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Compromised T Cell Immunity Links Increased Cutaneous Papillomavirus Activity to Squamous Cell Carcinoma Risk

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ABSTRACT

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer, with increased incidence in immunosuppressed patients. B-human papillomavirus (β-HPV) has been proposed as a contributor to cSCC risk partly based on increased β-HPV viral load and seropositivity observed among patients with cSCC. Experimental data in mice colonized with mouse papillomavirus (MmuPV1) suggest that T cell immunity against β-HPV suppresses skin cancer in immunocompetent hosts, and the loss of this immunity leads to the increased risk of cSCC. Herein, we demonstrate that CD8+ T cell depletion in MmuPV1-colonized mice that underwent skin carcinogenesis protocol led to increased viral load in the skin and seropositivity for anti-MmuPV1 antibodies. These findings provide evidence that compromised T cell immunity can be the link that connects increased β-HPV detection to cSCC risk.
INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer worldwide and has a rapidly increasing incidence rate (Lomas et al., 2012, Tokez et al., 2020). Ultraviolet (UV) radiation, immunosuppression, and β-human papillomavirus (β-HPV) have been proposed to be among the primary drivers of this cancer (Howley and Pfister, 2015, Rollison et al., 2019, Wang et al., 2014). Several studies have suggested that increased β-HPV replication in the skin and β-HPV seropositivity in cSCC patients is evidence of viral oncogenesis (Bouwes Bavinck et al., 2018, Bouwes Bavinck et al., 2010, Farzan et al., 2013, Genders et al., 2015, Hampras et al., 2014, Iannacone et al., 2014, Iannacone et al., 2012, Rollison et al., 2021, Waterboer et al., 2008). Countering this claim are the findings that, unlike high-risk α-HPVs, β-HPVs are not transcriptionally active in cSCC and no predominant β-HPV types have been found in skin cancers (Howley and Pfister, 2015). This has contributed to the “hit-and-run” hypothesis whereby β-HPV facilitates the initial development of UV-induced cSCC but is not required for subsequent cancer progression (Rollison et al., 2021).

We previously proposed an alternative explanation for the link between β-HPV and cSCC: T cell immunity against β-HPV suppresses skin cancer, and the loss of this immunity—rather than the oncogenic effect of HPVs—leads to markedly increased risk of skin cancer, which is observed in immunosuppressed patients (Strickley et al., 2019). In this study, the mean tumor count was significantly less in SKH-1 mice infected with MmuPV1 (3.6) compared with sham-infected control mice (9.1, \(P = 0.0169\)) at the completion of the DMBA/UV-induced carcinogenesis protocol (Strickley et al., 2019). Importantly, MmuPV1-infected SKH-1 mice that underwent CD8+ T cell depletion developed significantly more tumors (mean tumor count: 11.8) compared with immunocompetent MmuPV1-infected SKH-1 mice (mean tumor count: 3.6, \(P = 0.0009\)).
(Strickley et al., 2019). The efficiency of anti-CD8 antibody-based CD8\(^+\) T cell depletion was confirmed using flow cytometry on the skin (0 and 17% of total CD3\(^+\) T cells were CD8\(^+\) T cells in the anti-CD8 and IgG control antibody-treated group, respectively) and spleen (1 and 16% of total CD3\(^+\) T cells were CD8\(^+\) T cells in the anti-CD8 and IgG control antibody-treated group, respectively) at week 6 post-DMBA (Strickley et al., 2019).

