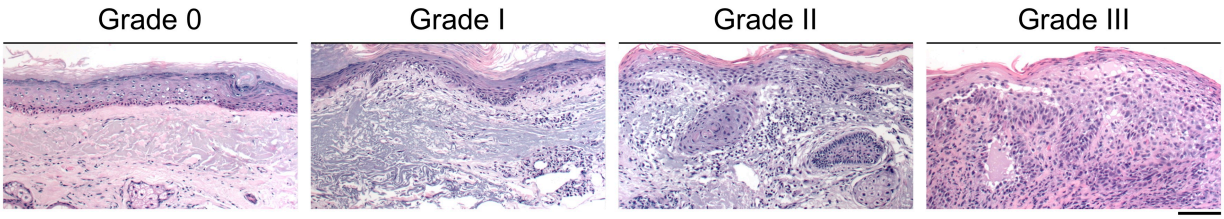
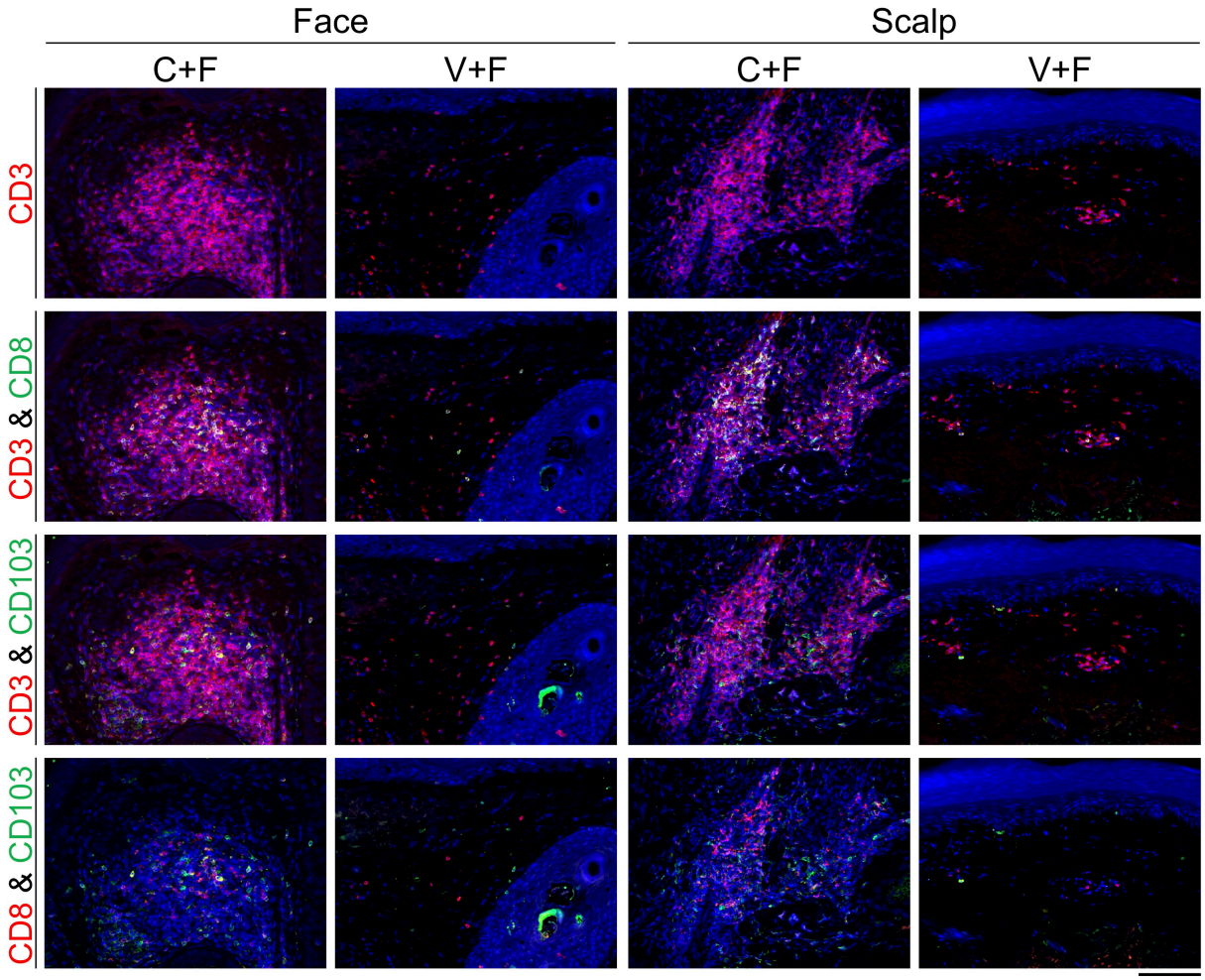
Supplemental Figures and tables for: Skin Cancer Precursor Immunotherapy for Squamous Cell Carcinoma Prevention

