Regulatory T Cells but Not IL-10 Impair Cell-Mediated Immunity in Human Papillomavirus E7 + Hyperplastic Epithelium

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High-risk human papillomavirus infection can induce cervical and other intraepithelial neoplasia and invasive cancers. A transgenic mouse expressing keratin 14 promotor-driven HPV16 E7 oncogene exhibits epithelial hyperplasia and mimics many features of human papillomavirus-related intraepithelial precancers. We have previously demonstrated that HPV16 E7–mediated epithelial hyperplasia suppresses T helper type 1 responses to intradermally delivered antigen and directs differentiation of CD4⁺ T cells towards a Foxp3⁺ regulatory phenotype (Treg). Here we establish that Foxp3⁺ Treg expansion from a transferred naïve T-cell population is driven directly by the hyperplastic skin and is independent of pre-existing immune-modulated lymphocytes. However, depletion of endogenous CD25⁺ Tregs before priming of adoptively transferred T cells significantly improves antigen-specific CD8⁺ T-cell responses but not T helper type 1 responses. Deletion of IL-10 had no effect on Treg expansion, epidermal dendritic cell alteration, and suppression of induced T helper type 1 immunity in HPV16 E7-driven hyperplastic mice. Thus, HPV16 E7–mediated epithelial hyperplasia promotes expansion of peripheral Tregs in response to intradermal immunization that suppress antigen-specific CD8⁺ T-cell responses independently of IL-10, but depletion of these Tregs is not sufficient to restore T helper type 1 immunity.

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INTRODUCTION
Infection with high-risk human papillomavirus (HPV) causes cervical and other anogenital and oropharyngeal cancers. HPV E6/E7 oncoproteins are constitutively expressed in epithelial cells of HPV-associated cancers (Frazer, 2004) and HPV E6/E7 oncoproteins are constitutively expressed in cervical and other anogenital and oropharyngeal cancers. Infection with high-risk human papillomavirus (HPV) causes adaptive immune cells into the skin and creates an inflammatory environment favoring local immunosuppression (Bergot et al., 2014; Choyce et al., 2013; Gosmann et al., 2014a, 2014b; Jazayeri et al., 2017). Recruitment of immune cells to the hyperplastic K14E7tg skin is not exclusively E7-specific, because K14E7tg skins transplanted onto immunocompetent hosts promote E7-specific and non-specific T-cell trafficking to the skin (Choyce et al., 2013; Jazayeri et al., 2017). T-cell responses to E7 and E7-unrelated antigens in K14E7tg animals are impaired after immunizations (Narayan et al., 2009; Tindle et al., 2001). Furthermore, the modulation of immune response to immunization is dependent on epithelial hyperplasia rather than on the expression of the E7 neoantigen itself, because mice expressing E7 antigen but not exhibiting epithelial hyperplasia are restored in their ability to prime T-cell responses to intradermally delivered antigens (Bashaw et al., 2019; Kuo et al., 2018).

Regulatory T cells (Tregs) mediate immunologic tolerance to self-antigens and prevent immunopathology caused by autoimmune diseases (Chaudhry et al., 2011; Jones et al., 2016). Tregs can suppress immunosurveillance by directly inhibiting effector cells or indirectly regulating antigen-presenting cells (Bauer et al., 2014; Shitara and Nishikawa, 2018). In chronic infection and tumors, the immunosuppressive cytokines produced by Tregs, including IL-10, have been linked to CD8⁺ T-cell exhaustion (Sawant et al., 2019; Shitara and Nishikawa, 2018). Treg-specific IL-10 deficiency in tumor-bearing mice improved T helper type (Th) 1 and Th17 responses (Wang et al., 2016), and production of IL-10 by antigen-driven Tregs through immunization can control T-cell immunity (Liu et al., 2009). In cervical neoplastic lesions, different types of cells, including dendritic cells (DCs),
monocytes, macrophages, and keratinocytes, have been reported to produce IL-10 in addition to Tregs (Bashaw et al., 2017, Prata et al., 2015). Although Tregs are not significantly increased in lymphoid organs of K14E7tg mice at resting state, depletion of CD4/CD25⁺ cells in K14E7tg mice improves endogenous CD8⁺ T-cell responses (Narayan et al., 2009). K14E7tg epidermis exhibits accumulation of pheno-typically and functionally altered DCs (Abd Wariff et al., 2015; Bashaw et al., 2019; Chandra et al., 2016). Intradermal immunization in K14E7tg mice leads to polarization of adaptively transferred ovalbumin (OVA)-specific CD4⁺ T cells into Foxp3⁺ Tregs and anergic T cells while impairing Th1 immunity (Bashaw et al., 2019). The modulation of immune response to immunization is dependent on epithelial hyperplasia rather than on the expression of the E7 neo-antigen itself (Bashaw et al., 2019).

