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Antigen Nonspecific Induction of Distinct Regulatory T Cell States in Oncogene-Driven Hyperproliferative Skin

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ABSTRACT
Regulatory T cells (Tregs) are recruited to nonlymphoid tissues in chronic disease, including cancer, and the tissue environment is held to shape the Treg phenotype diversity. Using single-cell RNA sequencing, we examined the transcriptomic and TCR profile of Tregs recruited to hyperproliferative HPV16 E7–expressing transgenic and control nontransgenic murine skin grafts. Tregs were more abundant in E7 transgenic skin grafts than control grafts, without evidence of E7 specificity. E7 transgenic grafts attracted both Klrg1* Tregs and Il1r2* Tregs, which were phenotypically distinct but shared a core gene signature with previously described tumor-infiltrating Tregs. Pseudotime trajectory analysis of Tregs of defined TCR clonotypes predicted phenotypic plasticity within the skin and between the skin and draining lymph nodes. Thus, oncogene-induced hyperproliferative skin expressing a single defined non–self-antigen can attract and induce non–Ag-specific Tregs that acquire distinct regulatory phenotypes characterized by specific effector gene signatures. ImmunoHorizons, 2021, 5: 102–116.

INTRODUCTION
Regulatory T cells (Tregs) are a heterogeneous group of T cells that play a crucial role in maintaining immune homeostasis. At barrier sites such as mucosa and skin, Tregs are present in increased frequencies (1) and express tissue-specific features (2) compared with their lymphoid organ counterparts, reflecting their key modulatory function under exposure to commensal microbiota and other innocuous or harmful environmental factors. Tregs also play a detrimental role in various types of infectious diseases and cancers. Patients with “high-risk” human papillomavirus (HPV)–associated diseases, such as cervical intraepithelial neoplasia III and cervical cancer, harbor increased frequencies of Tregs in peripheral blood and local lesions compared with patients who have cleared the infection (3, 4). Furthermore, Tregs specific for high-risk human papillomavirus oncoprotein Ags E6 and E7 are detected in cervical tumors and draining lymph nodes (DLNs) and were capable of suppressing proliferation and cytokine production of effector T cells ex vivo (5, 6). These findings suggest that a Treg response represents one
mechanism to suppress adaptive immunity against HPV. Phenotyping Tregs in HPV-induced lesions may thus guide the development of an effective immunotherapeutic regimen for patients with HPV-mediated diseases.

Recent advances in single-cell RNA-sequencing (scRNA-seq) technology have greatly extended our capacity to study the phenotype of tissue-infiltrating Tregs. scRNA-seq of Tregs from healthy mice reveals that colon- and skin-resident Tregs acquire a nonlymphoid tissue (NLT) gene signature and group into distinct subpopulations based on their degrees of tissue adaptation (7). scRNA-seq of Tregs in human tumors has demonstrated that tumor-infiltrating Tregs acquire tumor-specific features that are not observed in their normal-tissue counterparts (7–10). Nevertheless, many of these studies did not obtain a sufficient number of Tregs from pathological tissues to fully unravel their heterogeneity and Ag specificity.

To explore the phenotype and Ag specificity of Tregs in the context of HPV-induced lesions, we used a murine model expressing HPV16 E7 driven by the keratin 14 promoter (K14E7) to induce single oncogene-driven epithelial hyperplasia. K14E7 skin demonstrates an infiltration of T cells but is not rejected when being grafted onto immunocompetent nontransgenic mice, recapitulating HPV-induced premalignancy in human subjects (11, 12). We have previously shown that immunization in K14E7 skin induces expansion of regulatory and exhausted helper T cells (13). In this study, we examine single-cell transcriptomic and TCR profiles of skin graft–infiltrating T cells and compare those of K14E7 skin grafts with those of nontransgenic C57BL/6 skin grafts, demonstrating hyperplasia-associated–specific Treg phenotypes and plasticity within Treg TCR clonotypes. We further compared gene signatures of skin graft Tregs with those of Tregs previously reported across a range of tissues, including tumor, spleen, colon, and thymus, demonstrating overlapping transcriptomic features among these Tregs.

**MATERIALS AND METHODS**

**Mice**

K14E7 mice on an FVB background (14) were backcrossed to C57BL/6 mice for at least 12 generations for experiments and were obtained from the Animal Resource Centre (Perth, Australia). C57BL/6 and B6.SJL-Ptprca mice were also obtained from the Animal Resource Centre (Perth, Australia). All mice were bred and maintained in clean and specific germ–free conditions at the Translational Research Institute, Biological Resources Facility (Brisbane, Australia). For experimental work, female mice were used at 8–12 wk of age. All animal experiments and procedures were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia and approved by the University of Queensland Animal Ethics Committee (UQDI/367/13/NHMRC and UQDI/482/16).

**Skin grafting and sample processing**

Skin grafting was performed as previously described (11, 15). Briefly, skin graft recipients (B6.SJL-Ptprca) were shaved on the left thoracic flank region. Shaved skin was excised in the size that matches the donor skin. Ears from the donor mice (K14E7 and C57BL/6) were split into dorsal and ventral parts and grafted onto seven recipient mice for each donor group. Each recipient mice received two grafts that were placed side by side on the flank. After 28 d, skin grafts were well healed without macroscopic signs of inflammation. We collected skin grafts and axillary DLNs from each recipient. Skin grafts and DLNs from seven recipient mice were pooled for each genotype and tissue. Samples were cut into pieces using surgical scissors and then enzymatically digested with 1 mg/ml of collagenase D (Roche) and 0.2 mg/ml of DNase I (Thermo Fisher Scientific) for 1 h at 37°C. Digested samples were passed through a 70 μm filter (BD Falcon, Franklin Lakes, NJ) to generate a single-cell suspension for staining and cell sorting.

**FACS**

Single-cell suspensions of digested skin grafts and DLN samples were incubated with Fc Block (dilution 1:100; BD Pharmingen) diluted in PBS for 20 min on ice. Samples were subsequently incubated with PerCP-Cy5.5–conjugated rat anti-mouse CD45 Abs (clone 30-F11, dilution 1:200; BioLegend) and FITC-conjugated rat anti-mouse TCR β-chain Ab (clone H57-597, dilution 1:200; BioLegend) diluted in PBS plus 2% PBS plus 2 mmol/l EDTA for 30 min on ice. Before sorting, cells were labeled with propidium iodide (dilution 1:10,000) for viability staining. Live CD45+ cells of skin grafts and live CD45+ TCRβ+ cells of DLNs were sorted into 100% FBS containing 2 mmol/l EDTA using the BD FACSaria Fusion sorter. A total of 45,000 cells and a total of 15,000 cells were collected from K14E7 and C57BL/6 skin grafts, respectively. A total of 200,000 cells were collected from each DLN sample. Samples were kept on ice throughout the experiment prior to loading onto the 10x Genomics Chromium Controller.