Herein, we investigated whether compromised T cell immunity explains the increased β-HPV replication and seropositivity found in patients with increased cSCC risk. To address this hypothesis, we provide new analysis of the cohort of animals published previously (Strickley et al., 2019). We analyzed sera and skin biopsies collected from SKH-1 mice to determine the effect of CD8\(^+\) T cell depletion on papillomavirus replication in the skin and seropositivity to viral antigens in MmuPV1-colonized mice.
RESULTS

We investigated the impact of CD8$^+$ T cell depletion on MmuPV1 viral load in the skin and seropositivity for MmuPV1 antibodies in MmuPV1-colonized SKH-1 mice. All mice were colonized equally with MmuPV1 before CD8$^+$ T cell depletion and DMBA/UVB treatment. However, consistent with their significantly increased skin tumor burden (Strickley et al., 2019), CD8$^+$ T cell-depleted mice showed increased viral DNA detectable in the normal skin at the completion of skin DMBA/UVB skin carcinogenesis protocol ($P = 0.0229$, Figure 1). The normalized mean MmuPV1 DNA level in the normal skin of IgG-treated control mice ($n = 10$) was 0.2550 with a standard deviation of 0.2444, while normalized mean MmuPV1 DNA level in the normal skin of CD8$^+$ T cell-depleted mice ($n = 9$) was 0.7154 with a standard deviation of 0.5076. Using ELISA to detect mouse serum antibodies to MmuPV1 antigens, we found that CD8$^+$ T cell-depleted mice produced more serum antibodies to MmuPV1 E6 ($P = 0.0030$, Figure 2a), E7 ($P = 0.0220$, Figure 2b), and L1 ($P = 0.0041$, Figure 2c) antigens compared with IgG-treated control animals. The mean (M) and standard deviation (SD) of optical density of ELISA for the IgG-treated control mice ($n = 10$) was: E6 (M = 0.2296, SD = 0.1255), E7 (M = 0.3454, SD = 0.1859), and L1 (M = 0.2973, SD = 0.0943). The mean and standard deviation of optical density of ELISA for the CD8$^+$ T cell-depleted mice ($n = 9$) was: E6 (M = 0.6564, SD = 0.3782), E7 (M = 0.8701, SD = 0.6675), and L1 (M = 0.6458, SD = 0.3082).
DISCUSSION

Our findings suggest that T cell suppression explains the link between increased β-HPV load and seropositivity to cSCC risk. Immunosuppression is a major predisposing risk factor in skin cancer development and increases the likelihood of cSCC by > 100-fold (Bouwes Bavinck et al., 2018, Chockalingam et al., 2015, Genders et al., 2015, Nehal and Bichakjian, 2018). T cells are also preferentially reduced in the cancer-prone immunosenescent elderly population (Rodriguez et al., 2020). Likewise, there is a clear link between T cell-suppressed states and enhanced β-HPV replication in the human skin (Azzimonti et al., 2005, Dell'Oste et al., 2009, Landini et al., 2014, Quint et al., 2015, Zavattaro et al., 2008). Our study uses MmuPV1 to further validate this connection and extend it to seropositivity for anti-papillomavirus antibodies at an experimental level. T cells are paramount in immunity to mouse papillomavirus, which cross-protect the skin from UV-induced carcinogenesis (Strickley et al., 2019). Thus, increased papillomavirus replication and cSCC risk can both take place in hosts with compromised T cell immunity. Our findings indicate that the positive correlation between β-HPV load and cSCC risk may not be related to a causative association due to virus infection as suggested by previous studies (Bouwes Bavinck et al., 2018, Bouwes Bavinck et al., 2010, Farzan et al., 2013, Genders et al., 2015, Hampras et al., 2014, Iannacone et al., 2014, Iannacone et al., 2012, Rollison et al., 2021, Waterboer et al., 2008). Instead, this positive correlation may be brought about by immunosuppression as an independent variable that is unequally distributed between case and control groups.