Here, we investigate whether Tregs and IL-10 are involved in the suppression of antigen-driven immune responses in HPV-mediated epithelial hyperplasia, using intradermal immunization with the model antigen OVA and transfer of OVA-reactive T cells. K14E7tg hyperplastic skin directly induced Treg expansion from adaptively transferred T cells, independently of an endogenous adaptive immune system including pre-existing Tregs. Depletion of CD25⁺ Tregs before intradermal immunization significantly improved priming of CD8⁺ T-cell responses in K14E7tg mice. Depletion of IL-10 in K14E7tg mice had no effect on Treg induction and Th1 impairment. Thus, HPV-mediated epithelial hyperplasia promotes Treg polarization in response to intradermal immunization independent of pre-existing immune-modulated lymphocytes and IL-10, and depletion of Tregs can restore CD8⁺ T-cell responses but not Th1 immunity.

RESULTS
Immunization-induced Treg expansion in K14E7tg mice is independent of pre-existing immune-modulated lymphocytes
K14E7tg mice express E7 oncoprotein not only in skin but also in thymic epithelial cells, resulting in thymic hypertrophy and disrupted T-cell maturation (Malcolm et al., 2003). This intrinsically altered T-cell development might affect the priming of adaptively transferred T cells. To test whether endogenous T cells of K14E7tg mice promote polarization of adaptively transferred T cells to a regulatory phenotype, K14E7tgxRag1⁻/⁻ mice and Rag1⁻/⁻ controls were reconstituted with wild-type CD4⁺ T cells and, three weeks later, the frequency of CD4⁺Foxp3⁺ Tregs was analyzed. Accumulation of Foxp3⁺ Tregs was significantly increased in K14E7tgxRag1⁻/⁻ mice compared with Rag1⁻/⁻ controls (Figure 1a), demonstrating that host T cells are not required for induction of Tregs from T cells transferred to K14E7tg mice. To examine whether endogenous T cells of K14E7tg mice effect the generation of effector Th responses, K14E7tgxRag1⁻/⁻ mice were reconstituted with OVA-specific major histocompatibility complex II (MHCII)-restricted TCR-transgenic OT-II T cells and subsequently immunized intradermally with OVA and adjuvant Quil-A. Two weeks later, OT-II cells were analyzed for Foxp3 and cytokine expression. Immunization significantly increased numbers of Foxp3⁺ OT-II cells in K14E7tgxRag1⁻/⁻ mice compared with Rag1⁻/⁻ controls (Figure 1b), suggesting that immunization of HPV-mediated hyperplastic skin leads to Treg polarization without requirement for pre-existing Tregs. On in vitro restimulation with cognate peptide, OT-II cells in both K14E7tgxRag1⁻/⁻ and Rag1⁻/⁻ hosts secreted comparable amounts of IFN-γ and IL-17A (Figure 1b), indicating that increased numbers of Foxp3⁺ Tregs in K14E7tgxRag1⁻/⁻ mice did not affect the polarization of Th1/Th17 cells in a lymphopenic environment. Of note, lymphopenia-induced rapid T-cell proliferation can revert anergic CD4⁺ T cells into effector T cells (Kalekar et al., 2016) and improve anti-tumor Th1 effector functions (Xie et al., 2010). Collectively, these data indicate that K14E7-derived lymphocytes and their cytokine products are not required for Foxp3⁺ Treg polarization of transferred CD4⁺ T cells, but rather that Treg expansion can be initiated by components of the innate immune system or by the hyperplastic skin.

K14E7tg skin directly promotes Treg expansion after intradermal immunization
To understand whether K14E7tg skin promotes Treg expansion after intradermal immunization, we utilized a skin grafting model, in which ear skin of K14E7tg mice was transplanted to nontransgenic recipients. Graft recipients have an intact immune system and only carry a patch of K14E7tg skin, which models the relationship between HPV-associated lesions and the host immune system more closely than the K14E7tg mouse itself. Recipients received either two K14E7tg or two nontransgenic skin grafts to the flank, and the grafts were allowed to heal for 3 weeks before adoptive OT-II CD4⁺ T-cell transfer (Figure 2a). Mice were then immunized twice intradermally with OVA and Quil-A into either the graft or the ear pinnae. The phenotype of transferred CD4⁺ T cells was analyzed from postauricular ear-draining lymph nodes (LNs) and graft-draining axillary, brachial, and inguinal LNs. As expected, after immunization of ear skin, the frequency of Foxp3⁺ Tregs in the ear-draining LNs of recipients carrying a K14E7tg skin graft was comparable to the frequency in recipients carrying a nontransgenic skin graft (Figure 2b). However, graft-draining LNs of intra-graft immunized recipients carrying K14E7tg grafts showed enhanced frequencies of Foxp3⁺ Tregs compared with recipients carrying nontransgenic grafts (Figure 2b), demonstrating that immunization of K14E7tg skin directly promotes Treg expansion.

