). The IFN-γ-treated KCs produced the highest level of P-STAT1(Tyr701) at 2 h and downregulated expression of P-STAT1(Tyr701) from 24 to 48 h (Fig. S1). These data support the hypothesis that HPV16 E7 inhibits phosphorylation of STAT1 in keratinocytes.

**HPV16 E7 blocks the ability of IFN-γ to induce IRF-1 expression and the stability of IRF-1 expression in mouse keratinocytes**

We and others have previously reported that IFN-γ can increase expression of IFN regulatory factor-1 (IRF-1) mRNA in normal mouse and human keratinocytes (Nakanishi et al., 1997; Zhou et al., 2011). However, HPV16 E7 significantly blunts the increase in levels of irf-1 mRNA induced by IFN-γ in KCs (Zhou et al., 2011). Here, we examined the effect of IFN-γ on expression of IRF-1 protein in KCs and KCs–E7. IRF-1 protein was scarcely detectable in KCs and KCs–E7 without IFN-γ treatment (Fig. 2). IFN-γ treatment enhanced IRF-1 protein expression in KCs. However, very little IRF-1 was expressed in KCs–E7 treated with IFN-γ (Fig. 2). It could be concluded that HPV16 E7 substantially inhibits IFN-γ-induced expression of IRF-1 protein in mouse KCs.

Again, we carried out time-course experiments to determine whether or not HPV16 E7 inhibited expression of IRF-1 protein in IFN-γ-treated KCs–E7 over a 48 h period (Fig. S2). IRF-1 protein expression steadily increased in IFN-γ-treated KCs from 1 to 24 h, but was downregulated at 48 h (Fig. S2). In contrast, expression of IRF-1 protein was observed in IFN-γ-treated KCs–E7 from 1 to 2 h, but was undetectable from 24 to 48 h. These results suggested that HPV16 E7 inhibits IFN-γ-induced expression of IRF-1 protein in mouse KCs either by directly suppressing transcription of the irf-1 gene (Zhou et al., 2011) or by degrading the IRF-1 protein.
Transient expression of IRF-1 protein improves the ability of IFN-γ to upregulate CTL-mediated mouse KC lysis in vitro

We examined whether or not HPV16 E7 inhibited expression of the IRF-1 protein from an exogenous IRF-1 expression plasmid in KCs. KCs and KCs–E7 were transfected with plasmid pCDNA3 expressing IRF-1 (pIRF-1) or plasmid pCDNA3 expressing GFP (pGFP). Both KCs and KCs–E7 at 48 h post-transfection of the two plasmids were collected in order to detect IRF-1 protein expression by immunoblot assay (Fig. 3a) and GFP expression by immunofluorescence and flow cytometry analysis (Fig. 3b). Expression of IRF-1 protein could be detected not only in pIRF-1-transfected KCs, but also in pIRF-1-transfected KCs–E7 (Fig. 3a, c, left panel). These results, together with the data shown in Fig. S2, suggested that HPV16 E7 only inhibits expression of IRF-1 protein induced by IFN-γ, not its expression from the exogenous irf-1 gene in KCs.

Furthermore, immunofluorescence analysis showed that GFP was only expressed in KCs and KCs–E7 transfected with the pGFP plasmid (Fig. 3b). Nine per cent of KCs were GFP-positive cells after transfection of pGFP, and the percentage of GFP-positive cells was 18% for KCs–E7 treated with pGFP (Fig. 3c, left panel). Expression of IRF-1 in KCs and KCs–E7 transfected with the GFP plasmid was very weak, suggesting that plasmid transfection might have a slightly inducible effect on IRF-1 expression (Fig. 3a).

