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IL-17 Suppresses Immune Effector Functions in Human Papillomavirus-Associated Epithelial Hyperplasia

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Persistent infection with high-risk human papillomaviruses (HPV) causes epithelial hyperplasia that can progress to cancer and is thought to depend on immunosuppressive mechanisms that prevent viral clearance by the host. IL-17 is a cytokine with diverse functions in host defense and in the pathology of autoimmune disorders, chronic inflammatory diseases, and cancer. We analyzed biopsies from patients with HPV-associated cervical intraepithelial neoplasia grade 2/3 and murine skin displaying HPV16 E7 protein-induced epithelial hyperplasia, which closely models hyperplasia in chronic HPV lesions. Expression of IL-17 and IL-23, a major inducer of IL-17, was elevated in both human HPV-infected and murine E7-expressing lesions. Using a skin-grafting model, we demonstrated that IL-17 in HPV16 E7 transgenic skin grafts inhibited effective host immune responses against the graft. IL-17 was produced by CD3+ T cells, predominantly CD4+ T cells in human, and CD4+ and γδ T cells in mouse hyperplastic lesions. IL-23 and IL-1β, but not IL-18, induced IL-17 production in E7 transgenic skin. Together, these findings demonstrate an immunosuppressive role for IL-17 in HPV-associated epithelial hyperplasia and suggest that blocking IL-17 in persistent viral infection may promote antiviral immunity and prevent progression to cancer. The Journal of Immunology, 2014, 193: 2248–2257.

Human papillomaviruses (HPV) infect the epithelium of the genital tract and the skin. Persistent infection with high-risk HPV types can cause cancer, the most prevalent being cervical cancer with >0.5 million new cases and 0.2 million deaths per year (1, 2). Four major steps lead to the development of cervical cancer, as follows: 1) HPV transmission, 2) viral persistence, 3) progression of persistently infected epithelium to dysplastic epithelium, and 4) invasion through the epithelial basement membrane (3). Persistent infection with high-risk HPV is associated with the expression of the HPV proteins E6 and E7 in epithelial cells. E6 and E7 inhibit epithelial cell differentiation and apoptosis, thus promoting epithelial hyperplasia and transformation (4). To date, there is no cure for chronic HPV infection. Furthermore, little is known about the mechanisms that allow for persistent infection. However, a local immunosuppressive environment that prevents viral clearance by the host has been deemed critical for viral persistence (5).

IL-17 is an inflammatory cytokine (6) produced by CD4+ Th17 cells and CD8+ T cells as well as innate immune cells, including γδ T cells, NK cells, NKT cells, lymphoid-tissue inducer-like cells, and subsets of myeloid cells (7, 8). IL-17 induction in these cell types mostly requires signaling via the IL-23R, in addition to other cytokines, including IL-1β and IL-18 (8, 9). IL-17 has a key role in host defense against infections and the pathogenesis of some autoimmune and chronic inflammatory diseases (10–12). IL-17 has also been ascribed immune regulatory functions in diseases such as asthma and colitis (13), as well as antiand protumorigenic effects, depending on the disease context (14).

Recently, increased IL-17 production has been associated with persistent HPV16/18 infection and cervical epithelial neoplasia and transformation (15, 16). However, the function of IL-17 in viral persistence and premalignant epithelial disease is unknown.

In this study, we demonstrate a locally immunosuppressive function of IL-17 in HPV-associated premalignant disease. We observed elevated IL-17 production in human cervical precancerous lesions, as well as in skin in a mouse model of HPV16 E7 protein-induced epithelial hyperplasia. We identified CD4+ and γδ T cells in HPV16 E7 transgenic hyperplastic skin, and CD4+ T cells in human cervical neoplastic epithelium as the predominant cellular sources of IL-17. Using a skin transplantation model, we further demonstrate that the presence of IL-17 in E7 transgenic hyperplastic grafts suppresses graft rejection by an immunocompetent host. This identifies a local immunosuppressive role for IL-17 in a mouse model of HPV-associated premalignant disease.