DCs isolated from K14E7tg skin are efficient to induce Th1 responses ex vivo
Because Foxp3⁺ Treg expansion in K14E7tg mice can occur independently of host-derived lymphocytes and is directly promoted by the hyperplastic skin, we sought to determine if skin-resident DCs are programmed by hyperplastic skin to promote Treg polarization and impair Th1 responses. We isolated epidermal MHCIΙ⁺CD11c⁺ DCs of mice previously immunized intradermally with OVA and Quil-A and cocultured these with naive OT-II CD4⁺ T cells in vitro. CD4⁺ T cells incubated with DCs of K14E7tg epidermis were equally capable to secrete IFN-γ as those incubated with nontransgenic epidermal DCs. Also, the frequency of Foxp3⁺ Tregs in CD4⁺ T cells cocultured with DCs of K14E7tg and nontransgenic mice was similar (Figure 3a).
Different skin antigen-presenting cells, including macrophages, epidermal Langerhans cells, and dermal cDC1 and cDC2, can capture intradermally delivered antigens. However, only DCs that migrate to the draining LNs with the antigen can prime T cells (Tamoutounour et al., 2013). To test whether any skin-derived DCs contributed to Treg polarization in K14E7tg mice, we immunized mice intradermally with Alexa Fluor 488–conjugated OVA (OVA-AF488) and

Figure 1. Immunization-induced Treg expansion in K14E7tg mice does not require pre-existing lymphocytes. (a) K14E7xRag1−/− and Rag1−/− mice were adoptively transferred with 10 × 10^6 CD4+ T cells. Three weeks later, Foxp3+ CD4+ T cells in skin-draining LNs were determined by flow cytometry. (b) K14E7xRag1−/− and Rag1−/− mice were adoptively transferred with CD45.1+ 10^6 OT-II cells 1 day before intradermal immunization with 40 μg OVA and 5 μg Quil-A. Two weeks later, skin-draining LN cells were restimulated with 10 μg/ml OVA or OVA323-339 for 6 hours. Percentages of Foxp3+, IL-17A+, and IFN-γ+ CD4+ T cells were determined from live CD45.1+ adoptively transferred OT-II T cells. Data (mean ± SEM) are from one experiment with a total of 5–6 mice/group. Statistical significance was determined using unpaired t-test. **P < 0.01. K14E7tg, keratin 14–driven HPV16 E7 oncogene; LN, lymph node; ns, not significant; OVA, ovalbumin; Treg, regulatory T cell.
Quil-A. OVA-AF488+ DCs from draining LNs were then sorted and cocultured with naive OT-II CD4+ T cells. Relatively low numbers of Foxp3+ CD4+ T cells were observed in this coculture system. However, expression of Foxp3 on CD4+ T cells was significantly higher when cocultured with OVA-AF488+ DCs from K14E7tg animals than CD4+ T cells cocultured with OVA-AF488+ DCs from nontransgenic animals (Figure 3b). Surprisingly, K14E7tg OVA-AF488+ DCs induced IFN-γ–secreting CD4+ T cells significantly better than wild-type OVA-AF488+ DCs (Figure 3b). Together, these data demonstrate that DCs isolated from skin-draining LNs of immunized K14E7tg mice induce increased Foxp3+ CD4+ T cells but also efficiently activate Th1 responses ex vivo.