**scRNA-seq and TCR V(D)J sequencing**

The 10x Genomics Chromium Controller was used to partition each single cell with barcoded beads. This was followed by generation of single-cell barcoded 5′ cDNA library using the Chromium Single Cell 5′ Library and Gel Bead Kit (PN-1000014; 10x Genomics) per the manufacturer’s instructions. cDNAs were used for both RNA-sequencing (RNA-seq) library generation and TCR V(D)J-targeted enrichment followed by V(D)J library generation using the Chromium Single Cell V(D)J Enrichment Kit, Mouse T Cells (PN-100007; 10x Genomics) per the manufacturer’s instructions. Libraries for RNA-seq and V(D)J libraries were sequenced on the Illumina NextSeq500 platform using a 150 cycle and 300 cycle High Output Kit (Illumina), respectively. 5′ RNA-seq libraries obtained a minimum read depth of ~20,000 read pairs per cell. V(D)J-enriched libraries obtained a minimum read depth of ~5000 read pairs per cell.

**Preprocessing of scRNA-seq data**

The Illumina sequencing output was processed using the 10x Genomics Cell Ranger Software (v3.0.2). The command “cellranger mkfastq” was used to demultiplex raw binary base call files and generate FASTQ files for individual samples. The command
“cellranger count” was used to align RNA-seq reads to the prebuilt mouse reference genome GRCm38 (mm10) and to filter out low-quality cell barcodes and unique molecular identifiers. The resulting single-cell gene count matrices were further processed using the Seurat R package (v3.0.5) (16). We filtered out transcripts that were present in fewer than three cells. We removed outlier cells with fewer than 200 transcripts or more than 3800 transcripts. Cells expressing more than 10% mitochondrial genes were also excluded from downstream analysis. Cell–cell gene expression variation was normalized using the “LogNormalize” method with a scaling factor of 10,000 as implemented in the Seurat pipeline.

Gene expression datasets of individual samples were integrated using the standard Seurat integration workflow to correct batch effect. We generated two integrated datasets based on tissue of origin. The skin graft dataset consisted of gene expression profiles of 6779 cells from K14E7 skin grafts and 4816 cells from C57BL/6 skin grafts. The DLN dataset consisted of gene expression profiles of 5374 cells from K14E7 DLNs and 7585 cells from C57BL/6 DLNs. For each integrated dataset, principal component analysis was performed using the RunPCA function in Seurat. The first 20 principal components were used to explain the majority of variance in the dataset and to construct a k-nearest neighbor graph using the FindNeighbors function. Cells with similar gene expression profiles were clustered together using the Louvain algorithm in the FindClusters function at an optimal resolution (0.5 for clustering of CD45+ cells and T cells; the Louvain algorithm in the FindClusters function at an neighbor graph using the FindNeighbors function. Cells with the majority of variance in the dataset and to construct a in Seurat. The

Separation and annotation of T cells
We first identified superclusters using known markers characteristic to T cells, B cells, NK cells, and APCs. Cells in T cell but not in B cell, NK cell, and APC superclusters were considered as T cells and subsetted for further analysis. T cells that did not have TCR clonotype information were removed from the datasets. To annotate T cell clusters, we mainly relied on prior knowledge of representative T cell activation and differentiation features. Gene set enrichment analysis (see Gene set enrichment analysis) was applied to validate the annotations. For T cell clusters that did not express cell type-specific features, we performed differential expression gene analysis using a Wilcoxon rank-sum test implemented in the Seurat pipeline to explore highly expressed genes in these clusters. Highly expressed genes were considered significant if the Bonferroni-adjusted p value was below the threshold of 0.001 and the absolute log expression fold change was 0.25 or greater. To annotate NKT cells and MAIT cells, we analyzed their TCR clonotype information to test whether they expressed invariant TCR α-chains with the highly conserved CDR3 region, as indicated by previous literatures (17, 18).

Preprocessing of single-cell TCR V(D)J-sequencing data
Single-cell TCR (scTCR) V(D)J-sequencing data for each sample was processed using Cell Ranger software with the command “cellranger vdj” to perform sequence assembly and paired clonotype calling. For each sample, Cell Ranger generated an output file containing V(D)J information and CDR3 sequences of the TCR α- and β-chain for individual cells. We filtered out cells that did not contain full length and productive TCR α- and β-chain information based on “TRUE/FALSE” variables in the output files. We also excluded cells that expressed more than one distinct TCR α- or TCR β-chains; they are potential doublets and are likely to cause false clonotype counts in the downstream analysis, although the removal of these cells might also remove true singlets. Each cell was allocated to a clonotype defined as a unique combination of CDR3 nucleotide sequences from paired TCR α- and TCR β-chain. Finally, scTCR V(D)J-sequencing data were integrated with scRNA-seq data by matching the cell barcodes in the TCR clonotype tables and the gene expression matrices.

Gene set enrichment analysis
Gene set enrichment analysis was performed using the AUCell package (19). AUCell calculates the area under the curve score for each cell to determine whether the input gene signature is enriched within the top-ranking genes (ordered by their expression values) for each cell. Gene sets of naïve CD4⁺ T cells, Th1, Th2, Th17, and Treg cells were downloaded from the supplemental document named Additional File attached to the published RNA-seq study on CD4⁺ T cells (20). Gene sets of CD4⁺ effector and exhausted T cells were downloaded from Gene Expression Omnibus (GEO) DataSets (GSE30431). For details, a gene set (268 genes) of CD4⁺ effector T cells was obtained from differential expression analysis using the GEO2R tool by comparing “CD4 D8 Acute” datasets (GSM754811, GSM754812, GSM754813) to “CD4 naïve” datasets (GSM754804, GSM754805, GSM754806). A gene set (223 genes) of CD4⁺ exhausted T cells was obtained from differential expression analysis using GEO2R tool by comparing “CD4 D30 Chronic” datasets (GSM754835, GSM754836, GSM754837) to “CD4 naïve” datasets (GSM754804, GSM754805, GSM754806). Differentially expressed genes that had >2-fold increase and the adjusted p value <0.05 were used in our gene set enrichment analysis. The “tisTregST2” signature was extracted from Fig. 4B of the published study on mouse tissue-resident Tregs (21). The tumor-infiltrating Treg signature was obtained from the published tumor study (10), which summarized a list of conserved 31 Treg genes across four tumor studies in liver (10), melanoma (22), breast (9), colon, and lung cancers (23). Gene sets of six murine splenic Treg states were obtained from the published scRNA-seq study (24). For all gene sets used in this analysis, we selected upregulating genes with a false discovery rate <0.05 in each gene set. The gene set of Klrg1⁺ graft-infiltrating Tregs was based on single-cell expression data and obtained from differential expression analysis using the Seurat pipeline by comparing skin graft Treg states 2–6 to skin graft Treg states 1 and 7. Similarly, the gene set of Il1r2⁺ graft-infiltrating Tregs was obtained by comparing skin graft Treg state 7 to skin graft Treg states 1–6. Genes that have an adjusted p value <0.001 and the log
expression fold change >0.25 were used in gene set enrichment analysis.