Our study is limited to extrapolating findings from MmuPV1 in mice to β-HPV in humans. MmuPV1 is a well-established model for studying commensal HPV’s interaction with human hosts (Uberoij et al., 2017). Notably, the use of MmuPV1 in murine models allows for precise
interrogation of the T cell role as an independent variable affecting the β-HPV activity and cSCC risk, which cannot be directly done in a human study. Nonetheless, understanding β-HPV and human immune system interactions can improve cSCC prevention strategies by enhancing patients’ anti-HPV immunity. Previous research support either a passenger or pro-tumorigenic role for β-HPV in skin cancer and propose that a vaccine strategy may be efficacious in preventing tumorigenesis (Hasche et al., 2018, Weissenborn et al., 2005). However, the target(s) and the mechanism of action for such a vaccine are yet to be elucidated. Understanding the link between compromised T cell immunity, β-HPV, and cSCC risk is important for future efforts in developing a β-HPV vaccine. Further studies are warranted to examine the role of immunosuppression and immunosenescence in mediating the link between β-HPV and cSCC in humans. Future research on virus-associated diseases will benefit from considering the role of immunosuppression and immunosenescence on viral replication and seropositivity when examining a causative role for a virus in diseases.
MATERIALS & METHODS

All animal studies were reviewed and approved by the University of Louisville IACUC. Sera and skin biopsies were examined from the mice that were colonized with MmuPV1 and treated with 7,12-dimethylbenz[a]anthracene (DMBA) plus UVB in a skin carcinogenesis protocol described previously (Strickley et al., 2019). All mice were housed under pathogen-free conditions in the animal facilities at the University of Louisville in compliance with animal care and all relevant ethical regulations. Six-to-ten-week-old female SKH-1 Elite mice (Charles River; 477) were used. Mice back skin was scarified with a nail file, and 8x10⁹ VGE of MmuPV1 was applied. After four weeks, immune mice that did not develop warts or had warts that spontaneously regressed were used for the skin carcinogenesis experiment. Of the 9 mice in the CD8⁺ T cell depletion group, 4 mice had no warts and 5 had regressed warts after MmuPV1 infection. Of the 10 mice in the IgG control group, 7 mice had no warts and 3 mice had regressed warts after MmuPV1 infection.

CD8⁺ T cell depletion was performed by injecting anti-CD8 (rat anti-mouse CD8α; BioXCell; YTS 169.4) or IgG (rat isotype control; Sigma-Aldrich) antibodies intraperitoneally into mice. Starting a day before DMBA treatment, 750 µg antibody in 200 µl sterile PBS was injected per mouse (first dose) followed by 250 µg in 200 µl sterile PBS weekly injections following standard protocol (Li et al., 2021, Strickley et al., 2019). The efficacy of CD8⁺ T cell depletion was confirmed via flow cytometry of the harvested skin and spleen of a select mouse from test and control group at 6 weeks post-DMBA (Strickley et al., 2019).

The day after first antibody injection, mice were treated with 50 µg DMBA in 200 µl acetone on the back skin (Sigma-Aldrich; D3254). The mice were then irradiated with 100 mJ/cm² of narrow-band UVB (302–312 nm) 3 times weekly by a UVP Black-Ray Lamp UVB (VWR;
36575-052) for up to 25 weeks (Strickley et al., 2019). Mice were harvested as their tumor size reached terminal size (>1.5 cm in diameter) or developed ulcerated tumors. At the endpoint, skin and sera were collected. Skin biopsies were performed via removing the back skin that was colonized with MmuPV1 and received DMBA/UV. The skin used for PCR analysis was collected from normal skin without any lesion.

DNA isolation and PCR were conducted as described previously with modifications (Strickley et al., 2019). Semi-quantitative PCR of the skin was used because this method provided a more sensitive platform to detect MmuPV1 DNA in mouse skin after long-term colonization compared with DNA in situ hybridization assay (Strickley et al., 2019). DNA was extracted from mice back skin using the DNeasy Blood & Tissue Kit (Qiagen; 69506). MmuPV1-E2-Forward: 5’-CCTCCTCAGCCAAAGAAGGGC-3’ and MmuPV1-E2-Reverse:5’-GTCGTTCTCCTTGTCCGAGTCG-3’. Gapdh-Forward: 5’GGCCAGGATGTAAAGGTCATTAAG-3’ and Gapdh-Reverse: 5’-GTCCCTCGAACTAAGGGGAAAG-3’ primers were used for PCR. Antibodies to MmuPV1 antigens in mouse serum were detected using ELISA as described previously (Joh et al., 2014).

