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

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Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer, with increased incidence in immunosuppressed patients. β-Human papillomavirus has been proposed as a contributor to cSCC risk partly on the basis of increased β-human papillomavirus viral load and seropositivity observed among patients with cSCC. Experimental data in mice colonized with mouse papillomavirus type 1 suggest that T cell immunity against β-human papillomavirus suppresses skin cancer in immunocompetent hosts, and the loss of this immunity leads to the increased risk of cSCC. In this study, we show that $CD8^+$ T cell depletion in mouse papillomavirus type 1–colonized mice that underwent skin carcinogenesis protocol led to increased viral load in the skin and seropositivity for anti–mouse papillomavirus type 1 antibodies. These findings provide evidence that compromised T cell immunity can be the link that connects increased β-human papillomavirus detection to cSCC risk.

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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). UV radiation, immunosuppression, and β-human papillomavirus (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 patients with cSCC is evidence of viral oncogenesis (Bouwes Bavinck et al., 2018, 2010; Farzan et al., 2013; Genders et al., 2015; Hampras et al., 2014; Iannacone et al., 2014, 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 mouse papillomavirus type 1 (MmuPV1) (3.6) than in sham-infected control mice (9.1) ($P = 0.0169$) at the completion of the 7,12-dimethylbenz[*a*]anthracene (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) than immunocompetent MmuPV1-infected SKH-1 mice (mean tumor count = 3.6, $P = 0.0009$) (Strickley et al., 2019). The efficiency of anti-CD8 antibody (ab)-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 ab–treated group, respectively) and the spleen (1 and 16% of total $CD3^+$ T cells were $CD8^+$ T cells in the anti-CD8 and IgG control ab–treated group, respectively) at week 6 after DMBA (Strickley et al., 2019).

In this study, 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, to our knowledge, a previously unreported analysis of the cohort of animals published previously (Strickley et al., 2019). We analyzed sera and skin biopsies collected from SKH-1 mice to

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Abbreviations: ab, antibody; cSSC, cutaneous squamous cell carcinoma; DMBA, 7,12-dimethylbenz[a]anthracene; HPV, human papillomavirus; MmuPV1, mouse papillomavirus type 1; UVB, ultraviolet B

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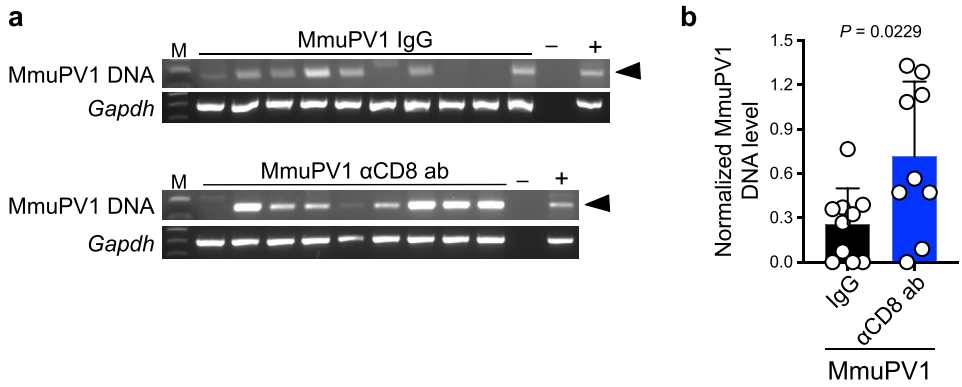

Figure 1. CD8$^+$ T cell depletion increases MmuPV1 DNA levels in the virus-colonized mouse skin. (**a**) Gel images of MmuPV1 E2 PCR on DNA isolated from MmuPV1-infected normal back skin of SKH-1 mice at the completion of the DMBA/UVB skin carcinogenesis protocol. Skin samples were collected at harvest, which took place from weeks 18 to 25 after DMBA as mice developed terminal tumors (n = 10 for IgG controls and n = 9 for anti-CD8 ab–treated MmuPV1-infected mice). Arrowheads indicate amplified MmuPV1 DNA bands (162 bp). M indicates molecular marker, – indicates negative control, and + indicates positive control. (**b**) Quantification of the PCR results in panel **a**. Viral DNA levels were normalized to mouse *Gapdh* using ImageJ (National Institutes of Health, Bethesda, MD) (two-tailed Mann–Whitney *U* test). The height of the bars is representative of the normalized mean of MmuPV1 DNA level, and the error bars represent the SD. ab, antibody; DMBA, 7,12-dimethylbenz[a]anthracene; MmuPV1, mouse papillomavirus type 1.

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/ultraviolet B (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 an SD of 0.2444, whereas the normalized mean MmuPV1 DNA level in the normal skin of CD8$^+$ T cell–depleted mice (n = 9) was 0.7154, with an SD 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 than IgG-treated control mice. The mean and SD of optical density of ELISA for the IgG-treated control mice (n = 10) were 0.2296 and 0.1255 for E6, 0.3454 and 0.1859 for E7, and 0.2973 and 0.0943 for L1, respectively. The mean and SD of optical density of ELISA for the CD8$^+$ T cell–depleted mice (n = 9) were 0.6564 and 0.3782 for E6, 0.8701 and 0.6675 for E7, and 0.6458 and 0.3082 for L1, respectively.