We next conducted a 51Cr release assay to examine whether or not IRF-1 protein expressed from the endogenous irf-1 gene plasmid facilitated CTL-induced KC lysis. Without IFN-γ treatment, OVA-specific T-cells failed to kill KCs and KCs–E7 treated with osmotic-loaded OVA and transfected with pIRF-1 or pGFP (Fig. 4). In contrast, IFN-γ treatment significantly improved the CTL-induced lysis to target KCs treated with osmotic-loaded OVA and transfected with pIRF-1 or pGFP (Fig. 4). The results of the 51Cr release assay further indicated that pIRF-1-transfected KCs–E7 treated with IFN-γ and osmotic-loaded OVA were effectively killed by OVA-specific T-cells, but OVA-specific T-cells failed to eliminate the pGFP-transfected KCs–E7 (Fig. 4). It could be concluded that HPV16 E7 suppresses CTL-mediated KC lysis by inhibiting the endogenous expression of IRF-1 induced by IFN-γ. The effect of HPV16 E7 on IFN-γ-mediated CTL killing can be overcome after IRF-1 expression is restored by transfection of an IRF-1 plasmid.

Transient expression of IRF-1 protein overcomes the effect of HPV16 E7 on MHC class I antigen presentation on mouse KCs

To investigate why transfection of the IRF-1 plasmid leads to improved CTL-induced KC lysis, we investigated whether or not IRF-1 protein expressed from the exogenous IRF-1 plasmid could overcome the inhibitory effects of E7 on IFN-γ-mediated MHC class I antigen processing and presentation on KCs–E7. Expression of the MHC class I H-2k b molecule and presentation of the CTL epitope (SIINFEKL/H-2k b) on KCs and KCs–E7 incubated with or without IFN-γ and osmotic-loaded OVA was detected by flow cytometry (Fig. 5). HPV16 E7 did not affect expression of MHC class I H-2k b on KCs and KCs–E7 upregulated by IFN-γ (Fig. 5a, b). Without IFN-γ treatment, neither IRF-1 nor GFP transient expression enhanced expression of MHC class I H-2k b on the two types of KCs (Fig. 5a, b), indicating that IFN-γ plays a crucial role in upregulation of MHC class I H-2k b expression on KCs.
Again, without IFN-γ treatment, neither IRF-1 nor GFP transient expression elicited presentation of the CTL epitope (SIINFEKL/H-2kb) on KCs (Fig. 5c). IFN-γ treatment enhanced CTL epitope presentation on KCs transfected with pGFP or pIRF-1 and osmotic-loaded OVA (Fig. 5c). Furthermore, IFN-γ treatment significantly facilitated presentation of the CTL epitope (SIINFEKL/H-2kb) on KCs–E7 transfected with pIRF-1, but this was not the case with KCs–E7 transfected with pGFP (Fig. 5d). Thus, it could be concluded that transient expression of IRF-1 overcomes the inhibitory effect of HPV16 E7 on IFN-γ-mediated MHC class I antigen presentation.

**HPV16 E7 inhibits IFN-γ-induced expression of TAP-1 in mouse keratinocytes**

On the basis of the aforementioned findings (Figs 1–5), we propose a model to show that HPV16 E7 inhibits IFN-γ-mediated MHC class I antigen processing and presentation in mouse KCs by blocking the JAK1/JAK2/STAT1/IRF-1 signalling pathway (Fig. 6). In this proposed model (Fig. 6), the presentation of endogenous peptides is associated with a group of proteins, including IF-1 and TAP-1. TAP-1, a target protein whose expression is induced by IRF-1, transports the antigenic peptide into a pre-Golgi region to assemble a stable MHC class I complex (Korkolopoulou et al., 1996). Thus, we examined whether or not HPV16 E7 affected expression of TAP-1 protein in mouse KCs (Fig. 7). Expression of TAP-1 protein was scarcely perceptible in KCs and KCs–E7 without IFN-γ treatment (Fig. 7a). IFN-γ treatment significantly enhanced expression of TAP-1 protein in KCs, but only slightly improved its expression in KCs–E7 (Fig. 7a). Expression of TAP-1 protein in IFN-γ-treated KCs was significantly higher than that in IFN-γ-treated KCs–E7 (Fig. 7b), suggesting that HPV16 E7 also inhibits TAP-1 protein expression induced by IFN-γ in mouse keratinocytes.
DISCUSSION

Viral products interfere with MHC class I antigen processing and presentation via multiple pathways, and the molecular mechanisms whereby HPV16 affects MHC class I antigen processing and presentation have not been fully elucidated (Zhou, 2009b).