Graphs and statistical analysis were performed using GraphPad Prism 9. Bar graphs show mean + standard deviation (s.d.). Two-tailed Mann-Whitney U test was used as the significance test between the two groups. A P value of less than 0.05 was considered significant. All error bars represent standard deviation.
DATA AVAILABILITY

The data supporting this study’s findings are available from the corresponding author upon reasonable request. No datasets were generated or analyzed during the current study.

CONFLICT OF INTEREST

SD is an inventor on a filed patent for the development of T cell-directed anti-cancer vaccines against commensal viruses (PCT/US2019/063172). Other authors declare no conflict of interest.

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AUTHOR CONTRIBUTION

Conceptualization: LHJ, HGS, SD; Formal Analysis: LHJ, HGS; Funding Acquisition: JJ, SD; Investigation: LHJ, HGS, DTH, JDS; Methodology: LHJ, HGS, DTH, JDS; Project Administration: JJ, SD; Resources: JJ, SD; Supervision: JJ, SD; Writing – Original Draft Preparation: LHJ, HGS, DTH; Writing – Review and Editing: JJ, SD.
REFERENCES


FIGURE LEGENDS

**Figure 1. CD8⁺ T cell depletion increases MmuPV1 DNA levels in the virus-colonized mouse skin.** (a) Gel images of MmuPV1 E2 PCR on DNA isolated from MmuPV1-infected normal back skin of SKH-1 mice at the completion of the DMBA/UVB skin carcinogenesis protocol. Skin samples were collected at harvest, which took place from week 18 to 25 post-DMBA as mice developed terminal tumors (n = 10 for IgG controls and n = 9 for anti-CD8 antibody-treated MmuPV1-infected mice). Arrowheads indicate amplified MmuPV1 DNA bands (162 bp). M: molecular marker, (-): negative control and (+): positive control. (b) Quantification of the PCR results in panel (a). Viral DNA levels were normalized to mouse Gapdh using ImageJ (two-tailed Mann–Whitney U test) The height of the bars is representative of normalized mean of MmuPV1 DNA level, and the error bars represent standard deviation.

**Figure 2. CD8⁺ T cell-depleted mice have higher antibody titers to MmuPV1 antigens.** (a-c) Enzyme-linked immunosorbent assay (ELISA) mean optical density for the detection of serum antibody to MmuPV1 E6 (a), E7 (b), and L1 (c) antigens in mice infected with MmuPV1 with or without CD8⁺ T cell depletion. Sera were collected at harvest, which took place from week 18 to 25 post-DMBA as mice developed terminal tumors (n = 10 for IgG control and n = 9 for anti-CD8 antibody-treated MmuPV1-infected mice, two-tailed Mann–Whitney U test). The height of the bars is representative of the average optical density of the ELISA, and the error bars represent standard deviation.
Figure 1.

(a) Western blot analysis showing the expression of MmuPV1 IgG and MmuPV1 αCD8 antibody with and without MmuPV1 DNA. The bands indicate the presence of MmuPV1 IgG and αCD8 antibody in the samples.

(b) Bar graph showing the normalized MmuPV1 DNA level. The data points indicate a significant difference in the normalized DNA level between the IgG and αCD8 antibody groups, with a p-value of 0.0229.
Figure 2.

(a) Serum Ab ELISA (OD at 405 nm) for MmuPV1 with IgG and αCD8 ab showing a significant difference with $P = 0.0030$ for E6.

(b) Serum Ab ELISA (OD at 405 nm) for MmuPV1 with IgG and αCD8 ab showing a significant difference with $P = 0.0220$ for E7.

(c) Serum Ab ELISA (OD at 405 nm) for MmuPV1 with IgG and αCD8 ab showing a significant difference with $P = 0.0041$ for L1.