Materials and Methods

Human cervical intraepithelial neoplasia and control biopsies

Biopsies from human cervical intraepithelial neoplasia (CIN2) or CIN3 and normal cervical tissue from the same patient were diagnosed and provided by L. Perrin and S. Cattanach (Mater Medical Centre, Brisbane, QLD, Australia). Women participating in the study were over the age of 20. Women who were pregnant, had evident cancer or cervical infection with another agent, or had had cervical treatment within the prior year were excluded from the study. All human subjects provided written informed consent prior to the procedure, and the study was approved by the institutional ethics committee (2009001060) and conducted in compliance with the Helsinki guidelines.
Mice
c57Bl/6J mice (designated wild type) and HPV16 E7 transgenic C57Bl/6J mice, which express the HPV 16 E7 oncoprotein under control of the keratin 14 (K14) promoter (designated K14E7) (17), were obtained from the Animal Resources Centre (Perth, WA, Australia). Colonies of K14E7 heterozygous mice were maintained by breeding K14E7 mice with C57Bl/6J females, rendering C57Bl/6J mice the appropriate control strain. TCR-8/− (18) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17−/− (19), IL-12p40−/− (20), and IL-1R1−/− (21) mice were obtained from Y. Iwakura (University of Tokyo, Tokyo, Japan), C. Farah (University of Queensland Centre for Clinical Research, Brisbane, QLD, Australia), and H. Thomas (University of Melbourne, Parkville, VIC, Australia). Il17a−/− knockout strains were grown on the C57Bl/6J background. All mice were maintained locally under specific-pathogen-free conditions. To generate K14E7 mice deficient in TCR-8, IL-17, IL-12p40, or IL-1R1, hemizygous K14E7 mice were crossed with the respective knockout mice and the E7-expressing progeny backcrossed with homozygous TCR-8−/−, IL-17−/−, IL-12p40−/−, and IL-1R1−/− mice. All mice were at 6–20 wk of age and were sex and age matched for all experiments.

All animal procedures were approved by the University of Queensland Animal Ethics Committee (AEC 290/10/NHMR/NIH).

Quantitative real-time PCR
For RNA extraction, frozen human biopsies and mouse tissues were homogenized in TRIzol (Invitrogen, Carlsbad, CA). RNA was purified using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). RNA was reverse transcribed using oligo (dT) (Applied Biosystems, Foster City, CA). Primers for SYBR Green quantitative real-time PCR were purchased from Integrated DNA Technologies (Coralville, IA) and were designed using the PrimerQuest PCR design tool (Integrated DNA Technologies). Primer sequences are as follows: murine hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward, 5′-CCCTTAAAATGGTTAAGGTGC-3′; murine HPRT reverse, 5′-AACAAGTCTGCGTGTACC-3′; human HPRT forward, 5′-TCAGGCTATACTCCTAACAGGTG-3′; human HPRT reverse, 5′-AGCTCCCTTACTGCTTTAT-3′; human IL-17 forward, 5′-GAAGTCACTCCCAGGTTCCA-3′; IL-17 reverse, 5′-GAGCTCCCTTATCACACAGAG-3′; human IL-12p40 forward, 5′-ATGGAGTTAAAGTCTGGCCTGTATCC-3′; and human IL-12p40 reverse, 5′-TCTCACTTACCCAATGGTTAAGGTC-3′. Predesigned TaqMan gene expression assays (Integrated DNA Technologies) were used for the quantification of mouse IL-23a mRNA expression. Quantitative real-time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using SDS Software 2.4 (Applied Biosystems). mRNA expression relative to the housekeeping gene HPRT was calculated as 2^ΔΔCT (cycle threshold [HPRT] − cycle threshold [gene of interest]).

Isolation and stimulation of cells
To isolate cells from CIN and control biopsies, the tissue samples were minced in 0.25% trypsin, 0.1% EDTA (Life Technologies, Mulgrave, VIC, Australia), 0.3 mg/ml collagenase, and 5 μg/ml DNase (Roche, Basel, Switzerland) in PBS and incubated with 1% penicillin/streptomycin/glutamine (1 mM), sodium pyruvate (1 mM) (Life Technologies), and HEPES (10 mM, final concentrations) (Invitrogen) for 4 h at 37°C. Cells were collected and filtered through a 70-μm cell strainer. Total protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Flow cytometry
mAbs against human CD45 (H130) and CD3 (OKT3), and anti-mouse CD8a (53-6.7) and CD4 (RM4-5) Abs were from eBioscence. Anti-human CD4 (RPA-T4) and IL-17A (BL168), anti-mouse IL-17A (TCC1-18H10.1), CD11b (M1/70), Gr-1 (RB6-8C5), and matched isotype control Abs were purchased from BioLegend. Anti-human TCR-8/γ (IMMU510) and matched isotype control Abs were obtained from Beckman Coulter (Brea, CA), and anti-mouse mAbs against CD45.2 (104), CD3e (145-2C11), and TCR-γ/δ (GL3) were from BD Biosciences. α-GaCer–loaded CD1d tetramer for detection of invariant NKT cells was provided by D. Godfrey (University of Melbourne). Cell suspensions were incubated with Fc block (BD Biosciences) for 15 min. Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) and Abs against cell surface markers were added for another 30 min of incubation. For interfering cytokine stimulation, cells were fixed and permeablized using the BD Biosciences Cytofix/Cytoperm kit, according to the manufacturer’s instructions. Abs against cytokines and matched isotype control Abs were added for 30 min. All steps were performed at 4°C. Flow Count Fluorospheres (Beckman Coulter) were used for calculation of total cell numbers. A Gallios flow cytometer (Beckman Coulter) was used to acquire samples. Data analysis was performed using Kaluza software 1.2 (Beckman Coulter). Doubled and dead cells were excluded from the analysis.