In the present study, we observed that CTLs do not kill KCs–E7 incubated with or without IFN-γ and osmotic-loaded OVA, consistent with a previously published study that CTLs fail to recognize epithelial cells in which viral gene is expressed (Leggatt et al., 2002). KCs–E7 escape from CTL recognition given that HPV16 E7 inhibits IFN-γ-mediated MHC class I antigen processing and presentation in keratinocytes. However, it remains unclear why HPV16 E7 inhibits IFN-γ-mediated MHC class I antigen processing and impairs CTL-induced target cell lysis (Zhou et al., 2011). IFN-γ is well known to have antiviral, immune-regulatory and anti-tumour properties (Schroder et al., 2004). IFN-γ facilitates the transcription of multiple genes by binding to its receptors to produce a variety of physiological and cellular responses, including increased expression of MHC class I molecules and upregulated CTL epitope presentation, especially activation of the JAK1/JAK2/STAT1 signalling pathway (Barnard & McMillan, 1999). Thus, STAT1 plays an important role in this signalling pathway. It has been reported that suppressed expression of STAT1 facilitates HPV genome amplification and maintenance of episomes in HPV16-infected HFK cells (Hong et al., 2011). However, it is still unclear how STAT1 plays a role in the mechanisms of HPV16 E7 to escape from CTL-mediated target cell lysis.

Here, we showed that HPV16 E7 does not inhibit IFN-γ-induced expression of STAT1 in keratinocytes but that it suppresses IFN-γ-induced phosphorylation of STAT1(Tyr701) in KCs–E7. Our results are similar to previously published studies showing that HPV16 E6 interferes with IFN-α signalling to inhibit phosphorylation of STAT1, STAT2 and Tyk2 in host cells (Li et al., 1999). Based on our studies and those of others, it is clear that STAT1 phosphorylation is a key process in the IFN-γ-mediated JAK1/JAK2/STAT1 signal transduction pathway. IFN-γ induces expression of phosphorylated STAT1, which binds to the promoter of the irf-1 gene to initiate IRF-1 expression (Lehtonen et al., 1997; Mori et al., 1999; Storm van’s Gravesande et al., 2002). IRF-1 as a secondary signal molecule in the IFN-γ-mediated JAK1/JAK2/STAT1 signal transduction pathway can induce transcription of multiple downstream genes such as tap-1, which are necessary for MHC class I antigen processing and presentation (Saito et al., 2000; Zhou, 2009a). In the present study, we showed that HPV16 E7 inhibits IFN-γ-induced expression of IRF-1 protein in KCs–E7 by blocking STAT1(Tyr701) phosphorylation, consistent with our previous
It is of interest to note that HPV16 E7 does not inhibit IFN-γ-induced MHC class I expression on KCs. Based on results of studies by other groups, MHC class I expression is induced by multiple IRFs including IRF-1, IRF-2, IRF-3, IRF-7, IRF-8 and IRF-9 (Drew et al., 1995; Jarosinski & Massa, 2002; Marquis et al., 2011; Tennant et al., 2007). HPV16 E7 may not affect expression of some of these IRFs, which are able to initiate MHC class I expression in KCs (Antonsson et al., 2006), which would explain why MHC class I molecules can still be expressed normally on KCs–E7 even when IFN-1 expression is blocked by HPV16 E7.

HPV16 E7 inhibits IFN-γ-mediated MHC class I antigen presentation and CTL-mediated KC lysis. MHC class I antigen presentation on cells is dependent on at least two pathways: TAP-dependent and TAP-independent pathways (Darji et al., 1997; Yewdell et al., 1998). For example, MHC class I antigen presentation has been found to be independent of TAP in human immunodeficiency virus-infected cells, macrophages and B-cells (Hammond et al., 1995). The recycling of MHC class I from cytosol to the surface of target cells through endocytosis is TAP independent (Merzougui et al., 2011; Tey & Khanna, 2012). However, it is unclear whether or not there is an epitope (peptide) on recycled MHC class I. MHC class I molecules might be expressed through the recycling pathway of endocytosis in keratinocytes that are not affected by HPV16E7.