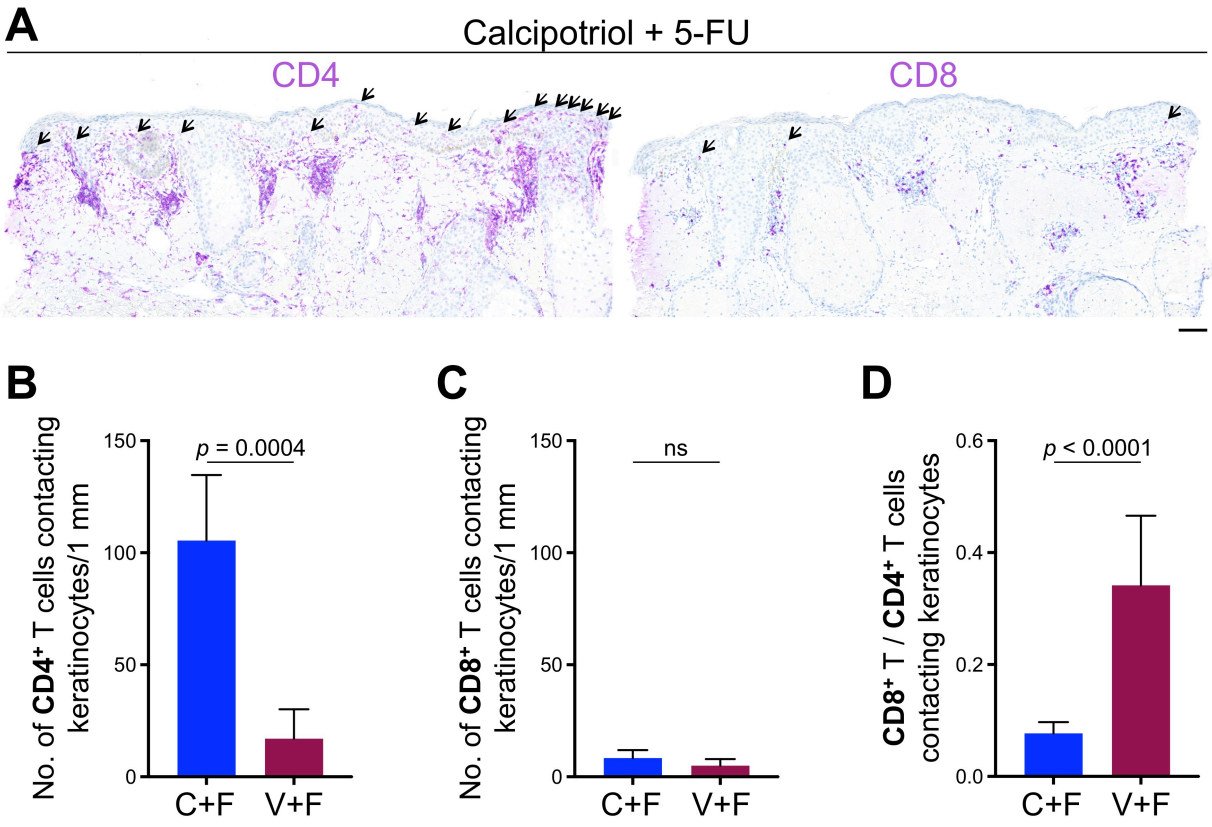


Cutaneous Inflammation Grades
Grade 0: No or rare inflammatory infiltrates.
Grade I: Mild perivascular immune cell infiltrate. No involvement of the overlying epidermis.
Grade II: Moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis). No epidermal dyskeratosis or apoptosis.
Grade III: Dense inflammation and epidermal involvement (significant spongiosis and exocytosis) with epithelial apoptosis, dyskeratosis and/or keratinolysis.