**Random resampling trial**

Random resampling trials were conducted when calculating T cell composition (Fig. 2A) and TCR diversity (Fig. 2B) to compensate the unequal sample size between K14E7 (2883 cells) and C57BL/6 (2145 cells) skin graft T cells. To calculate T cell composition, we set up 85% of the total cell number in C57BL/6 skin graft sample (0.85*2145 = 1824) as a fixed number. We randomly sampled 1824 cells from each of K14E7 and C57BL/6 skin graft samples and used these randomly sampled cells to calculate the percentage of each T cell cluster in each sample. Random sampling was performed 256 times, from which we obtained 256 sets of values. The mean and the SEM were then calculated and plotted using GraphPad Prism.

To calculate TCR diversity of each T cell cluster, we set up 85% of the total cell number in the T cell clusters with the lowest sample size as a fixed number (29 cells). We randomly sampled 29 cells from each T cell cluster and used these randomly sampled cells to calculate the ratio between clonotype number and total cell number. Random sampling were performed 10,000 times, from which we obtained 10,000 values. The mean and the SEM of these 10,000 values was calculated and plotted using GraphPad Prism.

**Pseudotime trajectory inference**

We subsetted all Tregs of K14E7 and C57BL/6 skin grafts and integrated these Tregs with DLN T cells that had clonotypes overlapped with clonally expanded Tregs in skin grafts. The integrated dataset served as input to Monocle 2 analysis (25, 26) to construct a DDRTree-based pseudotime trajectory. The pseudotime trajectory is Monocle-based, allowing the visualization of Treg clones on the trajectory. The same approach was used when displaying individual Treg clones on the trajectory.

**Gene coexpression network analysis**

Gene coexpression network analysis was performed using the bigScale 2 package (27). Basically, gene expression counts were transformed to Z-scores, which allows the measure of both linear and nonlinear correlations by simply calculating the Pearson correlation coefficients for each pair of genes. The top 0.25% correlated gene–gene networks were retained for visualization using Cytoscape software (v 3.6.1).

**Cell–cell communication analysis**

We constructed an integrated dataset consisting of single-cell gene expression profiles of both keratinocytes (KCs) and CD45+ immune cells in the context of E7-associated epithelial hyperplasia. Single-cell gene expression profiles of KCs were derived from the published scRNA-seq study on K14E7 and C57BL/6 ear skin (28). Single-cell gene expression profiles of CD45+ immune cells were generated in this study. We used the “merge” function in Seurat to integrate two datasets (log normalization included in the function) and used the “SubsetData” function to retain K14E7-derived but not C57BL/6-derived cells. For each ligand gene (Ccll, Ccl8, Ilt3, Il1b, Pvr, Nectin2, Tnfsf9) in Fig. 4A, we calculated the following: 1) the percentage of cells in each cluster (KC, APC, NK, and different T cell subclusters) in the integrated dataset that had >0 expression and 2) the average expression in cells that had >0 expression. The results were visualized in the heatmap using ggplot2 R package.

**Reanalysis of published scRNA-seq data of murine splenic, colon, and thymic Tregs**

The raw count matrix of splenic Tregs was downloaded from GEO DataSets (GSE110547) and processed using the standard Seurat pipeline as described above. Two independent sets of scRNA-seq data of murine colon Tregs were downloaded from https://figshare.com/projects/Treg_scRNA-seq/38864 and GEO DataSets (GSE110558), respectively, and were integrated with our skin Treg data using the standard data integration workflow in Seurat with default settings. The raw count matrix of murine thymic Tregs was downloaded from GEO DataSets (GSM3978655 in GSE134902) and processed using the standard Seurat pipeline.

**Statistical analysis**

Analysis of data containing two groups was performed using unpaired multiple t tests with the Holm–Šidák adjustment method. Analysis of data containing three or more groups was performed using unpaired one-way ANOVA with Tukey multiple-comparison post hoc test with a 95% confidence interval (Prism; GraphPad Software). Levels of significance are as follows: NS, \( p \geq 0.05 \), \( *p < 0.05 \), \( **p < 0.01 \), \( ***p < 0.001 \), and \( ****p < 0.0001 \).

**Data availability**

The accession number for the sequencing data reported in this paper is GSE156745 (https://www.ncbi.nlm.nih.gov/geo/).

**RESULTS**

**K14E7 skin grafts selectively attract diverse lymphoid cells**

To define how the E7-expressing hyperplastic epithelium directs the local phenotypic and clonotypic landscape of intraepithelial T cells and, specifically, Tregs, we collected the single-cell transcriptomic profile and TCR sequence of CD45+ cells in K14E7 skin grafts (6779 cells) and nontransgenic C57BL/6 skin grafts (4816 cells) (Fig. 1A). Comparison of transgenic and nontransgenic skin grafts, rather than skin of transgenic and nontransgenic animals, allows analysis of immune cells originating from animals whose immune repertoire has not been altered by transgene expression in the thymus (29). We similarly collected data from T cells in graft DLNs, to allow analysis of TCR clonotypes common between the skin graft and the DLN. Skin
grafts and DLNs were harvested 28 d after grafting, allowing the graft to heal beyond surgery-related inflammation and to establish immune modulation.

Using the Seurat R package (16) as a bioinformatics pipeline, we performed unsupervised clustering of skin graft CD45+ cells based on computed similarity of their transcriptomic profiles. We observed 4 superclusters (Fig. 1B) comprising T cells, B cells, NK cells, and APCs expressing marker genes for T (Cd3e), B (Cd19), NK (Fcgr3, Klrb1a, and Gzmb) cells, and APCs (H2-Aa and Fcgr3) (Fig. 1C). We integrated the single-cell gene expression data for the T cells with the TCR α- and β-chain sequences. After excluding T cells with low-quality TCR information, we obtained 5028 TCRαβ+ T cells (2883 cells from K14E7 skin grafts and 2145 cells from C57BL/6 skin grafts) that were divided into nine clusters (Fig. 1D). To annotate these clusters, we selected a list of representative T cell activation and differentiation features and investigated their

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**FIGURE 1.** K14E7 and C57BL/6 skin grafts attract diverse lymphoid cells.