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 antipapillomavirus 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 owing to virus infection as suggested by previous studies (Bouwes Bavinck et al., 2018, 2010; Farzan et al., 2013; Genders et al., 2015; Hampras et al., 2014; Iannacone et al., 2014, 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 interaction with human hosts (Uberoi et al., 2018). 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 protumorigenic 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

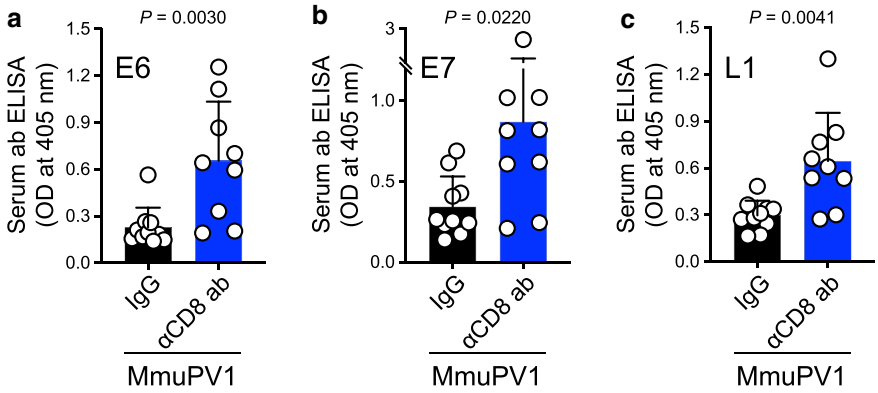

Figure 2. CD8$^+$ T cell–depleted mice have higher antibody titers to MmuPV1 antigens. (**a**–**c**) ELISA mean OD for the detection of serum ab to MmuPV1 (**a**) E6, (**b**) E7, and (**c**) L1 antigens in mice infected with MmuPV1 with or without CD8$^+$ T cell depletion. Sera were collected at harvest,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

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 disease.

MATERIALS AND METHODS

All animal studies were reviewed and approved by the University of Louisville institutional animal care and use committee. Sera and skin biopsies were examined from the mice that were colonized with MmuPV1 and treated with 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 (Louisville, KY) in compliance with animal care and all relevant ethical regulations. Female SKH-1 Elite mice aged 6–10 weeks (477, Charles River Laboratories, Wilmington, MA) were used. Mice's back skin was scarified with a nail file, and 8×10^9 viral genome equivalent of MmuPV1 was applied. After 4 weeks, immune mice that did not develop warts or had warts that spontaneously regressed were used for the skin carcinogenesis experiment. Of the nine mice in the CD8$^+$ T cell–depletion group, four mice had no warts, and five 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, YTS 169.4, Bio X Cell, Lebanon, NH) or IgG (rat isotype control, Sigma-Aldrich, St Loius, MO) antibodies intraperitoneally into the mice. Starting a day before DMBA treatment, 750 μg ab in 200 μl sterile PBS was injected per mouse (first dose) followed by 250 μg in 200 μl sterile PBS weekly injections following the standard protocol (Li et al., 2021; Strickley et al., 2019). The efficacy of CD8$^+$ T cell depletion was confirmed by flow cytometry of the harvested skin and spleen of a select mouse from the test and control groups 6 weeks after DMBA (Strickley et al., 2019).

The day after the first ab injection, mice were treated with 50 μg DMBA in 200 μl acetone on the back skin (D3254, Sigma-Aldrich). The mice were then irradiated with 100 mJ/cm^2 of narrow-band UVB (302–312 nm) three times weekly by a UVP Black-Ray Lamp UVB (36575-052, VWR International, Radnor, PA) 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 by 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). Semiquantitative 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 than DNA in situ hybridization assay (Strickley et al., 2019). DNA was extracted from mice's back skin using the DNeasy Blood & Tissue Kit (69506, Qiagen, Hilden, Germany). The primers 5′-CCTCCTCAGCCAAAGAAGGGC-3′ for MmuPV1-E2 forward and 5′-GTCGTTCTCCTTGTCCGAGTCG-3′ for MmuPV1-E2 reverse, 5′ GGCCAGGATGTAAAGGTCATTAAG-3′ for *Gapdh* forward and 5′-GTCCCTCGAACTAAGGGGAAAG-3′ for *Gapdh* reverse 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 (GraphPad Software, San Diego, CA). Bar graphs show mean + SD. Two-tailed Mann–Whitney *U* test was used as the significance test between the two groups. A $P < 0.05$ was considered significant. All error bars represent SD.

Data availability statement

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

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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.

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CONFLICT OF INTEREST

SD is an inventor on a filed patent for the development of T cell–directed anticancer vaccines against commensal viruses (PCT/US2019/063172). The remaining authors state no conflict of interest.

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