Skin explant culture
Ears were split into halves and cultured dermis side down on DMEM supplemented with FBS (10% v/v), penicillin/streptomycin/glutamine (1 mM), sodium pyruvate (1 mM) (Life Technologies), and HEPES (10 mM) (Invitrogen) in 24-well plates (37°C, 5% CO2). To remove any cytokine released as a result of tissue preparation, medium was exchanged after 1 h and again after 2 h. For ELISA and Western blot experiments. All animal procedures were approved by the University of Queensland Animal Ethics Committee (AEC 290/10/NHMR/NIH).

For cytokine neutralization in skin explant cultures, mAbs specific for the cytokine or matched isotype control Abs were added to the medium. Anti-mouse IL-17 and IL-23p19 neutralizing and control Abs were obtained from Lilly Research Laboratories (Indianapolis, IN). IL-12/23p40 (C17.8), IL-1β (B122), and matched control Abs were purchased from eBioscience (San Diego, CA), and neutralizing Ab against mouse IL-18 (8B11), and the corresponding type control Ab were purchased from MBL (Woburn, MA). The dorsal (or ventral) half from one ear was treated with isotype control Ab and compared with the dorsal (or ventral) half from the other ear of the same mouse, treated with neutralizing Ab. Supernatants were replaced after 20 h with Ab-supplemented medium and collected after another 20 h for analysis by ELISA.

Cytokine ELISA
ELISA measures the concentrations of mouse IL-17, IL-23 (p40/p19), and IFN-γ (eBioscience) was performed according to the manufacturer’s instructions. Samples undetectable within the range of the assay were plotted as the detection limit. Cytokine concentrations in supernatants were normalized to weight of the skin explant and are shown as pg/mg tissue.

Skin grafting
Donor ear skin was grafted onto recipient flanks, as previously described (22, 23). Briefly, skin from dorsal and ventral ear halves of donor mice (~1.5 cm2) was placed onto the flank region of an anesthetized recipient. Grafts were held in place with antibiotic-permeated gauze (Bactigras; Smith and Nephew, London, U.K.) and bandaged with micropore tape and Flex-wrap (Lyppard, QLD, Australia). Bandages were removed 7 d following grafting, and grafts were monitored three times weekly. Grafts were considered rejected if there was a loss of distinct border and necrosis to >80% of the graft area.

Western blot
Mouse ears were snap frozen and homogenized in 500 μl freshly prepared radioimmunoassay precipitation assay buffer (1% Triton X-100, 20 mM Tris [pH 7.5], 150 mM NaCl, protease inhibitors [Roche], in Multi-Q (H2O) 5 fixed using a T 10 basic ULTRA-TURRAX homogenizer (IKA). After 20 min of incubation (4°C), the homogenate was centrifuged (18,000 relative centrifugal force, 10 min, 4°C), and the supernatant was collected and filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA) to remove aggregates. Total protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).
primary Abs against mouse IL-1β (R&D Systems, Minneapolis, MN) or β-actin (Sigma-Aldrich, St. Louis, MO) (1:2000 dilution in blocking buffer, overnight, 4°C), followed by HRP-conjugated secondary Ab (1:5000 dilution in blocking buffer, 1 h, room temperature). Blots were developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA). Images were acquired with ChemiDoc (Bio-Rad, Hercules, CA).