(A) Graphical overview of the experimental setting. Single immune cells were collected from K14E7 and C57BL/6 skin grafts and DLNs 28 d after grafting for scRNA-seq and scTCR V(D)J sequencing. (B) Unsupervised clustering on 11,595 CD45+ cells from K14E7 and C57BL/6 skin grafts. Four superclusters were identified, including T cells, APCs, NK cells, and B cells. (C) Expression levels of key immune cell type-related genes across four CD45 superclusters were visualized. The gray color indicates zero expression. (D) Unsupervised clustering on 5028 TCRαβ+ T cells from K14E7 and C57BL/6 skin grafts. Nine T cell clusters were identified, including TNve (C3 and C4), CD4+ memory Tregs (C1), CD4+ Tex (C0), CD8+ TNve cells (C2), CD8+ Tmem (C5), CD8+ Tem (C7), type I IFN–stimulated T cells (C8), NKT cells (C6), and mucosal-associated invariant T (MAIT) cells (C6). (E) Percentage of cells expressing representative T cell activation and differentiation markers in each T cell cluster.
expression in each cluster (Fig. 1E). We thus identified clusters of naive CD4+ T cells (TNve) (C3 and C4: Cd44 low Ccr7 high), CD4+ memory Tregs (C1: Cd44+ IL7r high Foxp3+ Ccr7 high), and CD4+ memory exhausted-like T cells (Tex) (Tex/C0: Cd44+ IL7r high Ifng low Il4 low Il17a low) (Fig. 1D, 1E), the latter expressing high levels of coinhibitory molecules such as Ctla4, Pdcd1, and Tigit (Fig. 1E, Supplemental Fig. 1A). Gene set enrichment analysis of cluster-specific differentially expressed genes, using the AUCell package (19), showed that the Tex cluster C0 comprised cells enriched for a CD4+ T cell exhaustion signature (30) rather than a naive T, Th1, Th2, or Th17 signature (20), supporting classification of these cells as exhausted T cells (Supplemental Fig. 1B). Interestingly, these exhausted T cells were also enriched for a Treg signature, although their average enrichment scores were relatively low compared with the cells classified as Tregs in C1 (0.56 versus 1.61). This could be due to their expression of nonspecific genes in the Treg signature. In addition to CD4+ T cells, we identified CD8+ TNve cells (C2: Cd44 low Ccr7 high), CD8+ memory T cells (Tmem) (Tmem/C5: Cd44+ Ccr7 low IL7r high), CD8+ effector Tmem (Tem) (Tem/C7: Cd44+ Ccr7 low Il7r high Ifng high Gzmb high) (Fig. 1D, 1E), and additional minor nonconventional T cells (Supplemental Fig. 1C–E).

**K14E7 skin grafts are enriched in CD4+ Tregs and exhausted T helper cells**

We investigated whether K14E7 skin grafts were preferentially enriched for certain T cell phenotypes when compared with nontransgenic C57BL/6 skin grafts (Fig. 2A). K14E7 skin grafts, when compared with nontransgenic grafts, had an increased percentage of CD4+ Tregs (C1) (23.3 versus 13.7%) and Tex (C0) cells (22.8 versus 18.1%) and a decreased percentage of CD4+ TNve cells (C3) (8.6 versus 22.9%). K14E7 skin grafts similarly had increased percentages of CD8+ Tmem (C5) and Tem (C7) cells and a decreased percentage of CD8+ TNve (C2) cells compared with nontransgenic grafts. Overall, we observed that CD4+ Tregs and Tex cells comprised 46.1% (23.3% + 22.8%) of the total T cells in K14E7 skin grafts compared with 31.8% (18.1% + 13.7%) in C57BL/6 skin grafts, suggesting a role for Treg and Tex cells in local immune regulation.

To determine whether CD4+ Tregs and Tex cells were enriched in response to specific Ags, we examined the TCR diversity in K14E7 skin grafts when compared with nontransgenic C57BL/6 skin grafts (Fig. 2B). Low TCR diversity, indicated by a low ratio between the number of unique TCR clonotypes and the total cell number, demonstrates that more cells in that cluster share the same clonotype and therefore are more likely to respond to specific Ags. CD8+ Tmem (C5) and Tem (C7) cells had a much lower TCR diversity in K14E7 skin grafts when compared with nontransgenic grafts (C5: 0.828 versus 1.0; C7: 0.552 versus 0.966), suggesting that Ag-specific CD8+ T cells might be present in K14E7 skin grafts. In contrast to CD8+ T cells, we did not observe a lower TCR diversity in CD4+ Tregs (C1) and Tex (C0) cells in K14E7 skin grafts when compared with nontransgenic grafts (C1: 0.897 versus 0.862; C0: 0.978 versus 0.974), suggesting that enrichment of CD4+ Tregs and Tex cells in K14E7 skin grafts was not likely to be driven by expansion of Ag-specific clones.

**CD4+ Tregs in K14E7 skin grafts exhibit a regulatory phenotype absent in nontransgenic grafts**

To further categorize the phenotype of the various T cell clusters, we identified a set of genes differentially expressed in CD4+ Tregs (C1) when compared with CD4+ Tex (C0) and TNve (C3 and C4) cells (Supplemental Fig. 2A) and compared expression of these
genes between C57BL/6 and K14E7 skin grafts (Fig. 3A). We identified 12 genes selectively expressed in C57BL/6 Tregs and 66 genes selectively expressed in K14E7 Tregs (Fig. 3A, 3B), some of which (Hopx, Ikzf2, Tnfrsf9, Iilr2, Tigit, Ccr8, and Tff1) were also selectively expressed in Tregs when compared with other T cell subsets in the graft (Fig. 3C, 3D) and therefore likely linked to Treg-specific functions. Gene coexpression network analysis using the bigScalE 2 package (27) demonstrated a connection of a subset of the Treg-specific genes overexpressed in K14E7 Tregs (Iilr2, Tnfrsf9, Ccr8, and Tff1). These genes are all "ligand–receptor" immune-related genes, expression of which was strongly correlated (ρ > 0.7), forming an Iilr2-associated gene module (Supplemental Fig. 2B). Expression of the Hopx transcription factor further connected this Iilr2-associated module with expression of Tigit (ρ > 0.7). We investigated whether the ligands corresponding to the genes in this Iilr2-associated gene module were expressed by other cells in the K14E7 skin grafts (Fig. 4A). Single-cell gene expression profiles of K14E7-associated APCs, B cells, NK cells, and different T cell subsets in our dataset were combined with previously published scRNA-seq data of K14E7 KCs (28) to perform this analysis (see Materials and Methods). A large percentage of K14E7 KCs (33%) expressed Nectin2, which encodes a ligand for TIGIT, and ligation of TIGIT is associated with enhanced Treg-suppressive activity (31). In addition, we found that a large percentage (67%) of APCs highly expressed Il1b, which encodes the proinflammatory cytokine IL-1β. K14E7 Tregs

FIGURE 3. CD4+ Tregs in K14E7 skin grafts exhibit a regulatory phenotype absent in nontransgenic grafts. (A) K14E7 Treg-specific (red) and C57BL/6 Treg-specific (blue) genes were highlighted based on expression patterns of which gene showed large relative differences in terms of percentage of detection (>10%) and expression level (mean and median expression absolute log2 fold change >0.25) between K14E7 and C57BL/6 Tregs. (B) 12 C57BL/6 Treg-specific (blue) genes and 66 K14E7 Treg-specific (red) genes were identified based on whether their mean and median expression levels and percentage of detection all showed large relative differences (see A) between K14E7 and C57BL/6 Tregs. (C) Expression levels of representative K14E7 Treg-specific genes in K14E7 and C57BL/6 Tregs. Each black dot represents one Treg cell. (D) Expression levels of representative K14E7 Treg-specific genes across different T cell clusters. Each black dot represents one Treg cell.
FIGURE 4. Expression of Klrg1 and Il1r2 characterizes two functional Treg states.