Immune tolerance in K14E7tg mice is independent of IL-10

The cytokine environment is one of the major drivers of T-cell polarization (Jain et al., 2018; Kashiwagi et al., 2017). IL-10 production during antitumor therapeutic vaccination in preclinical and clinical studies of cancers is reported to promote tolerogenic DCs or directly inhibit antitumor effector T cells (Llopiz et al., 2017). Higher intralesional IL-10 expression levels correlated with tumor progression among HPV+ patients with cervical cancer (Bermúdez-Morales et al., 2008). K14E7tg skin expresses elevated levels of II-10 mRNA (Tuong et al., 2018). To test whether IL-10 signaling in K14E7tg skin contributes to the altered DC phenotype and tolerogenic CD4+ T-cell responses after intradermal immunization, we generated K14E7tgxIl-10−/− mice. Similar to K14E7tg mice, CD45+CD11c+EpCAM+ epidermal Langerhans cells of K14E7tgxIl-10−/− mice expressed significantly lower levels of surface MHCII, EpCAM, and CD11b (Figure 4a). We adaptively transferred OT-II T cells followed by intradermal immunization with OVA and Quil-A and analyzed the phenotype of endogenous and transferred T cells. Both K14E7tgxIl-10−/− and K14E7tg mice displayed significantly higher numbers of host-derived Foxp3+CD4+ T cells, which expressed high levels of the Treg activation markers CD25, CD44, CTLA-4, and CD73 (Supplementary Figure S1). In addition, the frequency of PD-1+positive Foxp3+CD4+ T cells was increased in K14E7tgxIl-10−/− and K14E7tg mice compared with C57BL/6 and II-10+/− mice (Supplementary Figure S1). Similar to K14E7tg mice, OT-II T cells transferred to K14E7tgxIl-10−/− mice acquired a Foxp3+ Treg phenotype (Figure 4b), and IFN-γ production was significantly impaired (Figure 4c). As expected, IL-10 deficiency in wild-type mice increased IFN-γ production by T cells (Figure 4c). These data demonstrate that IL-10 did not contribute to the phenotypic change in epidermal Langerhans cells and induction of Tregs in K14E7tg mice.

We further tested whether IL-10 is required for K14E7tg skin graft tolerance in immunocompetent hosts. IL-10–deficient mice were used as graft recipients to exclude the potential contribution of IL-10–producing host-derived cells infiltrating the donor skin. Both K14E7tg and K14E7tgxIl-10−/− grafts were tolerated by II-10+/− recipient mice (Supplementary Figure S2), demonstrating that IL-10 is not required in HPV E7–mediated hyperplasia-associated immunosuppression.

Depletion of CD25+ CD4+ Tregs improves CD8+ T-cell responses in K14E7tg mice

Tregs in peripheral tissues are known to modulate effector T-cell functions (Ganesan et al., 2013; Jacobs et al., 2010; Oweida et al., 2019). We investigated the ability of immunization-induced peripheral Tregs to impair T-cell responses in K14E7tg mice after intradermal immunization by depleting CD25+ Tregs using anti-CD25 antibody.

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single injection of anti-CD25 antibody effectively depletes Tregs up to 10 days after antibody injection (Supplementary Figure S3a). To test whether pre-existing Tregs in K14E7tg mice are responsible for suppression of effector T-cell responses, mice received antibody treatment followed by OT-I/OT-II cell transfer and intradermal immunization with OVA and Quil-A (Figure 5a). Six days later, draining LN cells were subjected to in vitro restimulation with cognate peptide, and IFN-γ production in OT-I/OT-II cells was analyzed. Adoptively transferred OT-I T cells were identified by coexpression of TCRVβ5.1-5.2 and TCRVα2 (Supplementary Figure S4) and percentage of IFN-γ+CD8+ T cells was determined among TCRVβ5.1-5.2/Vα2+CD8+ T cells. CD25+ cell depletion significantly reduced the frequency of host-derived Foxp3+CD25+CD4+ T cells in both K14E7tg and nontransgenic skin-draining LNs (Supplementary Figure S3). Depletion of CD25+ Tregs in K14E7tg mice significantly improved IFN-γ expression in OT-I CD8+ T cells (Figure 5b). We observed an increased frequency of IFN-γ+OT-II cells in CD25+ cell-depleted nontransgenic mice but not in K14E7tg mice (Figure 5c).

To further test whether immunization-induced Tregs in K14E7tg mice inhibit CD8+ T-cell immunity, transgenic and nontransgenic mice were immunized intradermally with OVA and Quil-A (Figure 5d). We then treated mice with Treg-depleting anti-CD25 antibodies followed by adoptive transfer of OT-I cells and a second intradermal immunization. (Figure 5d). Consistent with data in Figure 5b, depletion of Tregs significantly improved OT-I CD8+ T-cell immunity in K14E7tg mice, because OT-I T cells expressed significantly enhanced levels of proliferation marker Ki67 and effector cytokine IFN-γ when compared with PBS-treated K14E7tg mice (Figure 5e and f). Depletion of Tregs in nontransgenic mice had no effect on proliferation and IFN-γ expression by OT-I CD8+ T cells (Figure 5e and f). Thus, immunization of K14E7tg skin induces IL-10–independent Tregs that contribute to impairment of CD8+ T-cell responses, whereas impairment of Th1 responses are inhibited by other immunosuppressive mechanisms that remain to be defined.