Statistics

Statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA). Skin graft survival was plotted as Kaplan–Meier plots. Paired Student’s t test was used to assess differences between data sets from skin explant cytokine neutralization experiments. Wilcoxon matched-pairs signed rank test was applied to human data sets. For all other data, unpaired Student’s t test was performed. Differences were considered significant at \( p < 0.05 \) and are indicated as *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), and n.s., not significant.

Results

Elevated IL-17 expression in human cervical precancerous lesions and in a mouse model of HPV16 E7-induced epithelial hyperplasia

Recent studies showed an increase in IL-17 production in human CIN (15, 16). We similarly observed significantly elevated IL-17 mRNA expression in CIN2/3 lesions when compared with morphologically normal cervical control tissue from the same patients (Fig. 1A). Furthermore, mRNA expression of the p19 subunit of IL-23 (IL-23A), a major initiator of IL-17 production, was higher in precancerous compared with control specimens in 8 of 11 patients (Fig. 1B). Consistent with these findings, a higher percentage of cells isolated from the CIN2/3 specimens produced IL-17, with or without in vitro stimulation with PMA and ionomycin (Fig. 1C, 1D).

We next analyzed in a mouse model whether HPV16 E7-driven hyperplasia of skin epithelium (K14E7 mice) (17) is similarly associated with higher production of IL-17. Indeed, IL-17 concentrations were significantly elevated in culture supernatants of K14E7 compared with wild-type skin explants (Fig. 2A). In support of higher IL-17 production in K14E7 skin, we also detected significantly elevated IL-23A mRNA expression and significantly higher concentrations of IL-23 protein in supernatants of K14E7 compared with wild-type skin explants (Fig. 2B). Furthermore, significantly more cells in the dermis and epidermis of K14E7 mice produced IL-17 compared with wild-type dermis and epidermis (Fig. 2C, 2D). Whereas numbers of IL-17–producing cells were similar between dermis and epidermis of

**FIGURE 1.** Increased production of IL-17 in human CIN2/3 compared with control tissue. (A and B) IL-17 and IL-23A mRNA expression in human CIN2/3 and control cervical tissue from the same patient, measured by quantitative real-time PCR (\( n = 12 \) patients). (C and D) Flow cytometric analysis of cells isolated from CIN2/3 lesions and control tissue, unstimulated or stimulated with PMA and ionomycin. Numbers shown in (C) indicate the percentage of CD45+IL-17+ cells, or of cells treated with isotype Ab, of total live cells for one patient (representative analysis). The results obtained for four to five patients are summarized in (D). (In one of five patients, unstimulated cell suspensions contained too few IL-17+ cells to perform a meaningful analysis. Data not shown.) Groups were compared by Wilcoxon test; ***\( p < 0.001 \). n.s., not significant.
wild-type mice, significantly more IL-17–producing cells were detected in K14E7 transgenic epidermis compared with dermis from K14E7 mice. Together, these results demonstrate elevated production of IL-17 in HPV-associated premalignant disease in humans and a mouse model of HPV-induced hyperplasia.

**FIGURE 2.** Higher production of IL-17 and IL-23 in K14E7 compared with wild-type mouse skin. (A) IL-17 concentration in supernatants of C57BL/6 and K14E7 ear skin explants following 20 h of culture, assessed by ELISA (n = 4 mice per group). In supernatants from wild-type skin explant culture, IL-17 concentrations were below the detection limit of the assay (n.d., not detectable). Results shown are representative of two independent experiments. (B) IL-23A mRNA expression in C57BL/6 and K14E7 ear skin as determined by quantitative real-time PCR (n = 4 mice per group). IL-23 concentration in supernatants of C57BL/6 and K14E7 ear skin explants following 20 h of culture, assessed by ELISA (n = 4 mice per group). Results shown are representative of two independent experiments. (C and D) Flow cytometric analysis of cells isolated from dermis and epidermis of C57BL/6 and K14E7 mice, unstimulated or stimulated with PMA and ionomycin. Total live cells were gated for CD45.2+IL-17+ cells. Numbers in (C) indicate the numbers of CD45.2+IL-17+ cells per plot. Plots in (C) are representative of four independent experiments. The total numbers of CD45.2+IL-17+ cells in wild-type and K14E7 dermis and epidermis obtained from four independent experiments (n = 2 mice per strain per experiment) were calculated using Flow Count Fluospheres and are summarized in (D). Bars indicate the mean ± SEM. Groups were compared by unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.001.
IL-17 expression in K14E7 skin grafts suppresses an effective E7-directed immune response

Despite expressing a non-self Ag, skin transgenic for the HPV16 E7 protein is not rejected when transplanted onto otherwise syngeneic, nontransgenic recipient mice (24), most likely due to a local immunosuppressive environment. To functionally address the role of IL-17 production by K14E7 skin in K14E7 graft survival, we grafted E7 transgenic IL-17-deficient (E7⁻IL-17⁻) skin onto C57BL/6 recipients. As a control, skin from E7-negative IL-17⁰/⁰ recipients (Fig. 3B), confirming that local IL-17 production is key to regulating local immune effector function.

We have previously demonstrated an immunosuppressive role for IFN-γ in K14E7 skin grafts (25). As we obtained similar results for IL-17 in the current study, we sought to investigate whether or not there is a possible link between IFN-γ and IL-17 production in K14E7 skin. To this end, we neutralized IL-17 in K14E7 skin explant cultures with an anti–IL-17 mAb and observed no reduction in IFN-γ secretion (Fig. 3C). Furthermore, E7⁺IL-17⁻/⁻ and E7⁺IL-17⁺/⁺ control skin explants secreted similar amounts of IFN-γ (Fig. 3D), and E7⁺IFN-γ⁻/⁻ and E7⁺IFN-γ⁺/⁺ control skin explants produced similar amounts of IL-17 (Fig. 3E). These findings demonstrate that IL-17 and IFN-γ production in K14E7 skin are not directly linked, although coregulation of their production is not excluded, as absence of either is permissive for graft rejection.

**CD4 and γδ T cells in K14E7 skin, and CD4 T cells in human CIN are the predominant source of IL-17**

To identify the cellular sources of IL-17 in K14E7 skin, IL-17-expressing cells detected by intracellular flow cytometry were analyzed for expression of hematopoietic non-T cell (CD3⁻) and T cell (CD3⁺) markers (Fig. 4A, 4B). The majority of IL-17-producing cells in K14E7 skin were CD3⁺ T cells (epidermis, 85.8% ± 7.1%; dermis, 93.5% ± 2.4%). Among these, the largest populations of IL-17-producing cells were γδ T cells, with intermediate expression levels of CD3 and γδ TCR (referred to as CD3intγδ TCR⁺; epidermis, 32.6% ± 5.4%; dermis, 49.1% ± 4%) and CD4 T cells (epidermis, 19.9% ± 2.2%; dermis, 20% ± 3.1%). Low numbers of epidermal γδ T cells, a T cell population exclusively found in mouse epidermis and

**FIGURE 3.** IL-17 in K14E7 grafts suppresses graft rejection. (A) Kaplan–Meier survival curves of E7⁺IL-17⁻/⁻ (median graft survival: 15 d) and littermate control E7⁺IL-17⁺/⁺ and E7⁻IL-17⁻/⁻ grafts on C57BL/6 recipients. Numbers of donor and recipient mice per group are indicated. Pooled results from three independent experiments. (B) IFN-γ concentrations in the supernatants of K14E7 transgenic IL-17⁺/⁺ (n = 3 mice) and E7⁺IL-17⁻/⁻ (n = 3 mice) skin explants, measured by ELISA. Pooled results from four independent experiments. Bars indicate the mean ± SEM. (C) IFN-γ concentrations in the supernatants of K14E7 transgenic IL-17⁺/⁺ (n = 9 mice), IL-17⁻/⁻ (n = 11 mice), and IFN-γ⁻/⁻ (n = 3 mice; n.d., not detectable) skin explants, measured by ELISA. Pooled results from four independent experiments. Bars indicate the mean ± SEM. Groups were compared by paired t test. (D) IFN-γ concentration in the supernatants of K14E7 transgenic IL-17⁺/⁺ (n = 9 mice), IL-17⁻/⁻ (n = 11 mice), and IFN-γ⁻/⁻ (n = 3 mice; n.d., not detectable) skin explants, measured by ELISA. Pooled results from four independent experiments. Bars indicate the mean ± SEM. Groups were compared by paired t test. (n.s., not significant (p ≥ 0.05).
characterized by high CD3 and γδ-T cell expression (referred to as CD3highγδ-TCRhigh), CD8+ T cells, and invariant NKT cells were also found to produce IL-17 in K14E7 skin (Fig. 4A, 4B). IL-17 production in K14E7 transgenic γδ-T cell–deficient skin was reduced but not abolished compared with γδ T cell–replete skin (Supplemental Fig. 1A). Furthermore, K14E7 transgenic γδ T cell–deficient skin grafts were not rejected by nontransgenic recipients (Supplemental Fig. 1B), demonstrating that γδ T cells were not essential for K14E7 graft survival, and that IL-17 levels in the absence of γδ T cells were sufficient to allow for graft survival.

Similar to mouse E7 transgenic skin, IL-17–producing cells in human CIN2/3 lesions were mostly CD3+ T cells (89.0% ± 3.9%) (Fig. 4C, 4D). CD4+ T cells were the major producers of IL-17 in CIN2/3 lesions (58.2% ± 12.2%), whereas only a few γδ T cells produced IL-17 (Fig. 4C, 4D).

IL-17 production in K14E7 skin is induced by IL-23 and IL-1β, but not IL-18.

IL-23, IL-1β, and IL-18 are known inducers of IL-17 (8, 26). K14E7 skin expressed elevated levels of IL-23 (Fig. 2) and IL-18.
IL-17 production in hyperplastic, premalignant CIN2/3 lesions compared with control specimens in our study is corroborated by reports on elevated IL-17 concentrations in cervical secretion specimens of women with persistent high-risk HPV infection compared with specimens from HPV-negative controls (15), as well as increased numbers of IL-17+ cells in uterine cervical cancer and CIN tissue compared with cervical tissue from healthy controls (16). Baseline cytokine expression in human cervical tissue differs between individuals most likely due to genetic, environmental, and hormonal differences. To control for such variables in our study, we compared premalignant and histologically normal cervical specimens from the same patients, excluding an impact of subject-specific differences in baseline IL-17 expression between premalignant and control specimens. This observation contrasts with previous studies in which control specimens were sourced from unrelated healthy subjects (15, 16).

Persistent infection of basal epithelium with high-risk HPV is associated with the expression of the HPV protein E7, which drives epithelial hyperproliferation (28). To model HPV-associated disease in mice, which are not infected by HPV, transgenic mice that express HPV16 E7 under control of the K14 promoter in basal epithelial cells (designated K14E7) were used (17). These mice exhibit epithelial hyperplasia that closely models premalignant lesions in chronic HPV infection (17). Similar to our findings in human HPV-infected, hyperplastic cervical tissue, E7 transgenic, hyperplastic skin featured higher IL-17 production and elevated numbers of IL-17+ producing cells compared with wild-type skin. Furthermore, production of IL-23, which is a major inducer of IL-17, is produced at similar, low levels in both (Supplemental Fig. 2). To test a possible contribution of IL-23, IL-1β, and IL-18 to induction of the enhanced IL-17 production in K14E7 skin, we blocked cytokine function by addition of neutralizing mAbs to IL-23p19, IL-12/23p40, IL-1β, and IL-18 to skin explant culture medium (Fig. 5). Neutralization of IL-23p19, IL-12/23p40 (Fig. 5A), and IL-1β (Fig. 5B) led to a significant reduction of IL-17 concentrations compared with isotype Ab-treated explants of the same K14E7 mice. In contrast, neutralization of IL-18 did not affect IL-17 production (Fig. 5C). Together, these results indicate that IL-23 and IL-1β, but not IL-18, contribute to production of IL-17 in K14E7 skin.

We next sought to investigate whether IL-23 or IL-1β expressed in K14E7 skin is required for K14E7 graft survival. To this end, skin from K14E7 transgenic IL-12/23p40−/− mice, K14E7 transgenic IL-1R1−/− mice, and K14E7 transgenic littermate controls heterozygous for IL-12/23p40 or IL-1R1 was grafted onto wild-type recipients. None of the grafts were rejected (Fig. 6A, 6B), suggesting that IL-12/23p40 or IL-1R1 signaling alone, although contributing to IL-17 induction in K14E7 skin, is not sufficient to suppress an effective E7-directed immune response, most likely due to the residual IL-17 production observed in the E7 transgenic grafts (Fig. 6C).

**Discussion**

Local immunosuppression is considered a major contributing factor for persistent infection of epithelial tissues with high-risk HPV and associated epithelial hyperplasia (5), which is an essential step in the development of HPV-associated cancers (28). IL-17 is a pleiotropic cytokine whose role in HPV-associated premalignant disease is unknown. This study identifies IL-17 as a critical immunosuppressive determinant in HPV-associated, epithelial hyperplasia.

The observed increased IL-17 production in hyperplastic, premalignant CIN2/3 lesions compared with control specimens in our study is corroborated by reports on elevated IL-17 concentrations in cervical secretion specimens of women with persistent high-risk HPV infection compared with specimens from HPV-negative controls (15), as well as increased numbers of IL-17+ cells in uterine cervical cancer and CIN tissue compared with cervical tissue from healthy controls (16). Baseline cytokine expression in human cervical tissue differs between individuals most likely due to genetic, environmental, and hormonal differences. To control for such variables in our study, we compared premalignant and histologically normal cervical specimens from the same patients, excluding an impact of subject-specific differences in baseline IL-17 expression between premalignant and control specimens. This observation contrasts with previous studies in which control specimens were sourced from unrelated healthy subjects (15, 16).

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We have previously shown that immunocompetent hosts fail to reject K14E7 transgenic skin grafts (24), and that this is not dependent on Foxp3+ regulatory T cells (29). In this study, we demonstrate that IL-17 produced in K14E7 skin grafts suppresses...
graft rejection. Reduced levels of IL-17 in E7 transgenic grafts deficient in IL-12/23p40, IL-1R1, or γδ T cells were still sufficient to protect against graft rejection, indicating a strong potency of this cytokine in inhibiting host effector functions against E7 as a foreign Ag. These findings in the mouse model of HPV-induced hyperplasia suggest similar immunosuppressive functions for IL-17 in HPV-associated premalignant disease in humans. This hypothesis is further supported by studies reporting associations of polymorphisms in the genes encoding IL-17 and IL-12/23p40 with the phenotype, characterized by intermediate expression of CD3 and CD4 T cells. Notably, the majority of IL-17–producing cells were detected in the K14E7 transgenic epithelium, suggesting an IL-17–associated recruitment of myeloid cells and their subsequent development into MDSC as a possible mechanism responsible for impaired host effector T cell responses to K14E7 skin grafts, particularly as IDO expression is elevated in CIN2/3 lesions, and in K14E7 skin, and contributes to local immune regulation (43). However, numbers of CD11b+/Gr-1int MDSC were similar in K14E7 and E7+IL-17/− grafts (Supplemental Fig. 3), indicating that recruitment of MDSC into E7+ skin is IL-17 independent.

Similar to our findings for IL-17, we have previously reported that IFN-γ production in K14E7 skin had immune-suppressive effects in the skin-grafting model (25). This suggested that IL-17 and IFN-γ production in K14E7 skin might be functionally linked, as previously shown in other studies. The successful chemotherapeutic treatment of some transplantable and induced tumor models in mice required both IL-17 and IFN-γ, with IL-17 production preceding IFN-γ production (44, 45). In this study, however, we demonstrate that IFN-γ production in K14E7 skin is independent of IL-17 and vice versa. This conclusion is further corroborated by the finding that, in K14E7 skin, IL-18 is required for production of IFN-γ, but not IL-17, whereas IL-23 and IL-1β induce IL-17, but not IFN-γ production (Fig. 5) (27). The IL-17–mediated immune suppression thus represents a novel, IFN-γ–independent mechanism of immune suppression in HPV-associated hyperplasia.

The major producers of IL-17 in K14E7 skin were CD3int γδ-TCR−/− γδ T cells and CD4 T cells. Notably, the majority of IL-17–producing cells were detected in the K14E7 transgenic epidermis, despite IL-17–producing γδ T cells displaying a dermal phenotype, characterized by intermediate expression of CD3 and γδ-TCR (46). We hypothesize that HPV16 E7–induced hyperproliferation of epithelial cells is associated with the production of cytokines and chemokines that specifically recruit inflammatory treatments.