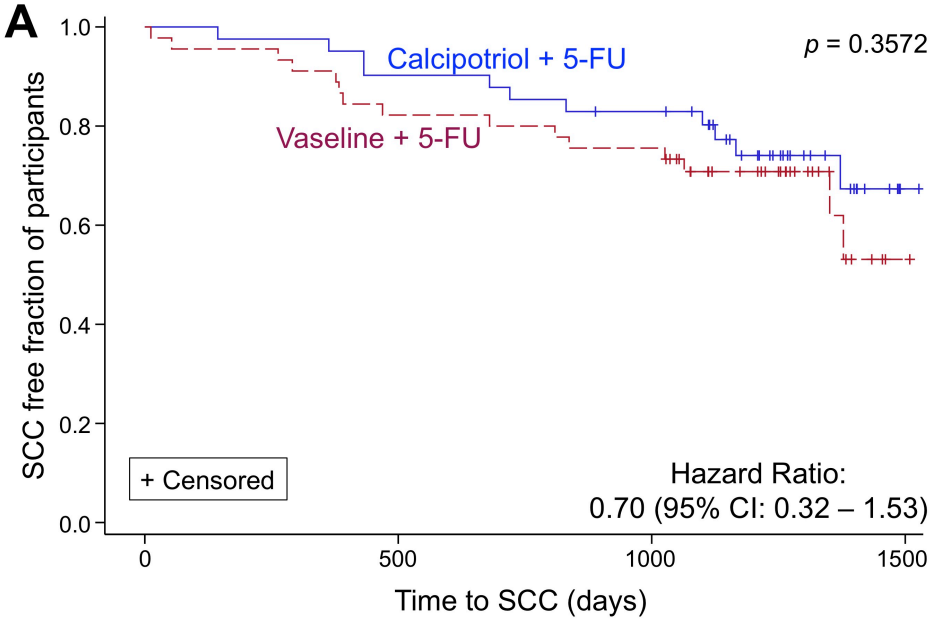
Supplemental Figure 1. Cutaneous inflammation grading system. The magnitude of T cell immunity in the AK biopsies obtained one day after treatment completion is measured by the criteria used for skin graft rejection (1-3). Representative histological images of AKs that correspond to each grade are shown.



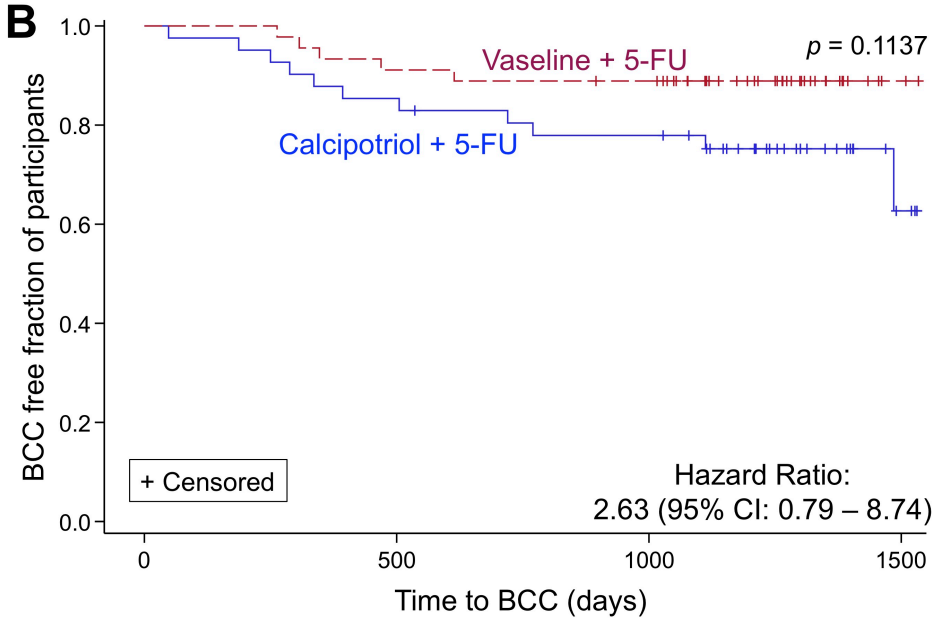
Supplemental Figure 2. CD8$^+$ T cell accumulation and CD8$^+$ T_{RM} induction in AKs treated with calcipotriol plus 5-FU. Representative images of CD3/CD8/CD103-stained AKs on the face and scalp biopsied one day after treatment with calcipotriol plus 5-FU (C+F) or Vaseline plus 5-FU (V+F) are shown (scale bar: 100 μm).



Supplemental Figure 3. CD4$^+$ T and CD8$^+$ T cell localization in AKs treated with calcipotriol plus 5-FU. (**A**) Representative images of CD4-stained or CD8-stained adjacent sections from an AK on the face treated with calcipotriol plus 5-FU combination are shown (arrows point to T cells contacting the premalignant keratinocytes, scale bar: 100 μm). Compared to CD4$^+$ T cells, note the low density of CD8$^+$ T cell infiltrates in the AK, which are mostly localized in the dermis and around the dermal blood vessels. (**B**) The number of CD4$^+$ T cells contacting epidermal keratinocytes in AKs are compared after treatment with calcipotriol plus 5-FU (C+F) versus Vaseline plus 5-FU (V+F). (**C**) The number of CD8$^+$ T cells contacting epidermal keratinocytes in AKs after calcipotriol plus 5-FU (C+F) versus Vaseline plus 5-FU (V+F) treatment are shown. (**D**) Bar graph shows the ratio of CD8$^+$ T cells to CD4$^+$ T cells contacting the AK keratinocytes in the test (C+F) and control (V+F) groups. The number of cells were counted across the length of the AK punch biopsy specimens obtained from face and scalp. The cell counts are presented per 1 mm length of the epidermis (n = *3* per group, ns: not significant by Wilcoxon rank-sum test).



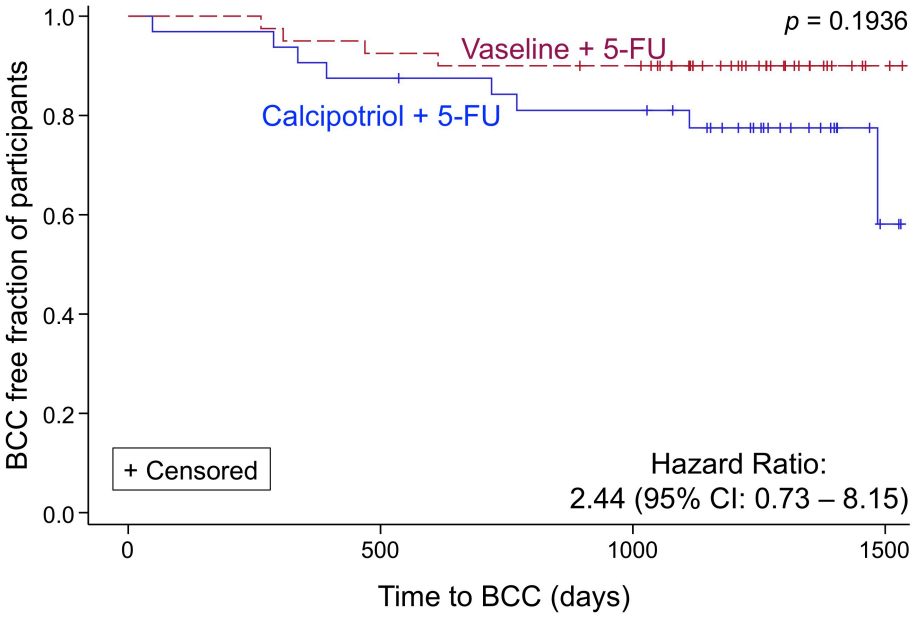
No. at risk	*0yr*	*1yr*	*2yr*	*3yr*
Calcipotriol + 5-FU	41	39	35	33
Vaseline + 5-FU	45	41	36	32



No. at risk	*0yr*	*1yr*	*2yr*	*3yr*
Calcipotriol + 5-FU	41	36	32	31
Vaseline + 5-FU	45	42	40	40

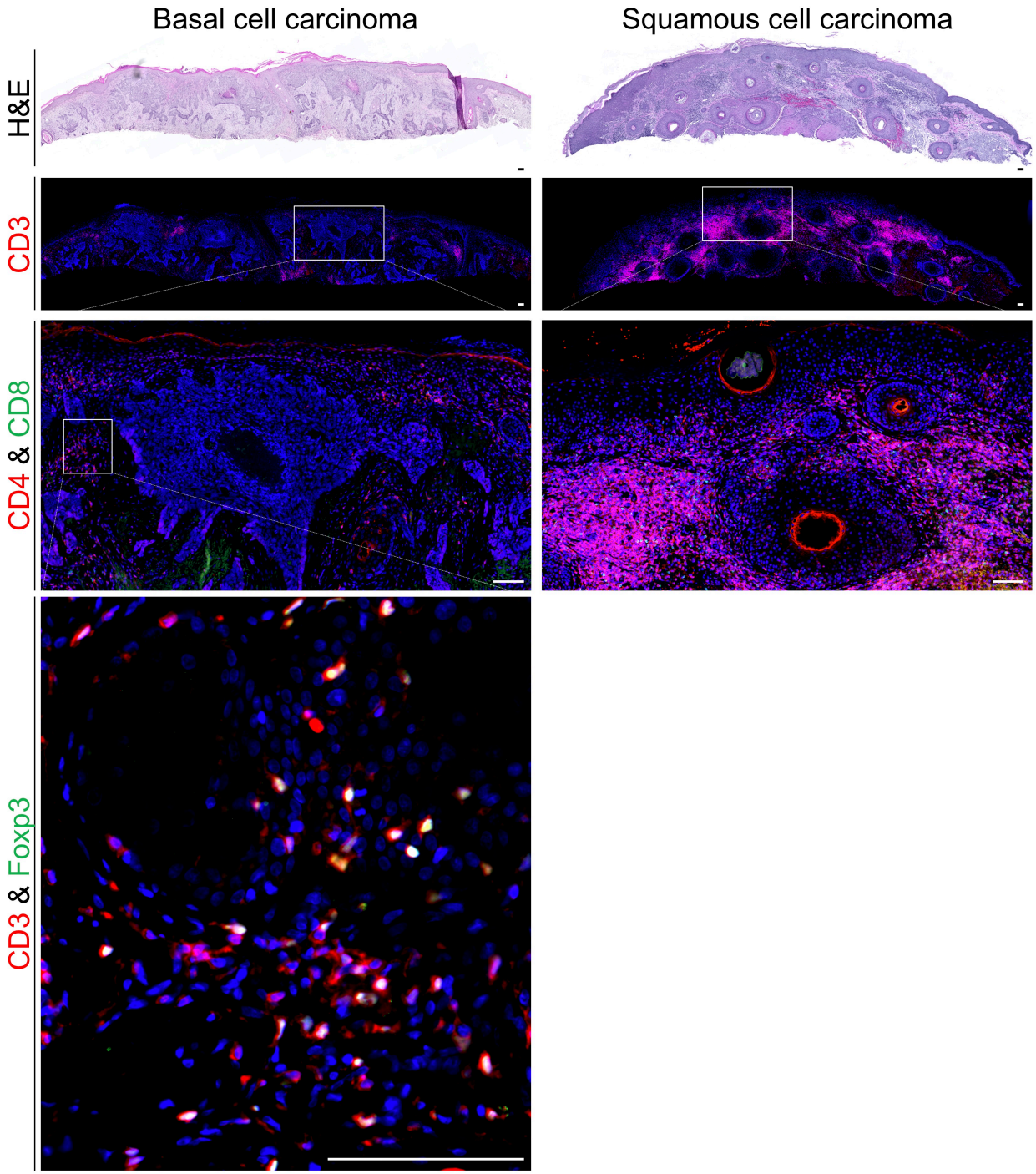
Supplemental Figure 4. Proportion of participants remaining free of SCC and BCC on any treated anatomical sites over time. Kaplan-Meier curves show the proportion of participants in the test and control group who remained (**A**) SCC and (**B**) BCC free on all their treated anatomical

sites during the >1500 days of follow-up after trial.

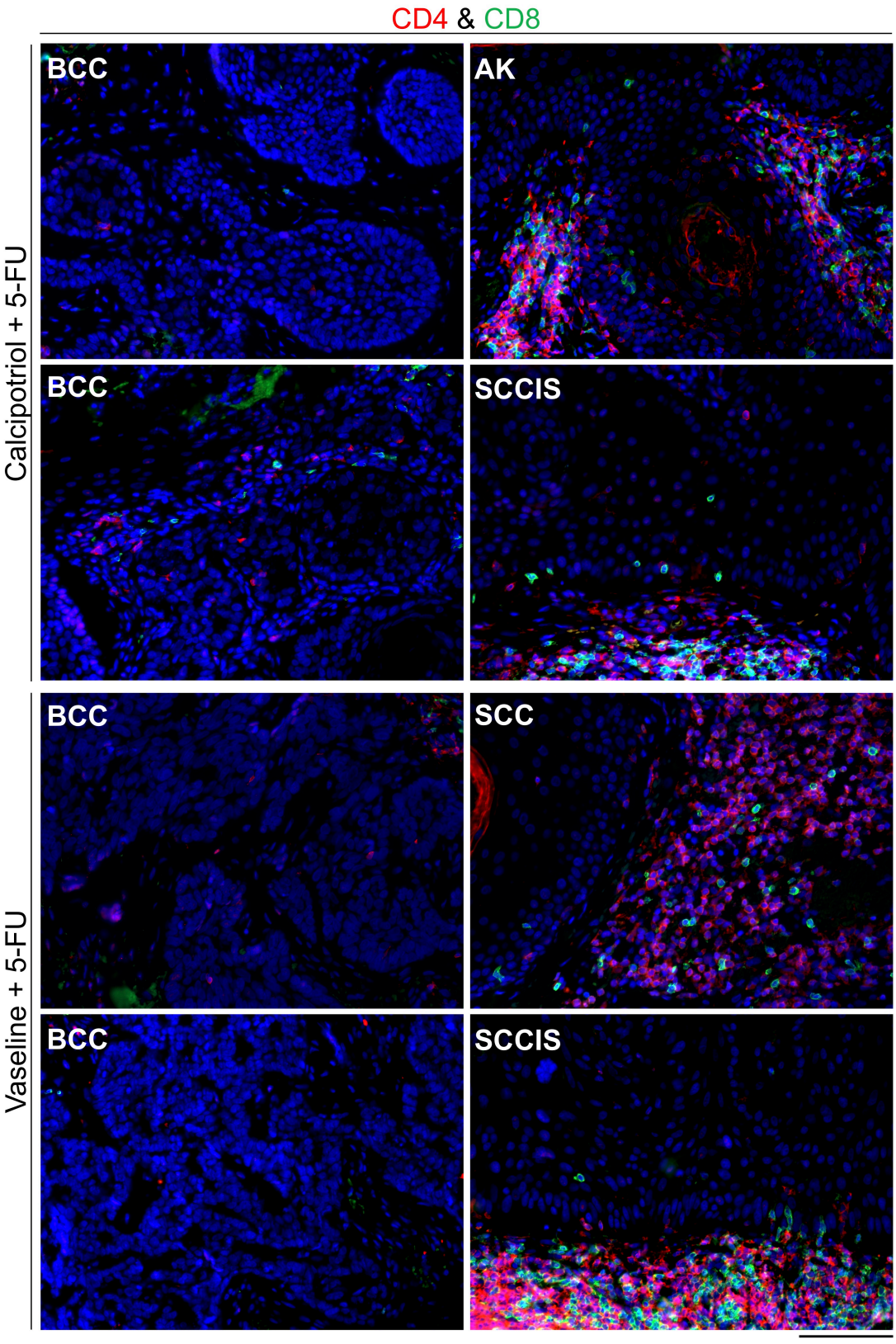


No. at risk	*0yr*	*1yr*	*2yr*	*3yr*
Calcipotriol + 5-FU	32	29	26	25
Vaseline + 5-FU	40	38	36	36

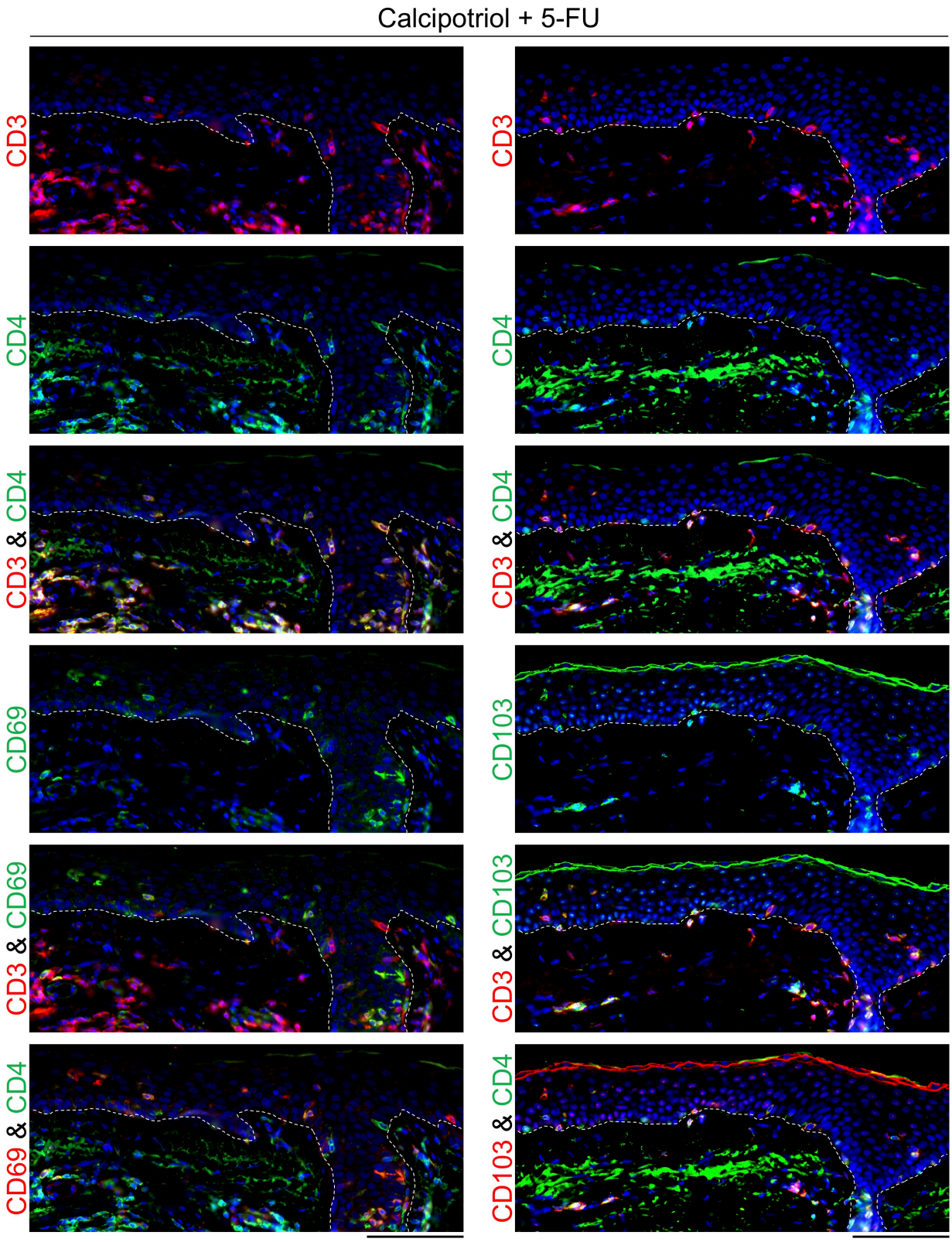
Supplemental Figure 5. Proportion of participants remaining BCC free on the treated face and scalp over time. Kaplan-Meier analysis shows the follow-up period of over >1500 days after trial.



Supplemental Figure 6. Differential immune microenvironments in BCC and SCC biopsied from the face of the same participant at a clinical visit after trial. H&E images depict the two KCs, a BCC and a SCC. Staining with CD3, CD4, CD8, and Foxp3 illustrates large T cell infiltrates in the SCC. In contrast, there is minimal T cell infiltrate at the periphery of the BCC, largely composed of immunosuppressive Foxp3^{+} regulatory T cells. Scale bars: 100μm.


CD4 & CD8
Calcipotriol + 5-FU
Vaseline + 5-FU
BCC
AK
BCC
SCCIS
BCC
SCC
BCC
SCCIS

Supplemental Figure 7. Differential immune microenvironments in BCC and AK-SCC spectrum lesions biopsied from the face and scalp of participants post-trial. KC biopsies from two participants in each treatment groups were stained for $CD4^+$ and $CD8^+$ T cells illustrating the magnitude of tumor infiltrating lymphocytes in BCCs compared to AK, SCCIS or SCC from the same participants. Each row represents KCs from a single participant. Scale bar: 100μm.


Calcipotriol + 5-FU
CD3
CD3
CD4
CD4
CD3 & CD4
CD3 & CD4
CD69
CD103
CD3 & CD69
CD3 & CD103
CD69 & CD4
CD103 & CD4

Supplemental Figure 8. T_{RM} surface marker expression in epidermal T cells. Representative image of normal skin from a post-trial biopsy specimen that was collected from a participant in the calcipotriol plus 5-FU treatment group is shown with staining for CD3/CD4/CD69 and CD3/CD4/CD103 (dashed lines highlight the basement membrane, scale bar: 100 μm).

Supplemental Table 1. Baseline demographic and clinical characteristics of participants treated on the face and scalp.

	Calcipotriol + 5-FU	Vaseline + 5-FU	*P value*
	(n=32)	**(n=40)**	
Age, mean (SD), y	67 (7)	70 (8)	*0.186*
(Range)	(56-81)	(52-84)	
Gender, *n* (%)			*0.451*
Male	25 (78)	34 (85)	
Female	7 (22)	6 (15)	
# Anatomical Sites Treated, *n* (%)			
Face	26 (81)	34 (85)	*0.671*
Scalp	18 (56)	24 (60)	*0.748*
Baseline AK Count on Each Anatomical Site, median (interquartile range)			
Face	18 (11)	15 (12)	***0.046***
Scalp	23 (17)	21 (22)	*0.565*
RUE	13 (14)	17 (29)	*0.354*
LUE	12 (9)	11 (22)	*0.797*
Skin Type, no. (%)			*0.154*
I	5 (16)	13 (33)	
II	22 (69)	23 (58)	
III	5 (16)	4 (10)	
Follow-up before trial, mean (SD), d	659 (477)	815 (382)	*0.126*
(Range)	(8-1366)	(24-1331)	
Follow-up after trial, mean (SD), d	1288 (200)	1255 (143)	*0.403*
(Range)	(536-1531)	(1016-1534)	
History of BCC before trial, *n* (%)			*0.829*

0	20 (63)	24 (60)	
≥1	12 (38)	16 (40)	
History of SCC before trial, *n* (%)			*0.826*
0	20 (63)	26 (65)	
≥1	12 (38)	14 (35)	
History of KC before trial, *n* (%)			*0.711*
0	15 (47)	17 (43)	
≥1	17 (53)	23 (58)	
History of 5-FU treatment before trial, *n* (%)			*0.203*
0	23 (72)	23 (58)	
≥1	9 (28)	17 (43)	
History of PDT treatment before trial, *n* (%)			*0.870*
0	19 (59)	27 (68)	
≥1	13 (41)	13 (33)	

5-FU, 5-fluorouracil; RUE, right upper extremity; LUE, left upper extremity; AK, actinic keratosis; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; KC, keratinocyte cancer; PDT, photodynamic therapy.

Supplemental Table 2. AK treatments that participants received after clinical trial.

	Calcipotriol + 5-FU	Vaseline + 5-FU	*P value*
	(n=41)	**(n=45)**	
Frequency of cryotherapy on any location, *mean (SD)*			
	26 (24)	22 (21)	*0.327*
5-FU monotherapy on any treated location, *n* (%)			
0	23 (56)	23 (51)	*0.643*
≥1	18 (44)	22 (49)	
5-FU monotherapy on treated face or scalp, *n* (%)			
0	17 (53)	20 (50)	*0.792*
≥1	15 (47)	20 (50)	
PDT on any treated location *n* (%)			*0.614*
0	29 (71)	34 (76)	
≥1	12 (29)	11 (24)	
PDT on treated face or scalp, *n* (%)			
0	21 (66)	31 (78)	*0.264*
≥1	11 (34)	9 (23)	
Field treatment on any treated location, *n* (%)			*0.607*
0	13 (32)	12 (27)	
≥1	28 (68)	33 (73)	
Field treatment on treated face or scalp, *n* (%)			*0.556*
0	10 (31)	10 (25)	
≥1	22 (69)	30 (75)	

5-FU, 5-fluorouracil. PDT, photodynamic therapy.

Supplemental Table 3. Cancer outcomes on all the treated anatomical sites.

		Calcipotriol + 5-FU	**Vaseline + 5-FU**	**Hazard Ratio**	***P Value***
	Type of lesion	Participants With ≥1 Lesion, *n* (%)	Participants With ≥1 Lesion, *n* (%)	HR (95% CI)	
Year 1					
	KC	7 of 41 (17)	6 of 45 (13)	1.306 (0.439 - 3.886)	*0.766*
	BCC	5 of 41 (12)	3 of 45 (7)	1.930 (0.461 - 8.075)	*0.470*
	SCC	2 of 41 (5)	4 of 45 (9)	0.531 (0.097 - 2.897)	*0.678*
Year 2					
	KC	12 of 40 (30)	12 of 45 (27)	1.034 (0.456 - 2.343)	*1.000*
	BCC	8 of 40 (20)	5 of 45 (11)	1.921 (0.628 - 5.871)	*0.367*
	SCC	5 of 40 (13)	9 of 45 (20)	0.577 (0.193 - 1.722)	*0.395*
Year 3					
	KC	16 of 39 (41)	15 of 45 (33)	0.837 (0.384 - 1.822)	*0.644*
	BCC	8 of 39 (21)	5 of 45 (11)	1.951 (0.638 - 5.965)	*0.365*
	SCC	6 of 39 (15)	13 of 45 (29)	0.488 (0.186 - 1.285)	*0.193*

5-FU, 5-fluorouracil; KC, keratinocyte cancer; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

References:

1. Lian CG, Bueno EM, Granter SR, Laga AC, Saavedra AP, Lin WM, et al. Biomarker evaluation of face transplant rejection: association of donor T cells with target cell injury. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc.* 2014;27(6):788-99.
2. Cendales LC, Kanitakis J, Schneeberger S, Burns C, Ruiz P, Landin L, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 2008;8(7):1396-400.
3. Cunningham TJ, Tabacchi M, Eliane JP, Tuchayi SM, Manivasagam S, Mirzaalian H, et al. Randomized trial of calcipotriol combined with 5-fluorouracil for skin cancer precursor immunotherapy. *The Journal of clinical investigation.* 2017;127(1):106-16.