**DISCUSSION**

In this study, immunosuppressive mechanisms of HPV-associated epithelial hyperplasia were examined in the
K14E7tg mouse model expressing HPV16 E7 oncoprotein, which mimics HPV-associated human precancerous lesions (Tuong et al., 2018). We demonstrate that antigen presentation in K14E7tg skin promotes expansion of Tregs and limits Th1 responses by a mechanism not mediated by pre-existing immune-modulated lymphocytes or IL-10. Removing Tregs improves antigen-specific CD8\(^+\) T-cell but not Th1 immunity. We have previously shown that K14E7tg epithelial

![Figure 4](image-url)

**Figure 4. Altered LC phenotype, enhanced Treg polarization, and impaired Th1 response in K14E7tg mice are IL-10 independent.** (a) Shown are MFI of surface features on epidermal CD45\(^+\)CD11c\(^+\)EpCAM\(^+\) LCs. (b, c) A total of 5 × 10\(^6\) CD45.1\(^+\)OT-II T cells were adoptively transferred into mice and immunized twice with 10 μg OVA and 1 μg Quil-A at days 1 and 7. One week later, percentage of Foxp3\(^+\)Tregs and IFN\(\gamma\) \(^+\) T cells in skin-draining LNs were analyzed of live CD45.1\(^+\)CD4\(^+\) OT-II T cells. IFN-\(\gamma\) secretion was determined after in vitro restimulation with 10 μg/ml OVA\(_{323-339}\). Numbers in the bar graphs indicate percent reduction of IFN-\(\gamma\) \(^+\) T cells. Data (mean ± SEM) are representative of two independent experiments with 4 mice/group. Statistical significance was determined by one-way ANOVA followed by Sidak multiple comparison test. ***P < 0.001; ****P < 0.0001. K14E7tg, keratin 14-driven HPV16 E7 oncogene; LC, Langerhans cell; LN, lymph node; MFI, mean fluorescence intensity; MHCII, major histocompatibility complex II; OVA, ovalbumin; Th, T helper; Treg, regulatory T cell.
Depletion of Tregs increases CD8⁺ but not Th1 immunity in K14E7tg mice. (a, d) Mice were treated with anti-CD25 antibody (PC61) or PBS, received OT-I or OT-II cells, and were intradermally immunized with 10 µg OVA and 1 µg Quil A at indicated time points. Seven days after T-cell transfer, skin-draining LN cells were restimulated with OVA257-264 or OVA323-339 and analyzed by flow cytometry. The percentage of (b, f) IFN-γ⁺CD8⁺ or (e) Ki67⁺CD44⁺CD8⁺ cells was calculated of TCRβ1.5.2⁺ TCRβ2⁺CD8⁺ OT-I T cells, and (c) IFN-γ⁺CD4⁺ cells of CD45.1⁺CD4⁺ OT-II cells. Numbers in the bar graphs indicate percent reduction of IFN-γ⁺ T cells. Data (mean ± SEM) from (a–c) are representative of one experiment with 3–4 mice/group and (d–f) from three independent experiments with 4–6 mice/group. Statistical significance was calculated by unpaired t-test analysis. *P < 0.05; **P < 0.01; ***P < 0.001. K14E7tg, keratin 14-driven HPV16 E7 oncogene; LN, lymph node; ns, not significant; OVA, ovalbumin; Th, T helper type; Treg, regulatory T cell.

hyperplasia but not E7 neoantigen itself modulates the immune responses to OVA (Bashaw et al., 2019). The immunosuppressive effects of E7-induced epithelial hyperplasia are antigen independent. Besides OVA, K14E7tg mice immunized with other antigens, including E7, Sendai virus nucleoprotein, and influenza virus proteins, also induced impaired CD8⁺ T-cell responses (Narayan et al., 2009; Tindle et al., 2001). Even without antigenic challenge, K14E7tgxRag1⁻/⁻ mice reconstituted with CD4⁺ T cells resulted in increased Treg polarization, demonstrating an antigen-unspecific immunosuppression. Although K14E7tg skin suppressed OVA-specific immune responses, K14E7tg skin grafts coexpressing OVA transgene are still rejected (Jazayeri et al., 2017). This implies that K14E7tg skin does not induce OVA-specific immune tolerance, and failure to K14E7tg skin graft rejection is mediated, in part, by limited E7 antigen presentation (Kuo et al., 2018). Epithelial hyperplasia employs multiple mechanisms of immunosuppression that contribute to tolerance of K14E7tg skin grafts on immunocompetent recipients (Bergot et al., 2014; Choyce et al., 2013; Gosmann et al., 2014b). Genetic deletion of suppressive components such as NK T cells, IFN-γ, and IL-18 triggers K14E7tg skin graft rejection without disrupting the epidermal hyperplasia (Choyce et al., 2013; Gosmann et al., 2014a; Mattarollo et al., 2010). Although systemic administration of proinflammatory endotoxin overcomes immunosuppression in K14E7tg mice (Frazer et al., 2001), intragraft immunization with OVA and the proinflammatory adjuvant Quil A did not affect immune tolerance of K14E7tg skin grafts transferred OVA-reactive T cells.