Given that expression of subunits of TAP is initiated by IRF-1 (Brucet et al., 2004), MHC class I expression mediated by the TAP-dependent pathway can be affected by IRF-1, but IRF-1 cannot affect MHC class I expression mediated by the TAP-independent pathway. This is probably the reason why HPV16 E7 cannot block MHC class I expression mediated by the IRF-1/TAP-independent pathway on KCs, although HPV16 E7 can inhibit IRF-1 expression in KCs. This subject needs further study. Moreover, the recycling capacity of MHC class I in mouse keratinocytes may be superior to that of other cells. This hypothesis should also be investigated in the future.

In conclusion, HPV16 E7 inhibits IFN-γ-induced STAT1 phosphorylation to block expression of IRF-1 and TAP-1 proteins in keratinocytes. CTLs cannot effectively kill KCs–E7 incubated with or without IFN-γ and osmotic-loaded OVA. HPV16 E7 substantially suppresses peptide loading and epitope presentation on KCs. Transfection of plasmid expressing IFN-1 overcomes the inhibitory effect of HPV16 E7 on CTL-mediated KC lysis and MHC class I antigen processing and presentation. Our results imply a complex viral strategy whereby HPVs escape from immune surveillance and establish a persistent viral infection in host cells (keratinocytes) by interfering with the IFN-γ-mediated JAK/STAT1/IRF-1 signal transduction pathway.

METHODS

Immunogen and peptide. An 8mer peptide (SIINFEKL), the major CTL epitope of OVA, was provided by AusPep, which binds to MHC class I H-2Kb (aa 258–266 of OVA).

Mice. C57 BL/6J mice and E7 transgene from keratin-14 (K14) mice generated by Dr Paul Lambert (Lambert et al., 1993) were bred under conventional conditions in specific-pathogen-free holding rooms in the Princess Alexandra Hospital Biological Resources Facility (Brisbane, QLD, Australia). The protocols for these experiments were approved by the institutional Animal Ethics Committee.

Generation of effector cells in vitro. C57Bl/6J female mice (6–8 weeks of age) were immunized once with 100 μg SIINFEKL/30 μg keyhole limpet haemocyanin (KLH, Sigma Pharmaceuticals)/30 μg QuilA (Spikoside, ISOTEC A B). Lymph node cells were collected from immunized mice at day 4 after immunization. Lymphocytes were grown in Click’s medium containing 10 % FBS, 200 mM L-glutamine and 5 × 10⁻⁷ M 2-mercaptoethanol, supplemented with 1 ng mouse interleukin-2 (Pharmingen) ml⁻¹ and 0.05 μM SIINFEKL peptide for 4 days before using.

Generation of target cells in vitro. Isolation and culture of KCs from fresh skin has been previously described (Zhou et al., 2009, 2011). Briefly, keratinocytes derived from both WT and E7-transgenic mice were cultured in 3:1 medium as described previously for 48 h and then transferred into serum-free keratinocyte medium (Gibco, Invitrogen) for 2 days. The keratinocytes were put in 96-well plates at 2 × 10⁵ cells per well for a 5¹Cr release assay or 5 × 10⁵ cells per tube for the flow cytometry experiment.

Osmotic loading of OVA or MYO. As described previously (Ahmad et al., 1995), 5 × 10⁵ cells were suspended in 1 ml RPMI 1640 osmotic loading buffer [25 mM HEPES (pH 7.2), 0.5 M sucrose, 10 % (w/v) polyethylene glycol,] including OVA (Sigma) or MYO (Sigma) at 10 mg ml⁻¹ and incubated at 37 °C for 2 min. The loaded KCs were washed and centrifuged at 300 g for 7 min. Cells were then resuspended with RPMI 1640, flushed and centrifuged at 300 g for 5 min. Finally, KCs were resuspended into culture medium and incubated with or without treatment of IFN-γ at 100 U ml⁻¹ for 24 h.

CTL assay (⁵¹Cr release assay). A standard 5 h ⁵¹Cr release assay was conducted as described previously (Zhou et al., 2009, 2011). CTL assay data were expressed as percentage of specific lysis.

Real-time reverse transcriptase PCR (RT-PCR). mRNA was extracted and reverse transcriptional PCR was carried out as described previously (Zhou et al., 2009). cDNA samples were dissolved in PCR mix buffer (FastStart SYBR Green Master; Qiagen) and real-time PCR
Fig. 5. Restoration of MHC class I antigen presentation on KCs–E7 by IRF-1 protein expressed by the pIRF-1 plasmid. Both KCs and KCs–E7 were transfected with pIRF-1 or pGFP. KCs were then treated with osmotic-loaded OVA (10 mg ml$^{-1}$) and incubated with or without IFN-γ stimulation (100 U ml$^{-1}$) for 48 h. A flow cytometry experiment was conducted. (a, b) Left panel: FACS histograms showing expression of MHC class I H-2Kb on KCs and KCs–E7. Right panel: Results represent mean ± SD of triplicate determinations of ΔMFI of MHC class I expression on KCs and KCs–E7 in three independent experiments. (c, d) Left panel: FACS histograms showing the presentation of CTL epitope/MHC class I complexes (SIINFEKL/H-2Kb) on KCs and KCs–E7. Right panel: Results represent mean ± SD of triplicate determinations of ΔMFI of CTL epitope presentation on KCs and KCs–E7 in three independent experiments (*P<0.05, Student’s t-test). In the left column of panels, samples are displayed as follows: pink line, KCs or KCs–E7 treated with IFN-γ and transfected with pIRF-1; blue line, KCs or KCs–E7 treated with IFN-γ and transfected with pGFP; red line, KCs or KCs–E7 transfected with pIRF-1; green line, KCs or KCs–E7 transfected with pGFP.
was conducted under the following conditions: 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 94 °C for 1 min and 72 °C for 1 min. The following primers were used in this project: for *stat1*, forward: 5'-TCTGGTGCTTCCTTTGGTCT-3', reverse: 5'-GGAGGTGAACCTGACTTCCA-3'; for mouse housekeeping protein (reference gene), forward: 5'-GGAGGGCCCATCAGTTTAAT-3', reverse: 5'-AAACAATTGCATTGCATAGTGC-3'. Data were analysed by Rotor-Gene 6000 software.

**Immunoblot assay.** Protein samples were extracted from keratinocytes as described previously (Wang *et al.*, 2009). Protein samples (50 μg) were separated by SDS-PAGE and blotted onto PVDF membrane (Millipore). The protein blots were first probed by primary antibodies against different proteins including STAT1, phosphorylated-STAT1 (Tyr701), IRF-1, TAP-1 and β-actin overnight at 4 °C. The blots were then probed with either HRP-conjugated sheep anti-rabbit antibody (Chemicon Pty Ltd) or HRP-conjugated donkey anti-goat antibody (Santa Cruz) for 1 h at room temperature and detected using a chemiluminescence system.

The primary antibodies used for probing different proteins included: (i) STAT1 polyclone antibody (Cell Signaling Technology); (ii) phosphorylated STAT1 (to target Tyr701 in STAT1) polyclone antibody (Cell Signaling Technology); (iii) IRF-1 polyclone antibody (Santa Cruz); (iv) TAP-1 polyclone antibody (Santa Cruz); and (v) β-actin polyclonal antibody (Cell Signaling Technology).

**Flow cytometry.** KCs cells (5 x 10⁵) were incubated with the following primary antibodies: mouse anti-SIINFEKL/H2Kb, clone D25.1.1.16, 50 μl per sample (provided by Dr Purcell, University of Melbourne, Australia); mouse anti-H-2Kb antibody, clone AF6-88.5, diluted 1 : 50 in 5 % FCS in PBS, 50 μl per sample (Pharmingen); and mouse anti-rat IgM antibody, isotype control for KCs, diluted 1 : 50 in 5 % FCS in PBS, 50 μl per sample (Pharmingen) for 1 h at 4 °C.

Cells were then washed twice and incubated with the secondary antibody, FITC-rabbit anti-mouse Ig antibody, diluted 1:25 in 5 % FCS in PBS, 50 μl per sample (Dako Cytomation) for 1 h at 4 °C.

Fig. 6. Model showing that HPV16 E7 inhibits MHC class I antigen processing and presentation by blocking the IFN-γ-mediated JAK-STAT1/IRF-1 signal transduction pathway. IFN-γ binds to its cognate receptor subunit to activate JAKs, which leads to expression and phosphorylation of STAT1. STAT1 phosphorylation induces expression of IRF-1 and TAP-1 to enhance peptide loading on MHC class I molecules. HPV16 E7 protein blocks STAT1 phosphorylation and causes inhibition of IRF-1 and TAP-1 expression. Reduction of TAP-1 expression decreases the amount of peptide loading and CTL epitope presentation on target cells, so that KCs–E7 can escape from recognition by CTLs.

Fig. 7. HPV16 E7 blocks IFN-γ-induced TAP-1 expression in KCs. (a) Expression of TAP-1 protein in KCs and KCs–E7 incubated with or without IFN-γ treatment for 48 h was detected by immunoblot assay. Expression of β-actin was detected as a loading control. (b) Relative intensity of TAP-1 protein expressed in KCs and KCs–E7 incubated with or without IFN-γ treatment. Results represent mean ± SD of triplicate determinations of relative intensity of TAP-1 expression in three independent experiments (*P*<0.05, Student's *t*-test).
Cells were washed twice again before fixation with 5% formaldehyde in PBS, and detected using a flow cytometer (FACS Calibur; BD Pharmingen). Flow cytometry data were analysed with WinMDI 2.8 version (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

During the flow cytometry assay, viable KCs were selected by gating on the appropriate cell size, using forward and side scatter properties to exclude any small, non-viable cells.

ΔMFI means the difference in mean fluorescence intensity (MFI) between test and isotype control.

**Keratinocyte transfection.** As described previously (Wang et al., 2009), buffer A (24 μl lipofectamin in 600 μl serum-free KC medium; Gibco, Invitrogen), and buffer B, containing either 12 μg pCDNA3-IRF-1 plasmid DNA (kindly provided by Dr Pramod Rath, Jawaharlal Nehru University, India) or pCDNA3-GFP DNA, mixed with 9 μl Lipofectamin Plus reagent (Gibco, Invitrogen) in 600 μl serum-free KC medium, were incubated at 25 °C for 20 min. Buffer A was then mixed with buffer B and incubated at 25 °C for 30 min. KCs were grown in six-well plates and washed with PBS before transfection. Two hundred microliters of buffer A and B mixture was added to each well of KCs. KCs were incubated at 25 °C for 10 min and 1.3 ml per well of serum-free KC medium was added. Transfected KCs were incubated at 37 °C for 17 h and then further incubated for 48 h with or without treatment of IFN-γ (100 U ml⁻¹) in keratinocyte medium. Cells were then treated with osmotic-loaded OVA or MYO.

**Statistical analysis.** All experimental data including percentage of CTL lysis, relative transcription of target genes in RT-PCR, ΔMFI of target molecules in the flow cytometry assay and relative intensity of protein expression in the immunoblot assay were analysed with Student’s t-test. Error bars represent mean ± SD or ± SEM. Differences were considered significant for P<0.05.

**ACKNOWLEDGEMENTS**

This research was funded by the Cancer Cooperative Group (CCG) and ANZ Trustee PhD scholarships. We are very grateful to Dr Ian Frazer for providing HPV16 E7 transgenic mice and to Mrs Allison Choyce for providing samples of keratinocytes for this research. We thank Mrs Katherine Regan for editing the manuscript. The authors of this paper have no financial conflict of interest.

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