FIGURE 6. IL-1 and IL-23 are not required for survival of K14E7 skin grafts. Kaplan–Meier survival curves of (A) E7+IL-12p40+/− and littermate control E7+IL-12p40+/+ grafts and (B) E7+IL-1R1+/− and littermate control E7+IL-1R1+/+ grafts on C57BL/6 recipients. Numbers shown indicate the numbers of donor and recipient mice. Pooled results from three and two independent experiments, respectively. (C) IL-17 concentrations in the supernatants of K14E7 transgenic IL-12p40−/− (n = 6 mice), IL-12p40−/− (n = 6 mice), IL-1R1−/− (n = 6 mice), IL-1R1−/− (n = 5 mice), IL-17−/− (n = 6 mice), and IL-17−/− (n = 5 mice) skin explants, measured by ELISA. Pooled results from two independent experiments per group. Bars indicate the mean ± SEM. Groups were compared by unpaired t test; *p < 0.05, **p < 0.01. n.s., not significant.
cells to the epidemics. These include T cells that express CCR6 (47), a chemokine receptor known for its role in epidermal trafficking (48). In line with this, the migration of dermal γδ T cells to the epidemics in an IL-23–induced mouse model of psoriasiform dermatitis, which also features a hyperplastic epithelium, was also mediated by CCR6 (48). It is conceivable that similar mechanisms mediate the recruitment of inflammatory cells to human hyperplastic cervical tissue. Similar to E7 transgenic skin, in human cervical HPV-associated premalignant lesions, CD4+ T cells and γδ T cells produced IL-17; however, CD4+ T cells were more prominent among the IL-17–producing cell population. This finding is corroborated by a recent study reporting increased numbers of CD4+IL-17+ cells in CIN and cervical cancer compared with control cervical tissue from healthy donors (16).

As the E7 transgene is expressed as an autoantigen in the thymus of K14E7 mice (17), IL-17 production by T cells in K14E7 skin is expected to be E7 Ag independent. Furthermore, the IL-17 production in K14E7 skin occurred downstream of IL-23 and IL-21. Ag-independent IL-17 production by memory CD4+ T cells and by γδ T cells has been previously described and required IL-23 in combination with IL-1β (49, 50). More recently, γδ T cells purified from lymph nodes of mice with experimental autoimmune encephalitis were also found to produce IL-17 following stimulation with IL-1β or IL-18 in combination with IL-23 (9). In E7 transgenic, hyperplastic skin, however, IL-18 was not required for IL-17 production, reinforcing the idea of IL-17 production being specifically regulated according to tissue and cellular origin.

In summary, our results demonstrate that IL-17 mediates immunosuppression in HPV-associated epithelial hyperplasia, suggesting that blocking of IL-17 could be of therapeutic use in persistent infection with high-risk HPV and thus aid prevent progression of premalignant lesions to cancer.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1: γδ T cells are not required for K14E7 graft survival. A IL-17 concentrations in the supernatants of K14E7 transgenic TCRδ+/− (n=5 mice), TCRδ−/− (n=7 mice) and IL-17−/− (n=3 mice, n.d.: not detectable) skin explants, measured by ELISA. Pooled results from 2 independent experiments. Bars indicate the mean±SEM. Groups were compared by unpaired t test; n.s.: not significant (p≥0.05). B Kaplan-Meier survival curves of E7+TCRδ+/− and littermate control E7+TCRδ+/− grafts on E7TCRδ+/− littermate recipients. Numbers shown indicate the numbers of donor and recipient mice. Pooled results from 3 independent experiments.
Supplemental Figure 2: Similar levels of mature IL-1β in wild type and K14E7 skin. A IL-1β concentration in supernatants of C57BL/6 and K14E7 ear skin explants following 20 h of culture, assessed by ELISA (n=4 mice per group). Results shown are representative of two independent experiments. B Western Blot showing pro-IL-1β (31 kDa) and mature IL-1β (17 kDa) in skin lysates of individual wild type (n=2) and K14E7 (n=3) mice. The same blot was probed for β-actin as a loading control. Signal intensities of immature and mature IL-1β are shown relative to signal intensities for β-actin, normalized to wild type. Lines indicate means±SEM. Groups were compared by unpaired t test; n.s.: not significant (p≥0.05).
Supplemental Figure 3: Similar numbers of CD11b^+Gr-1^{int} cells in K14E7 and E7^{IL-17^-/-} grafts. Skin of K14E7 (E7^{IL-17^{+/+}}) and E7^{IL-17^-/-} mice (n=3 donors per group) was grafted onto C57BL/6 recipients (n=6 recipients per group). Grafts were collected 11 days post grafting and cell suspensions prepared for flow cytometric analysis. A CD45.2^+ cells were gated for CD11b^+Gr-1^{int} cells. B CD11b^+Gr-1^{int} cells from grafted tissue were quantified as percentage of CD45.2^+ cells and as numbers of cells per mg tissue. Bars indicate means±SEM. Groups were compared by unpaired t test; n.s.: not significant (p≥0.05).