Immune DCs play a critical role in induction of Tregs, which are detrimental to antitumor immunity (Togashi et al., 2019), and depletion of Tregs improves therapeutic vaccine efficacy in preclinical and clinical studies, for example, in patients with melanoma (Jacobs et al., 2010) and lung adenocarcinoma (Ganesan et al., 2013) and murine head and neck cancer models (Oweida et al., 2019). Tregs can be generated from naive T-cell precursors in the thymus or from conventional CD4⁺ T cells in peripheral tissues. Antigen presentation in the context of MHCII is required to induce both thymus-derived and peripheral Tregs (Togashi et al., 2019). Tregs regulate antigen-priming and effector T-cell responses and promote expansion of Tregs or anergic T cells. Although depletion of CD25⁺ Foxp3⁺ cells did not facilitate K14E7tg skin graft rejection (Mattarollo et al., 2011), grafts of lymphocyte-deficient K14E7tgxRag1⁻/⁻ mice are rejected by
immunocompetent mice (Choyce et al., 2013), which indicates the presence of immune-suppressive lymphocytes within the hyperplastic K14E7tg skin. Our results suggest that Treg polarization is independent of endogenous immune-modulated lymphocytes in K14E7tg animals, because K14E7tgxRag1–/– mice reconstituted with purified CD4+ T cells still led to enhanced expansion of peripheral Foxp3+ Tregs. The frequency of Foxp3+CD25+CD4+ T cells in K14E7tg lymphoid organs is similar to wild-types (Narayan et al., 2009). However, significantly increased frequencies of Tregs were observed in skin-draining LNs of K14E7tg mice following immunization (Bashaw et al., 2019), suggesting that the drivers of Treg expansion after intradermal immunization are produced in the periphery. Of importance, K14E7tg skin transplanted onto nontransgenic hosts induced increased numbers of Tregs in graft-draining LNs, demonstrating a direct role of K14E7tg hyperplastic skin in peripheral Treg expansion. It remains unknown if Treg polarization is mediated by the hyperproliferative epithelium itself or by components of the innate immune system and will be technically difficult to delineate.

Innate cytokines concurrently or in sequence with the signals generated by DCs can dictate the type of effector T cells. We have observed increased IL-10 mRNA in K14E7tg skin (Tuong et al., 2018) and epidermal Langerhans cells (Chandra et al., 2016). Additionally, increased levels of IL-10 (Kobayashi et al., 2008) and a shift of Th1 to Th2 and Treg cytokine profiles including IL-10 have been reported to positively correlate with HPV-associated neoplastic progression in humans (Pegbini et al., 2012). A strong correlation between increased serum IL-10 levels and poor clinical prognosis in HPV+ patients with cervical cancer was also recently reported, signifying a role for IL-10 in HPV-related immunosuppression (Wang et al., 2018). IL-10 can directly suppress effector T cells or condition DCs to promote Treg proliferation (Girard-Madoux et al., 2012; Llopiz et al., 2017; Sawant et al., 2019); however, in this study, deletion of IL-10 in K14E7tg mice had no impact on Treg induction and Th1 impairment. Of note, multiple factors, including TGFβ, IL-2, and retinoic acid, either alone or in combination can promote induction of Foxp3+ Tregs (Togashi et al., 2019; Yamazaki et al., 2007) and suboptimal antigen presentation in the presence of TGFβ can convert conventional CD4+ T cells to Foxp3+ Tregs (Yamazaki et al., 2007). In vivo induction of Tregs in K14E7tg skin might therefore require IL-2 and/or TGFβ in addition to altered DC antigen presentation, and further studies are needed to mechanistically dissect the drivers of Tregs and the subsequent peripheral immune tolerance in HPV-induced epithelial hyperplasia.

Our current data provide evidence for the functional relevance of peripheral antigen-driven Tregs. Depletion of CD25+ Tregs during priming of T cells in K14E7tg mice significantly improved the proliferation and effector cytokine secretion by CD8+ T cells but was unable to restore antigen-specific Th1 immunity. Although a causal relationship has not been demonstrated, Th1 immunity during therapeutic vaccination has been a good prognostic predictor for regression of HPV-associated disease in humans (van der Burg and Melief, 2011; Welters et al., 2008). Disease progression has been associated with the presence of Tregs and Th2 responses (Shah et al., 2011; Shang et al., 2015). Our study indicates that overcoming the suppressive effect of Tregs alone in K14E7tg skin is insufficient to fully restore cell-mediated immune responses. T-cell help is required to generate efficient effector and memory cytotoxic T lymphocyte responses and activate other tumoricial immune cells (Borst et al., 2018). Besides providing help, CD4+ T cells themselves can acquire antitumor cytotoxic functions (Quezada et al., 2010). An efficient therapeutic HPV vaccine should therefore induce Th1 and CD8+ cytotoxic T lymphocyte responses in combination with treatment to break local immunosuppression.