(A) Cell–cell communication map predicts which surrounding cells interact with Tregs in K14E7 skin grafts. Tnfrsf9, Tigit, Il1r2, and Ccr8 were representative receptor genes highly expressed in K14E7 Tregs. Expression patterns of their corresponding ligand genes (Ccl1, Ccl8, Il1a, Il1b, Pvr, Nectin2, and Tnfsf9) in K14E7 KCs, APCs, B cells, NK cells, and different T cell subsets were visualized using the “viridis” color scale (mean expression) and the size scale (percentage of detection). (B) Unsupervised clustering of CD4+ Tregs in K14E7 and C57BL/6 skin grafts shows three Treg subclusters, including Treg.1 (Klrg1+), Treg.2 (Klrg1− Il1r2−), and Treg.3 (Il1r2+). Expression levels of Klrg1 and Il1r2 across Treg subclusters were visualized. (C) Monocle 2 pseudotime trajectory cataloged Tregs in K14E7 and C57BL/6 skin grafts into seven states. The absolute number of Treg.1, Treg.2, and Treg.3 cells in each state was summarized. (D) Expression levels of Klrg1 and Il1r2 and enrichment scores (AUCell) of CD4+ TNve signature across different Treg states were visualized on the trajectory. (E) The 79 published tisTregST2-specific genes and the

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that express \(I\text{Ilr}2\) could produce both a membrane and a soluble receptor that competes with effector T cells for IL-10, thereby suppressing T cell activation (32). Thus, Tregs from K14E7 skin grafts, unlike those from nontransgenic skin grafts, have a phenotype with the potential for local regulation of innate and adaptive immune effector responses.

**Expression of \(Klrg1\) and \(I\text{Ilr}2\) characterizes two functional Treg states**

Many of the genes overexpressed in K14E7 Tregs when compared with C57BL/6 Tregs (e.g., \(H\text{opx}, Tnfrsf9, I\text{Ilr}2, Ccr8,\) and \(Tjfb\)) were not expressed in all Tregs. We therefore investigated whether the CD4+ Treg cluster (C1) demonstrated further heterogeneity by performing unsupervised clustering (16) on this subset of cells. We obtained 3 subclusters (Treg.1, Treg.2 and Treg.3) from a total of 965 Tregs (671 in K14E7 and 294 in C57BL/6 skin grafts) (Fig. 4B). Differential expression analysis revealed the genes that were most significantly upregulated in each Treg subcluster (Supplemental Fig. 2C). \(Klrg1\) and \(I\text{Ilr}2\) enabled further phenotypic categorization of Treg.1 (\(Klrg1^+\), Treg.2 (\(Klrg1^- I\text{Ilr}2^+\)), and Treg.3 (\(I\text{Ilr}2^+\)) (Fig. 4B).

Uniform Manifold Approximation and Projection visualization indicated that these Treg subclusters were closely linked, implying that they might represent different cell states instead of distinct subtypes. We therefore performed pseudotime trajectory analysis on the total Treg population using the Monocle 2 package (25, 26). The resulting DDTree-based trajectory, which orders cells based on global gene expression changes, cataloged Tregs into seven states and placed each Treg subcluster (Treg.1, Treg.2 and Treg.3) onto a different branch (Fig. 4C). State 1 consisted predominantly of cells from Treg.2 but also contained cells from Treg.1 and Treg.3. We categorized cells in state 1 as functionally immature Tregs because they had low expression of effector molecules and were enriched with a CD4+ TNve gene signature (20) using the AUCell package (Fig. 4D). Cells in states 2–6 were mainly derived from \(Klrg1^+\) Treg.1. Cells in state 7 consisted mostly of \(I\text{Ilr}2^+\) Treg.3. Overall, the different Treg states on the trajectory were interconnected and grouped into three phenotypic regions (immature Tregs, \(Klrg1^+\) Tregs, and \(I\text{Ilr}2^+\) Tregs). Notably, we observed that a number of \(I\text{Ilr}2^+\) Tregs adjacent to states 5 and 6 also highly expressed \(Klrg1\) (Fig. 4D, Supplemental Fig. 2D). These cells were likely to display phenotypes characteristic of both \(I\text{Ilr}2^+\) Tregs and \(Klrg1^+\) Tregs.

\(Klrg1\)-expressing Tregs have been previously reported as a tissue-resident Treg population, termed tisTregST2, in virtually all NLTs in healthy mice (21). We therefore compared the 200 genes characteristically expressed by K14E7 and C57BL/6 Tregs with the 79 published tisTregST2-specific genes, and found 27 genes common to both gene sets (Fig. 4E). These included \(Klrg1\), and other immune-related molecules, including \(I\text{Ilr}1l, Cd44, Gata3, T\text{ig}ft,\) and two tisTregST2-associated transcription factors \(Baf\text{f}4\) and \(N\text{fli}3\) (21, 33). Expression of these 27 tisTregST2-specific genes was enriched (AUCell package) in \(Klrg1^+\) Tregs (states 2–6) in our study, when compared with the other Tregs (Fig. 4F). Furthermore, a gradual increase of the tisTregST2 enrichment score was observed in Tregs from states 2 and 3 to states 4–6, suggesting that \(Klrg1^+\) Tregs in states 4–6 display increased maturity compared with Tregs in states 2 and 3. We then selected 31 genes previously identified as a signature of tumor-infiltrating Tregs across different clinical studies in liver (10), melanoma (22), breast (9), colon, and lung cancers (23). Among these 31 genes, 15 genes were highly expressed in the graft-infiltrating Tregs (Fig. 4E), including many Treg-specific genes from K14E7 skin grafts (\(I\text{Ilr}2, Tnfrsf9,\) and \(Ccr8\)). Expression of the 15 tumor Treg-specific genes was enriched in both \(Klrg1^+\) Tregs (state 4–6) and \(I\text{Ilr}2^+\) Tregs (state 7) (Fig. 4F). However, \(I\text{Ilr}2^+\) Tregs had increased enrichment scores and were enriched with a different set of tumor Treg-specific genes (e.g., \(Ncf4\), \(Baf\text{f}4\), \(I\text{Ilr}2^+,\) and \(Ccr8\)) when compared with \(Klrg1^+\) Tregs (Supplemental Fig. 2). Overall, these data suggest that the \(I\text{Ilr}2^+\) Tregs in our study resemble tumor-infiltrating Tregs, whereas \(Klrg1^+\) Tregs resembled tisTregST2 cells but have the potential to acquire tumor Treg-specific features. To investigate which Treg states were more enriched in K14E7 skin grafts, we calculated the percentage of total for each Treg state in each graft type (Fig. 4G). C57BL/6 skin grafts contained a higher proportion of immature Tregs (47%) when compared with K14E7 skin grafts (24%). In contrast, K14E7 skin grafts, when compared with C57BL/6 skin grafts, contained higher proportions of both \(I\text{Ilr}2^+\) Tregs (state 7; 16 versus 10%) and \(Klrg1^+\) Tregs (states 3–6; 18, 25, 2, and 7% versus 14, 16, 1, and 1%, respectively), indicating that K14E7 Tregs are more mature and activated compared with C57BL/6 Tregs.

Previous studies have reported various Treg states within lymphoid tissues (LTs), NLTs, or thymus. We examined how these studies relate to our findings on \(Klrg1^+\) and \(I\text{Ilr}2^+\) Tregs. scRNA-seq of murine splenic Tregs revealed six Treg states (24). We obtained gene signatures (see Materials and Methods) of each of these states along with their proposed annotations. Both \(Klrg1^+\) and \(I\text{Ilr}2^+\) Tregs in our data were enriched (AUCell package) for signatures of splenic Treg state 1 and 2 (activated Tregs) but not for signatures of splenic Treg state 3 (early activated Tregs) and states 4–6 (resting Tregs) (Fig. 5A). This indicates that both \(Klrg1^+\) and \(I\text{Ilr}2^+\) graft-infiltrating Tregs resemble activated Tregs in LTs. We then compared scRNA-seq data of murine colon Tregs (7, 34) with our
FIGURE 5. Comparison of skin graft–derived Klrg1+ and Il1r2+ Tregs with previously reported Treg states in spleen, colon, and thymus.

(A) Gene set enrichment analysis examined which of the six splenic Treg states most resemble skin graft–derived immature Tregs, Klrg1+ Tregs, and Il1r2+ Tregs. Gene signature and a proposed annotation was obtained for each splenic Treg state from published scRNA-seq data (24). Each bar represents the mean enrichment score of all Tregs in that state with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey multiple comparison test. ns, p ≥ 0.05, ****p < 0.0001. (B) Two published scRNA-seq data of murine colon Tregs (7, 34) were integrated with our data of skin graft Tregs. Unsupervised clustering was performed, and this grouped Tregs from different studies together based on their transcriptomic similarities. Each study has a different set of annotated Treg clusters that was plotted separately. (C) Reanalysis of the published scRNA-seq data of adult murine thymic Tregs (35). Expression levels of Ccr7, Ccr6, Klrg1, and Il1r2 and enrichment scores (AUCell package) of Klrg1+ and Il1r2+ graft-infiltrating Treg signatures across all thymic Tregs were examined.
data using the data integration pipeline in Seurat. The two independent colon datasets exhibited similar Treg composition but with different annotation methods (Fig. 5B). Using colon Tregs as a reference, we found that immature Tregs in our data resembled circulating thymus-derived Tregs or LT-homing Tregs in the colon. Klrg1⁺ and Il1r2⁺ Tregs in our data displayed overlapping phenotypes with multiple colon Treg populations, including Klrg1⁺ tissue-repair thymus-derived Tregs or NLT Tregs, and Rorc⁺ peripherally induced Tregs or Il10⁺ suppressive Tregs. We also confirmed the presence of Klrg1⁺ and Il1r2⁺ Tregs in the thymus. scRNA-seq data of adult murine thymic Tregs (35) grouped Tregs into Ccr7⁺ Ccr6⁻ newly generated Tregs and Ccr7⁻ Ccr6⁻ recirculating Tregs (36, 37) (Fig. 5C), the latter of which represents Tregs that re-enter the thymus from the

FIGURE 6. Clonally expanded Tregs in K14E7 skin grafts displayed phenotypic plasticity.
(A) Underrepresented Treg clones (clonotype count = 1) and overrepresented Treg clones (clonotype count >1) in K14E7 and C57BL/6 skin grafts were visualized on the pseudotime trajectory. (B) Distribution (absolute number and percentage) of clonally expanded Tregs across different Treg substates was calculated in each graft type. (C) Left, Clonotype overlap analysis between LN T cell clones and clonally expanded skin-resident Tregs in K14E7 and C57BL/6 samples. Right, LN clones overlapped with clonally expanded skin-resident Tregs were visualized on the trajectory. (D) Clonally expanded skin-resident Tregs (orange) together with LN-resident Tregs (purple) were plotted on the trajectory.
Interestingly, differential expression of Klrg1 and Il1r2 defined two substates of recirculating Tregs, whereas their expression was negligible in newly generated Tregs, suggesting that emergence of Klrg1+ and Il1r2+ Tregs are likely the outcome of peripheral activation rather than thymic selection. Moreover, Klrg1+ and Il1r2+ recirculating Tregs were enriched for signatures of Klrg1+ and Il1r2+ graft-infiltrating Tregs, respectively, suggesting a potential migratory route of Klrg1+ and Il1r2+ Tregs from skin grafts to thymus.

**Clonally expanded Tregs in K14E7 skin grafts displayed phenotypic plasticity**

To investigate whether Tregs of the same clonotype acquire a common phenotype, we aligned all clonally expanded Tregs (TCR clonotype count >1) with the pseudotime trajectory (Fig. 6A). Clonally expanded Tregs were generally proportionally distributed across the different Treg states (Fig. 6B). The majority of clonally expanded Tregs in state 1 were localized in a region adjacent to Tregs in states 2 and 3 on the trajectory (Fig. 6A), suggesting that they acquired the phenotype of Klrg1+ Tregs upon clonal expansion. We then similarly analyzed T cells from graft DLNs from the same scRNA-seq experiment and asked whether TCR clonotypes were shared across tissues. Clonotype overlap analysis based on paired TCRα and β sequences revealed eight lymph node (LN) TCR clones that were also clonally expanded skin-resident Tregs in K14E7 samples (Fig. 6C). These LN clones showed diverse phenotypes across the pseudotime trajectory, including the phenotypes typical of immature Tregs, Il1r2+ Tregs, and Klrg1+ Tregs. The two LN clones from the C57BL/6-grafted animals demonstrated a Klrg1+ Treg phenotype. (Fig. 6C; K14E7, red; C57BL/6, blue). To visualize the phenotypic diversity of individual clones, we further plotted the clonally expanded skin-resident Tregs together with LN-resident Tregs of the same clonotype on the trajectory (Supplemental Fig. 4; K14E7, clonotypes 1–8; C57BL/6, clonotypes 9 and 10). For each of these TCR clonotypes, the phenotypic diversity of individual cells could differ within the skin graft as well as between the skin graft and the LN. For example, cells from a particular clonotype demonstrated an immature Treg phenotype when present in the LN and a Klrg1+ Treg phenotype when present in the skin graft, suggesting local phenotypic maturation within the tissue (Fig. 6D; clonotypes 1, 4, and 8). Cells with clonotype 6 displayed phenotypes of either Il1r2+ Tregs or Klrg1+ Tregs within the same tissue compartment (skin graft or LN), demonstrating that acquisition of different Treg phenotypes can occur within a single TCR-defined clone.

**DISCUSSION**

The current study provides insight into the development of phenotypic heterogeneity of tissue-infiltrating Tregs in consequence of interaction with HPV-induced premalignant skin lesions. Using scRNA-seq with integrated TCR sequencing, we profiled Tregs from both K14E7 and nontransgenic C57BL/6 skin grafts. We demonstrated that Tregs had some conserved gene expression in either graft type. Although Tregs in K14E7 skin grafts were enriched and acquired enhanced effector regulatory phenotypes in comparison with those in C57BL/6 skin grafts, we found no evidence for these Tregs to be Ag-specific. Our analysis further revealed phenotypic diversity of Tregs within a single T cell clonotype.

Unsupervised clustering of single-cell transcriptomic profiles of T cells revealed that Tregs in both graft types had high expression of conventional Treg markers, such as Cd4, Foxp3, Ctla4 and Cd25, and displayed two major functional states characterized by high expression of Klrg1 or Il1r2. Previous studies have described the presence of Tregs expressing Klrg1+ or Il1r2+, although none have considered the possibility that these define two functionally distinct Treg populations. Transcriptomic profiles of Klrg1+ Tregs from our dataset resembled those of a recently characterized Treg subset (tisTregsST2) naturally present in nearly all NLTs (21). TisTregsST2 cells express high levels of KLRL1 and ST2 and respond to the alarmin cytokine IL-33, which can induce AREG, a ligand for epidermal growth factor receptor that plays a nonredundant role in tissue repair (38). In our dataset, high ST2 gene expression was found predominantly in Klrg1+ Tregs, although AREG gene expression was found in all Treg states, implying that IL-33 signaling but not AREG expression is limited to Klrg1+ Tregs. IL-33 signaling has been shown to expand ST2-expressing Tregs both in vitro and in vivo (39) and possibly plays a tumor-promoting role, as evidenced by the increased number of ST2-expressing Tregs in tumor lesions of cancer patients (40, 41). Given that Klrg1+ Tregs were enriched in K14E7 skin grafts compared with C57BL/6 skin grafts, Klrg1+ Tregs in K14E7 skin grafts might either receive a steady source of IL-33 or display higher sensitivity to IL-33 compared with those in C57BL/6 skin grafts.

Increased expression of Il1r2 in Tregs has been previously reported at tumor sites but not in healthy tissues, blood, and tumor-adjacent LNs (9, 10, 22, 23). Our data suggest that Il1r2 expression could help define a Treg functional state distinct from Klrg1+ Tregs. In our study, Il1r2+ Tregs were relatively increased in K14E7 skin grafts when compared with isogenic skin grafts. This observation, when combined with the findings of previous studies, suggests that induction of Il1r2+ Tregs might correlate with the degree of tissue inflammatory response. Pseudotime trajectory mapping, combined with TCR-based clonotype analysis, demonstrated phenotypic plasticity of Klrg1+ Tregs, which can acquire the phenotype of Il1r2+ Tregs. Besides Il1r2, other effector molecules, such as Tnfrsf9 (4-1BB) and Ccr8, were highly expressed in Il1r2+ Tregs in both the present and previous studies, indicating that these molecules represent a common consequence of Treg maturation, migration, and suppression of effector T cell function in premalignant and malignant lesions. Costimulation by 4-1BB is a therapeutic strategy used to stimulate strong cytotoxic CD8+ T cell response (42). The significance of 4-1BB signaling in Tregs remains less clear, although some studies have demonstrated that 4-1BB agonist can promote Treg expansion and suppressive capacity (43). Notably, downregulation of IL-1 signaling has been demonstrated as one essential mechanism that prevents spontaneous rejection of K14E7 skin grafts and inhibits priming of E7-specific CD8 T cell
responses (44). As IL-1R2 acts as a negative regulator of IL-1 signaling, it is probable that Il1r2+ Tregs also play an important role in inducing K14E7 graft tolerance.

The capacity of IL-1R2–expressing Tregs to inhibit IL-1 signaling has been recently demonstrated in the context of intrathymic Treg development (37). IL-1R2+ Tregs preferentially recirculated to the thymus from the periphery, whereas newly generated thymic Tregs negligibly express IL-1R2. Under inflammatory conditions, these IL-1R2+ Tregs are capable of neutralizing intrathymic IL-1β, thereby preventing IL-1β from blocking intrathymic Treg development. Our data shows that IL-1R2+ thymic Tregs share phenotypic similarities with Il1r2+ graft-infiltrating Tregs, and Il1r2+ Tregs in skin grafts may have a similar functional capacity on regulating IL-1 signaling locally. This pathway might further enhance systemic Treg levels by enhancing intrathymic Treg development via IL-1β neutralization, thereby promoting K14E7 graft tolerance and cancer progression.

Previous studies of Treg phenotypes have primarily focused on steady-state conditions across a range of tissues. We assume that Tregs can present in a defined number of phenotypic states, whether examined in LTs, NLTs, or thymus and under steady-state and perturbed conditions, although some phenotypes may become dominant and/or display unique features in different environments. Based on this assumption, we have used previous studies to better interpret our observation on Klrg1+ and Il1r2+ Tregs in oncogene-driven hyperproliferative skin. LTs, such as spleen, predominantly are held to have Tregs of a resting phenotype, with a minor population of activated Tregs (24) that is likely to migrate to NLTs and acquire NLT-specific features (7). Our results are consistent with this interpretation, showing that Klrg1+ and Il1r2+ graft-infiltrating Tregs resemble activated rather than resting splenic Tregs. Klrg1+ and Il1r2+ graft-infiltrating Tregs do not match perfectly with previously reported NLT Treg states, likely because of the difference between the altering environment of a recently placed skin graft compared with the steady-state conditions in the colon. Interestingly, we found that the thymus, when compared with LTs and NLTs, preferentially recruits Klrg1+ and Il1r2+ Tregs, even under steady-state conditions, indicating that these two Treg states are present under physiological conditions, albeit with incompletely defined roles.

Induction of functionally diverse Treg cell states locally in tissues is probably associated with colocalization with not only immune cells but also various types of nonimmune cells, including epithelial cells and stromal cells, which are exposed and respond to signals from microbial communities and environmental stress. Several studies have observed that Tregs can populate specific cellular niches and function in a niche-specific manner. Tregs at tumor sites are observed to directly contact dendritic cells and form tertiary lymphoid structures to promote a tolerogenic dendritic cell phenotype (45–47). Tregs in hair follicles uniquely express Jagl, which facilitates the proliferation and differentiation of hair follicle stem cells via the Notch pathway (48, 49). Within these niches, Tregs and their surrounding cells undergo bi-directional communications. Our ligand–receptor, cell–cell interaction demonstrates a mechanism by which different Treg phenotypes can interact with different types of immune cells and nonimmune cells in K14E7 skin grafts to influence immune effector functions. Furthermore, we suggest that the enrichment of both Klrg1+ and Il1r2+ Tregs in K14E7 skin grafts is more likely due to E7-induced epithelial hyperplasia rather than E7 expression itself. K14E7 skin with a mutated Rb gene (K14.E7xRbΔL/ΔL), which expresses equivalent levels of E7 mRNA to K14E7 skin but lacks the hyperplastic phenotype, downregulates a significant number of inflammatory molecules and fails to induce a strong immune cell infiltrate (50). A reduction in local immune signaling and immune cell recruitment leads to a lack of cell–cell interactions that might be essential for the induction of diverse Treg phenotypes.

Clonotype analysis revealed that Tregs in both K14E7 and nontransgenic skin grafts displayed a large diversity in their TCR repertoire with little indication of clonal expansion. Our finding does not rule out the presence of E7–specific Treg clones in K14E7 skin grafts. Instead, we would like to emphasize the complexity of the TCR repertoire in the inflammatory environment expressing a single defined non–self-antigen. Previous studies using adoptive cell transfer have shown that activated OVA–specific and E7–specific CD8+ T cells were attracted to K14E7 grafts simultaneously (51). Together with the findings presented in this study, we thus hypothesize that many Tregs recruited to K14E7 grafts are likely not E7–specific, although further studies are needed to confirm their ability to suppress effector T cells in an Ag–independent fashion. Tregs displaying different phenotypes and different Ag specificities might induce Ag–independent immune tolerance, a concept previously described as “bystander” T cell response (52, 53). In cancer patients, tumor-infiltrating T cells were found to recognize a variety of Ags unrelated to the tumor, many of which are associated with viral pathogens (53). A number of studies further demonstrated that innate-like receptors, such as TLRs expressed on T cells, can act as costimulatory receptors for TCR–independent activation (54–56). Interestingly, Tregs in our study expressed various types of innate sensors, such as antiviral restriction factors and peptidoglycan recognition protein, whose expression was increased in K14E7 skin grafts compared with nontransgenic skin grafts. This implies that local innate immune signals can directly influence Treg activation regardless of the Treg Ag specificity.

In conclusion, our study has generated a comprehensive overview of the diversity of Treg response induced within HPV16 E7–induced premalignant lesions, when compared with the Treg population in control skin. Our findings provide opportunities to target functionally active Tregs in an immunosuppressive environment with higher specificity.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES


ONCOGENE-DRIVEN EPITHELIAL HYPERPLASIA INDUCES Treg RESPONSE


Figure S1: Annotation of T cell clusters in K14E7 and C57BL/6 skin grafts. (A) Percentage of cells expressing inhibitory receptor genes in each T cell cluster. (B) Distribution of AUC enrichment scores (x-axis) of individual cells in each CD4+ T cell clusters (C0, C1, C3, C4) for signatures of CD4+ T cell exhaustion, TNve, Th1, Th2, Th17 and Tregs (see methods). (C) DEGs (adj p<0.001, Red) upregulated in IFN-stimulated T cells (C8) compared to all other T cell clusters. The labelled genes were IFN-stimulated genes. (D) Expression levels of representative IFN-stimulated genes (Ifit3, Isg15 and Ifit1) across different T cell clusters. (E) TCR V(D)J usage of cells in cluster 6 (NKT and MAIT cells) in K14E7 (29 cells) and C57BL/6 (34 cells) skin grafts. The invariant NKT cells expressed TCRα chains comprising a Vα11-Jα18 rearrangement. The MAIT cells expressed TCRα chains comprising a Vα1-Jα33 rearrangement.
Figure S2: Analysis of Treg phenotype and sub-states. (A) CD4+ Tregs but not CD4+ Tex and TNve cells highly expressed a notable number of immune activation and regulatory genes. Left: DEG analysis between CD4+ Tex and CD4+ TNve cells identified genes highly expressed in CD4+ Tex cells (adj p<0.001, logFC>1). Red dots highlighted DEG encoding “ligand and receptor” molecules (Upper) and transcription factors (Lower). Middle: DEG analysis between CD4+ Tregs and CD4+ TNve cells identified genes highly expressed in Tregs (adj p<0.001, logFC>1). Right: DEG analysis between CD4+ Tregs and CD4+ Tex cells identified genes highly expressed in Tregs (adj p<0.001, logFC<0) and CD4+ Tex cells (adj p<0.001, logFC>0). (B) Gene co-expression network shows a connection among Il1r2, Tigit, Hopx, Tnfrsf9, Ccr8 and Tff. The numbers displayed on edges represent Pearson correlation coefficients. (C) DEGs (adj p<0.001, Red) upregulated in Klrk1+ Tregs (Treg.1), Klrk1 Il1r2+ Tregs (Treg.2) and Il1r2+ Tregs (Treg.3). (D) Tregs that co-expressed Il1r2 an Klrk1 were highlighted (brown) on the pseudotime trajectory.
Figure S3: Enrichment of tisTregST2 and tumour Treg signature genes in different Treg sub-states. (A) Percentage of cells that have non-zero expression of each tisTregST2-specific gene and their mean expression were calculated for each Treg states. Solid circles indicate Treg states with the lowest (Blue) and the highest (Purple) mean enrichment scores for the tisTregST2-specific genes (see Figure 4F). (B) Percentage of cells that have non-zero expression of each tumour Treg-specific gene and their mean expression were calculated for each Treg states. Solid circles indicate Treg states with the lowest (Blue) and the highest (Brown) mean enrichment scores for the tumour Treg-specific genes (see Figure 4F).
Figure S4: Phenotypic plasticity of clonally expanded Tregs. Clonally expanded skin-resident Tregs (Orange) together with LN-resident Tregs (Purple) of the same clonotypes were plotted on the trajectory. Eight representative clonotypes in K14E7 sample and two representative clonotypes in C57BL/6 sample were visualized individually.