In conclusion, we demonstrate that immunization to K14E7tg hyperplastic skin polarizes antigen-specific transferred T cells to Tregs while limiting Th1 responses, which is independent of endogenous Tregs and IL-10. Treg deficiency improves CD8+ T-cell immunity but not Th1 responses. These findings expand our understanding on the mechanisms involved in the immune modulation in HPV-associated neoplastic diseases. However, further studies are required to explore the exact drivers of Treg polarization in the HPV16 E7–driven hyperplastic environment to identify targets for cancer immunotherapy.

MATERIALS AND METHODS

Mice
C57BL/6, K14E7tg, Rag1–/–, K14E7tgxRag1–/–, OT-I, B6.SJLxOT-II, Il-10–/–, and K14E7tgxII–/– mice were bred and housed under specific pathogen-free conditions at the Translational Research Institute biological research facility. Six- to twelve-week-old female mice were used for all experiments. All experiments were approved by the University of Queensland Animal Ethics Committee (UQDI/452/16).

Single-cell suspension preparation
Single-cell suspensions were prepared following procedures described previously (Bashaw et al., 2019). Briefly, mice were killed and ear skins, LNs, and spleens were harvested. Ears were split into ventral and dorsal sides and incubated dermis-side down in 2.5 mg/ml dispase II (Roche, Basel, Switzerland) in PBS for 1 hour at 37 °C in 5% CO2 to separate epidermis from dermis. Tissues were chopped tiny pieces with scissors and incubated in 0.2 mg/ml DNase I (Thermo Fisher Scientific, Waltham, MA) and 1 mg/ml collagenase D (Roche) for 30 minutes at 37 °C. Digested samples were filtered through a 70-μm cell strainer with 10 ml PBS and centrifuged at 350g for 5 minutes. Splenocytes were additionally incubated with Ammonium-Chloride-Potassium lysis buffer for 2 minutes to remove erythrocytes. Cell pellets were resuspended in flow cytometry staining buffer (2% fetal bovine serum, 2 mM EDTA, PBS).

Flow cytometry analysis
Cell suspensions were incubated with Fcγ receptor blocking and live/dead stain in PBS on ice for 20–30 minutes. After washing at 300g for 5 minutes, cells were stained with fluorescence-conjugated antibodies against cell surface antigens by incubating for 30 minutes on ice. To analyze cytokine or Foxp3 expression, cells were fixed and permeabilized using Foxp3 Transcription Factor Fixation and Permeabilization buffer (eBioscience, San Diego, CA) for 30 minutes at 18 °C. After two washes, cells were stained with antibodies for 30 minutes at 18 °C. All antibody clones are listed in Supplementary Table S1. Data acquisition was performed on BD LSRFortessa X-20...
flow cytometers (BD Biosciences, San Jose, CA), and analysis was performed using Flowjo software (LLC, Ashland, OR). Adoptively transferred OT-I T cells or OT-II T cells were set as parent gate to calculate the percentages of Foxp3+ Tregs and T-cell responses.

Adoptive transfer experiments

OT-I or OT-II cells were obtained from spleens and LNs and were transferred into recipient mice through intravenous tail injection. One day later, recipient mice were immunized intradermally with OVA (Sigma Life Science, St. Louis, MO) and Quil-A (InvivoGen, San Diego, CA) into the ear pinnae (Bashaw et al., 2019). Alternatively, mice were immunized twice into the ear pinnae or grafted skin with a 1-week interval. After 7 days, single-cell suspensions were obtained as described earlier, and AF488 OVA and Quil-A at the ear pinnae. After 48 hours, mice were killed and their technical assistance.

Skin grafting
Skin grafting was performed following a procedure described previously (Yu and Chandra, 2021). Briefly, dorsal and ventral donor ear skins were grafted onto flanks of anesthetized mice and mice were bandaged. Bandages were removed after 7 days. Graft size was determined from photographs including a ruler and imaging Fiji ImageJ software (National Institutes of Health, Bethesda, MD).

In vitro DC/T-cell coculture assays

Mice were immunized intradermally with OVA-AF488 or unlabelled OVA and Quil-A at the ear pinnae. After 48 hours, mice were killed and single-cell suspensions from ear skin epidermis or draining LNs were obtained as described earlier, and AF488+MHCII+CD11c+ DCs were sorted using a BD FACS Aria Fusion Sorter (BD Biosciences). CD4+OT-II cells were sorted on the basis of TCRβ and CD4 expression. A total of 15 × 10^3 to 20 × 10^3 DCs were incubated with 50 × 10^3 CD4+OT-II T cells in a 96-well U-bottom plate with 50 ng/ml IL-2 for 5 days. Cells were analyzed by flow cytometry after restimulation with 10 μg/ml OVA257–264 (InvivoGen) or OVA323–339 (InvivoGen) and intracellular staining. Percent reduction in IFN-γ production was calculated using the following formula:

\[
\% \text{ reduction} = \left(\frac{\% \text{ IFN-} \gamma^+ \text{ T cells control} - \% \text{ IFN-} \gamma^+ \text{ T cells K14E7}}{\% \text{ IFN-} \gamma^+ \text{ T cells control}}\right) \times 100
\]

Depletion of Tregs

Mice received 500 μg anti-CD25 antibody (clone PC-61, Bio X Cell, Lebanon, NH) intraperitoneally. CD25+ Treg depletion was evaluated in peripheral blood by flow cytometry using anti-CD25 antibody (7D4; BioLegend, San Diego, CA).

Statistical analysis

Statistical differences between two groups were analyzed by two-tailed unpaired t-test. One-way ANOVA followed by Sidak’s multiple comparison test was used to compare the mean of three or more groups. P-values ≤ 0.05 with 95% confidence interval were considered statistically significant.

Data availability statement

All data generated during this study are included in this article and its supplementary material file.

REFERENCES


Supplementary Figure S1. IL-10−/− deficient K14E7tg hyperplastic skin displays increased Tregs. C57BL/6, K14E7, Il-10−/−, and K14E7xIl-10−/− mice received intradermal immunization with OVA (10 μg) and Quil-A (1 μg). One week after the second immunization, single-cell suspensions were analyzed by flow cytometry. Dot plots and bar graphs show the frequencies of Foxp3⁺CD4⁺ T cells coexpressing CD44, CD25, CTLA-4, CD73, and PD-1 pre-gated on live CD3⁺ CD4⁺ skin cells. Statistical significance was determined by one-way ANOVA followed by Sidak multiple comparison test. ***P < 0.001; ****P < 0.0001; error bar, mean ± SEM. Data shown are one of two experiments with 4 mice per group. K14E7tg, keratin 14—driven HPV16 E7 oncogene; OVA, ovalbumin; Treg, regulatory T cell.
Supplementary Figure S2. IL-10–deficient K14E7tg skin is not spontaneously rejected by IL-10−/− recipients. IL-10−/− mice received adjacent K14E7xIl-10−/− and Il-10−/− ear skin grafts as indicated. Graft rejection was assessed weekly starting from day 7 after grafting. (a) Representative images of skin grafts. (b) Shrinkage of the grafts over time was calculated using day 7 values as baseline. Statistical significance was calculated by two-way ANOVA analysis. Error bar, mean ± SEM. Data shown are from one experiment with 5–6 mice per group. K14E7tg, keratin 14–driven HPV16 E7 oncogene; ns, not significant.

Supplementary Figure S3. In vivo depletion of CD4+ CD25+ Tregs. (a) C57BL/6 mice received 250 µg or 500 µg anti-CD25 antibody (clone PC6) in 200 µl PBS or PBS alone (control). The frequency of blood CD25+ CD4+ T cells was analyzed at the indicated days after treatment. (b) K14E7 and C57BL/6 mice were treated with PC61 anti-CD25 antibody (500 µg) or PBS (control) two days before OT-II cell adoptive transfer. The next day, mice were immunized intradermally with OVA (10 µg) and Quil-A (1 µg). One week after OT-II cell transfer, skin-draining LNs were harvested and the frequencies of Foxp3+ CD25+ CD4+ T cells were analyzed by flow cytometry. Statistical significance was determined with unpaired t-test (**P < 0.01). K14E7tg, keratin 14–driven HPV16 E7 oncogene; LN, lymph node; OVA, ovalbumin.
Supplementary Figure S4. Markers for distinguishing OT-I CD8+ T cells.
C57BL/6 and OT-I mice splenocytes were stained with anti-CD3, anti-CD8α, anti-Vα2, and anti-Vβ5.1.5.2 antibodies and analyzed on CD3+ CD8+ gated cells.

Supplementary Table S1. Lists of Antibodies Used for Flow Cytometry

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Abbreviations: APC, allophycocyanin; BV, brilliant violet; Cy, cyanine; FITC, fluorescein; MHC II, major histocompatibility complex II; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein.