

1 **OX40⁺ regulatory T cells in cutaneous squamous cell carcinoma suppress**
2 **effector T cell responses and associate with metastatic potential**

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1 **Abstract**

2 **Purpose:** Cutaneous squamous cell carcinoma (cSCC) is the most common
3 human cancer with metastatic potential. Despite T cells accumulating around cSCCs,
4 these tumors continue to grow and persist. To investigate reasons for failure of T
5 cells to mount a protective response in cSCC, we focused on regulatory T cells
6 (Tregs) as this suppressive population is well represented among the infiltrating
7 lymphocytes.

8 **Experimental Design:** Flow cytometry was conducted on cSCC lymphocytes and
9 *in vitro* functional assays were performed using sorted tumoral T cells. Lymphocyte
10 subsets in primary cSCCs were quantified immunohistochemically.

11 **Results:** FOXP3⁺ Tregs were more frequent in cSCCs than in peripheral blood
12 ($p < 0.0001$, $n = 86$ tumors). Tumoral Tregs suppressed proliferation of tumoral effector
13 CD4⁺ ($p = 0.005$, $n = 10$ tumors) and CD8⁺ T cells ($p = 0.043$, $n = 9$ tumors) and inhibited
14 interferon- γ secretion by tumoral effector T cells ($p = 0.0186$, $n = 11$ tumors). The
15 costimulatory molecule OX40 was expressed predominantly on tumoral Tregs
16 ($p < 0.0001$, $n = 15$ tumors) and triggering OX40 with an agonist anti-OX40 antibody
17 overcame the suppression exerted by Tregs, leading to increased tumoral effector
18 CD4⁺ lymphocyte proliferation ($p = 0.0098$, $n = 10$ tumors). Tregs and OX40⁺
19 lymphocytes were more abundant in primary cSCCs which metastasized than in
20 primary cSCCs which had not metastasized ($n = 48$ and $n = 49$ tumors respectively).

21 **Conclusions:** Tregs in cSCCs suppress effector T cell responses and are
22 associated with subsequent metastasis, suggesting a key role for Tregs in cSCC
23 development and progression. OX40 agonism reversed the suppressive effects of

1 Tregs *in vitro*, suggesting that targeting OX40 could benefit the subset of cSCC
2 patients at high risk of metastasis.

3

4 **Translational relevance**

5 Cutaneous squamous cell carcinoma (cSCC) is a highly prevalent cancer which is
6 capable of metastasizing despite treatment by surgical excision. Alterations in
7 immunity greatly influence cancer development, especially cSCC development. As
8 cSCCs are heavily infiltrated with T cells, cSCCs form an attractive model to study
9 cancer T cell immunology. In this study, increased Treg frequencies were identified
10 in cSCCs compared with peripheral blood. Moreover, tumoral Tregs suppressed
11 tumoral effector T cell responses *in vitro*, indicating a role for Tregs in preventing
12 effector T cell response against this tumor. Significantly more Tregs were observed
13 in primary cSCCs which had metastasized than in primary cSCCs which had not
14 metastasized, suggesting that tumoral Tregs may influence clinical outcome in this
15 tumor. Additionally, an agonistic anti-OX40 antibody enhanced tumoral CD4⁺ effector
16 T cell responses *in vitro*, supporting a rationale for investigating OX40 activation as a
17 potential immunotherapeutic strategy for cSCC.

18

19

1 **Introduction**

2 Non melanoma skin cancer (NMSC), which comprises cutaneous squamous cell
3 carcinomas (cSCCs) and basal cell carcinomas (BCCs), is the most common type of
4 cancer and greatly impacts on global health, with estimated annual incidences of 2.8
5 million cSCCs and 10 million BCCs worldwide (1). In the USA alone, 3.5 million
6 NMSCs arise each year, with cSCCs accounting for 700,000 of these tumors (2).
7 Skin cancer creates a substantial economic burden and, in 2004, the estimated cost
8 of skin cancer and precancerous skin lesions to US health services was \$6.6 million
9 (3). Despite treatment with surgery, cSCC poses problems because of its propensity
10 to metastasize, occurring in 16 - 30% of tumors which have invaded 6mm in depth or
11 over 2 cm in diameter (4, 5).

12 cSCCs are keratinocyte-derived neoplasms associated with chronic exposure to
13 ultraviolet radiation, which causes genetic alterations within the epidermis and
14 promotes squamous cell carcinogenesis and subsequent metastases (6-8).
15 Alterations in immunity have a key role in regulating cutaneous carcinogenesis, as
16 evidenced by immunosuppression greatly increasing the incidence of cSCCs, with
17 these tumors 65-250 fold more frequent in organ transplant recipients (9). Indeed,
18 cSCC constitutes an ideal tumor model for investigating cancer immunology because
19 most cSCCs are surrounded by an immune cell infiltrate, yet this infiltrate is generally
20 incapable of mounting a successful anti-tumor response. Tregs have a physiological
21 function in maintaining immunological self-tolerance and homeostasis, however, in
22 certain cancers increased numbers of tumor infiltrating Tregs are associated with
23 poorer clinical outcome, suggesting that Tregs suppress immune responses within
24 the tumoral environment (10-13). As a result of this, there has been considerable
25 interest in developing immunotherapeutic strategies that target Tregs and boost

1 effector immune responses in cancer (14-18). One such approach involves the
2 provision of costimulatory signals through receptors that belong to the tumor
3 necrosis receptor superfamily, including OX40. Engagement of this receptor has
4 been shown to promote T cell activation through effects on different subpopulations
5 of T cells, for example by promoting proliferation and survival of effector memory T
6 cells following antigenic activation, as well as by suppressing regulatory T cell
7 activity (16-19). These effects highlight OX40 as a promising target for tumor therapy,
8 and cases of tumor regression have been recently reported in a phase I clinical trial
9 with anti-OX40 agonistic antibody (20).

10 In this study, we show that cSCCs contain a higher proportion of Tregs than
11 peripheral blood from the same individuals, and that these tumoral Tregs suppress
12 tumoral effector T cell proliferation and interferon- γ secretion. In addition, increased
13 expression of the costimulatory receptor OX40 was noted on cSCC Tregs and
14 activation of OX40 enhanced tumoral CD4⁺ effector T cell responses. Furthermore,
15 increased frequencies of Tregs and OX40⁺ lymphocytes are observed in primary
16 cSCCs which subsequently metastasize. The results demonstrate that OX40⁺ Tregs
17 in cSCCs are immunosuppressive and are associated with a propensity of cSCCs to
18 metastasize.

19

20 **Materials and Methods**

21 **Immunohistochemistry**

22 Ethical approval for the study was provided by the South Central Hampshire B
23 NRES Committee (reference number 07/H0504/187). Archived formalin-fixed

1 paraffin-embedded (FFPE) cSCC samples were obtained from University Hospital
2 Southampton NHS Foundation Trust. Immunohistochemistry was performed as
3 described previously (21), with microwave antigen retrieval performed in either 10
4 μ M citrate or 1.6 μ M EDTA buffers. Primary antibodies included anti-CD3 (1:200,
5 DAKO), anti-CD4 (1:100, DAKO), anti-CD8 (1:20, Abcam), anti-CD25 (1:50,
6 Novocastra), anti-FOXP3 (1:50, Abcam) and anti-OX40 (1:50, BD Biosciences), and
7 biotinylated goat anti-mouse (1:200, DAKO) or swine anti-rabbit (1:400, DAKO) were
8 used as secondary antibodies. For cell quantification, 5 representative images at 40x
9 magnification were taken from each immunostained cSCC section and analysed
10 using ImageJ software.

11

12 **Immunofluorescence / confocal microscopy**

13 Tissue from freshly excised cSCC was obtained from patients during surgery at the
14 Dermatology Department, University Hospital Southampton NHS Foundation Trust.
15 The tissue samples (n=15 tumors) were snap frozen in liquid nitrogen, embedded in
16 OCT medium, cryosectioned and immunostained as described previously (22).
17 Primary antibodies included anti-CD3 (1:200, DAKO), anti-CD4 (1:50, Abcam), anti-
18 CD8 (1:20, Invitrogen), anti-FOXP3 (1:20, eBioscience), anti-cytokeratin 16 (1:20,
19 Thermo Scientific), anti-cytokeratin 17 (1:20, DAKO), anti CD31 (1:200, eBioscience),
20 anti-CLA (1:200, Biolegend), anti-E-selectin (1:20, R & D Systems) and anti-OX40
21 (1:200, BD Biosciences). Fluorophore conjugated secondary antibodies comprised
22 Alexa Fluor 488 goat anti-mouse IgG1a, Alexa Fluor 488 goat anti-rat IgM, Alexa
23 Fluor 555 goat anti-rabbit IgG, Alexa Fluor 555 goat anti-rat IgG and Alexa Fluor 633
24 goat anti-mouse IgG2a (all from Invitrogen). Tissue sections were counterstained

1 with DAPI (Sigma) before being mounted in Mowiol 4-88 (Harco) and imaged using a
2 Zeiss Axioskop 2 fluorescence microscope or a Leica SP5 confocal microscope.

3

4 **Lymphocyte isolation / flow cytometry**

5 Following formal surgical excision of the cSCC (n=93 tumors), part of the fresh
6 tumor sample was taken and cut into small pieces, then enzymatically digested with
7 1 mg/ml collagenase I-A (Sigma) and 10 µg/ml DNase I (Sigma) in RPMI medium
8 (Gibco) at 37°C for 1.5 hours. The resulting suspension was passed through 70µm
9 cell strainers (BD) and centrifuged in Optiprep (Axis-Shield). Peripheral blood
10 mononuclear cells (PBMCs) were isolated from venous blood from the cSCC
11 patients by centrifugation with Lymphoprep (Axis-Shield). The following fluorophore
12 conjugated antibodies were used for flow cytometry: anti-CD3 (APC Cy7, Biolegend),
13 anti-CD4 (PerCP Cy5.5 or FITC, Biolegend), anti-CD8 (PE Cy7, Biolegend), anti-
14 CD25 (PE, Invitrogen), anti-CD127 (Brilliant Violet 421, Biolegend), anti-FOXP3
15 (APC, eBioscience), anti-CD45RO (PerCP Cy5.5, Biolegend), anti-Helios (PerCP
16 Cy5.5, Biolegend), anti-CLA (Brilliant Violet 421, BD Biosciences), anti-CCR4
17 (PerCPCy5.5, Biolegend or FITC, R&D Systems) and anti-OX40 (PE, eBioscience).
18 For cell surface staining, cells were incubated with the antibodies in the dark for 30
19 minutes at 4°C in PBS + 1% BSA + 10% FBS. For intracellular markers, a FOXP3
20 staining buffer set (eBioscience) was used to fix and permeabilize cells prior to
21 staining (23, 24). An aqua live/dead viability stain (Invitrogen) was added before flow
22 cytometry using a BD FACSAria.

23

1 **Generation of anti-human OX40 monoclonal antibodies (mAb)**

2 For the production of anti-hOX40 mAb, mice were immunized with hOX40-hFc
3 fusion protein and 293F cells transfected with hOX40 (primary fusion protein in
4 complete Freund's adjuvant, s.c.; secondary transfected cells in incomplete, s.c.;
5 final boost fusion protein, i.v.). Spleen cells from the immunized mice were fused
6 with NS-1 cells using conventional hybridoma technology. Plates were screened by
7 ELISA using hOX40-hFc fusion protein and positive wells tested for binding to
8 hOX40-transfected 293F cells. Agonist mAb were identified by their ability to
9 enhance T cell proliferation using cultured peripheral blood mononuclear cells
10 obtained from healthy volunteers that were stimulated with sub-optimal
11 concentrations of anti-CD3 mAb.

12

13 **Functional assays**

14 Tumoral CD3⁺CD4⁺CD25^{high}CD127^{low} Tregs and CD3⁺CD4⁺CD25^{low} or CD3⁺CD8⁺
15 effector T cells were sorted by FACS using the "purity" setting. 96 well U-bottomed
16 plates were used for co-culture assays and all functional experiments were
17 performed using triplicate wells. Tumoral effector T cells (2,500 per well) were co-
18 cultured with/without tumoral Tregs (1,250 per well) in the presence of irradiated
19 (47Gy) autologous PBMCs (25,000 per well), stimulated with 1 µg/ml
20 phytohemagglutinin (PHA, Sigma) or 1 µg/ml soluble anti-CD3 (eBioscience). After
21 culture for 48 hours, tritiated thymidine based lymphocyte proliferation assays were
22 performed as described previously (25); this method of measuring cell proliferation
23 was used because the limited numbers of lymphocytes isolated from the tumor
24 samples meant that CFSE dilution experiments were not feasible. Interferon-γ

1 enzyme-linked immunospot (ELISPOT) assays were conducted as before (26) with
2 co-cultures of tumoral Tregs, effector T cells and CD3⁺ cell depleted irradiated
3 autologous PBMCs stimulated as above. For experiments with anti-OX40 antibody,
4 tritiated thymidine uptake and interferon- γ ELISPOT assays were performed with
5 tumoral CD4⁺ T cells cultured with/without anti-OX40 (5 μ g/ml) or mouse IgG1
6 (Biolegend) and stimulated as above in the presence of accessory cells.

7

8 **Statistical analysis**

9 Statistical analysis was conducted using GraphPad Prism software. For functional
10 assays, paired Wilcoxon rank tests were used to assess significance, and for
11 immunohistochemical and flow cytometric quantification of normally distributed data,
12 paired T tests or one way ANOVA with Tukey's test for multiple comparisons were
13 used.

14

15 **Results**

16 **Quantification of Tregs in cSCCs**

17 cSCCs are infiltrated by immune cells, with immunohistochemistry conducted on
18 archived FFPE cSCCs showing accumulation of CD3⁺ T cells in the stroma around
19 islands of tumor keratinocytes (i.e. peritumoral), whilst CD3⁺ T cells within tumor cell
20 nests (i.e. intratumoral) were found less frequently (figure 1A, B and supplementary
21 figure 1A, B). In addition to CD4⁺ T cells and CD8⁺ T cells within the tumoral infiltrate
22 (i.e. combination of peritumoral and intratumoral infiltrate), frequent numbers of
23 CD25⁺ and FOXP3⁺ Treg populations were also present (figure 1C, D;

1 supplementary figure 1C, D). Quantification of the T cell subsets that comprised the
2 tumoral immune infiltrate in 24 cSCCs demonstrated that CD3⁺, CD4⁺ and CD8⁺ T
3 cells accounted for 40.5% ± 10.3%, 36.6% ± 7.1% and 22.0% ± 7.5% of the infiltrate
4 respectively, whilst FOXP3⁺ Tregs comprised 11.0% ± 4.4% of the immune infiltrate,
5 indicating a Treg to CD4⁺ T cell ratio of approximately 1:3 and a Treg: CD8⁺ T cell
6 ratio of 1:2 (figure 1C). To determine whether there were differences in Treg
7 numbers between precancerous and cancerous skin lesions, immunohistochemistry
8 was performed which demonstrated that actinic keratoses (AKs) contained similar
9 FOXP3⁺ Treg frequencies to cSCCs (24.1% ± 14.2% of AK immune infiltrate versus
10 29.2% ± 19.4% of cSCC immune infiltrate, n=44 AKs, n=79 cSCCs, p=0.131, figure
11 1D).

12 Using anti-cytokeratin 16 to delineate the cSCC tumor islands,
13 immunofluorescence microscopy showed the presence of FOXP3⁺ Tregs and CD3⁺
14 T cells in the stroma surrounding the cSCCs with fewer of these cells within the
15 tumor islands (figure 1E, F). Tregs exert suppressive function via contact dependent
16 mechanisms (27), although non-contact mechanisms may also play a role in some
17 instances (28). Confocal microscopy of cSCCs indicated close approximation of
18 tumoral Tregs (identified by nuclear FOXP3 and cell membrane CD4) and
19 CD4⁺FOXP3⁻ T cells and, separately, CD8⁺ T cells (figure 1G), suggesting contact
20 dependent interactions between Tregs and effector T cells in this tumor. Flow
21 cytometry of tumoral immunocytes from freshly-excised cSCCs demonstrated that
22 cSCCs contained lower CD4⁺ T cell frequencies than peripheral blood of the same
23 individuals (55.7% ± 18.7% versus 70.9% ± 17.8% of the live CD3⁺ lymphocyte
24 population respectively, p=0.0211, n=17 tumors, figure 1H, I). By contrast, Tregs
25 were more numerous in cSCCs than the corresponding peripheral blood and non-

1 lesional skin ($19.8\% \pm 8.6\%$ versus $5.5\% \pm 4.1\%$ and $7.3\% \pm 4.1\%$ of the $CD4^+$
2 population respectively expressed FOXP3, $p < 0.0001$ for both comparisons, $n = 86$
3 tumors, figure 1J, K), indicating that in cSCC there is an accumulation of tumoral
4 Tregs, potentially contributing to the protumorigenic microenvironment. Tregs were
5 also significantly more frequent in BCC than in corresponding peripheral blood (16.7%
6 $\pm 7.7\%$ versus $2.8\% \pm 2.2\%$ of $CD4^+$ population for BCC and blood respectively,
7 $p = 0.0045$ $n = 6$, figure 1L). Although Tregs seemed more frequent in
8 keratoacanthoma (KA, a skin lesion that clinically resembles cSCC but is known to
9 regress spontaneously) than in corresponding blood ($11.2\% \pm 7.5\%$ versus $4.4\% \pm$
10 3.3% of $CD4^+$ population for KA and blood respectively, $n = 6$, figure 1L), there were
11 significantly overall fewer Tregs in KA than in cSCC ($p = 0.0321$, supplementary figure
12 1E).

13

14 **Tregs in cSCCs are memory T cells which express the skin homing marker** 15 **CLA**

16 Many of the T cells within normal skin are effector memory T cells which express
17 CD45RO (29). In the current study the majority of FOXP3⁺ Tregs ($87.8\% \pm 11.7\%$)
18 and $CD4^+FOXP3^-$ T cells ($85.7\% \pm 7.3\%$) in cSCCs were $CD45RO^+$ ($n = 8$ tumors),
19 whereas $77.6\% \pm 16.7\%$ of FOXP3⁺ Tregs and $52.7\% \pm 19.3\%$ of $CD4^+FOXP3^-$ T
20 cells in peripheral blood were $CD45RO^+$ ($p = 0.0171$ and $p = 0.0013$ respectively for
21 FOXP3⁺ Tregs and $CD4^+FOXP3^-$ T cells comparison between cSCCs and peripheral
22 blood from same individuals, figure 2A-C). The T cell marker Helios may denote
23 thymically derived natural Tregs (24) but can also be upregulated on activated T
24 cells (30). Flow cytometry established that Helios was expressed at higher levels in

1 tumoral FOXP3⁺ Tregs (47.2% ± 7.9%, n=9 tumors) than peripheral blood FOXP3⁺
2 Tregs (26.7% ± 8.5%, p=0.0022) and tumoral non-regulatory CD4⁺FOXP3⁻ T cells
3 (9.6% ± 5.8%, p<0.0001) from the same subjects, (figure 2D, E).

4 Clark et al (29) have reported that T cells, including Tregs, from cSCCs lack
5 expression of the skin homing addressins, cutaneous lymphocyte antigen (CLA) and
6 C-C chemokine receptor 4 (CCR4). We observed that CLA was expressed by 78.8%
7 ± 13.0% of the FOXP3⁺ Tregs population in cSCCs, which was higher than the
8 proportion of FOXP3⁺ Tregs (40.0% ± 13.7%) expressing CLA in peripheral blood
9 (p<0.0001, n=19 tumors, figure 2F, G). The latter is similar to that reported by Booth
10 et al in blood (23). Likewise, greater numbers of CD4⁺FOXP3⁻ T cells (58.2% ±
11 15.1%) in cSCC expressed CLA than those in peripheral blood (p<0.0001, n=19
12 tumors, figure 2F, H). Furthermore, experiments performed in a separate institution
13 on a different set of cSCCs (n=13) identified CLA positivity in 64.7% ± 31.2% of
14 tumoral CD3⁺ T cells (supplementary figure 2A, B). CCR4 expression was detected
15 on 44.1% ± 32.7% of FOXP3⁺ Tregs in cSCCs, although this was lower than the
16 number of FOXP3⁺ Tregs expressing CCR4 in peripheral blood (72.7% ± 17.8%),
17 (p=0.0139, n=17 tumors, figure 2F, I). Equal numbers of tumoral CD4⁺FOXP3⁻ T
18 cells (25.4% ± 21.4%) and peripheral blood CD4⁺FOXP3⁻ T cells (27.6% ± 19.8%)
19 expressed CCR4 (figure 2J). Further experiments were performed to confirm the
20 expression of CLA and CCR4 in cSCC Tregs; CCR4 was detected on cSCC Tregs at
21 similar levels with an alternate anti-CCR4 antibody and immunofluorescence /
22 confocal microscopy using a different anti-CLA antibody showed CLA⁺ cells in
23 cSCCs (figure 3A) and that the tumoral FOXP3⁺ cells expressed CLA (figure 3B and
24 supplementary figure 2C).

1 CLA permits lymphocyte trafficking to the skin through its binding to E-selectin on
2 cutaneous endothelial cells (29). Immunofluorescence microscopy was performed
3 using cytokeratin (CK) 16 or 17 co-staining which allows the cSCC tumor cells to be
4 visualized. Blood vessels (highlighted by CD31⁺ endothelial cells) were detected in
5 the peritumoral areas of cSCCs with no evidence of intratumoral vessels (figure 3C
6 and supplementary figure 3A, B), and that most of the peritumoral vasculature
7 expressed E-selectin (figure 3D and supplementary figure 4), suggesting that CLA⁺
8 Tregs can readily be directed to the site of the tumor from the blood via interaction
9 with E-selectin on these endothelial cells.

10

11 **cSCC Tregs suppress tumoral effector T cell responses**

12 *In vitro* co-culture experiments with Tregs and effector T cells were performed to
13 investigate cSCC Treg function. cSCC Tregs and effector T cells were co-cultured in
14 a 1:2 ratio based on their relative frequencies observed in the prior
15 immunohistochemical quantification experiments (figure 1C). Tumoral Tregs were
16 identified by expression of CD3, CD4, high levels of CD25 and low levels of CD127
17 and isolated using fluorescence activated cell sorting (figure 4A). Sorted tumoral
18 CD4⁺ effector T cells identified as CD3⁺CD4⁺CD25^{low} and CD8⁺ effector T cells were
19 CD3⁺CD8⁺ (figure 4A). After sorting, a sample of the cells were fixed and
20 permeabilized for analysis of FOXP3 expression, confirming that most of the sorted
21 CD3⁺CD4⁺CD25^{high}CD127^{low} cells were Tregs (figure 4B and supplementary figure
22 5A). In addition, interferon- γ was produced by <4% of tumoral
23 CD3⁺CD4⁺CD25^{high}CD127^{low} cells following PMA and ionomycin stimulation,
24 suggesting that this CD3⁺CD4⁺CD25^{high}CD127^{low} population was minimally

1 contaminated by effector T cells (figure 4C). Tritiated thymidine-based lymphocyte
2 proliferation assays showed that tumoral CD3⁺CD4⁺CD25^{high}CD127^{low} Tregs were
3 able to suppress PHA-induced proliferation of tumoral CD3⁺CD4⁺CD25^{low} effector T
4 cells (median suppression 41.7%, n=10 tumors, figure 4D) and, to a lesser extent,
5 CD3⁺CD8⁺ effector T cells (median suppression 12.6%, p=0.043, n=9 tumors, figure
6 4E). Tumoral Tregs also suppressed proliferation of anti-CD3 stimulated tumoral
7 CD4⁺ effector T cells (median suppression 46.2%, n=4 tumors, supplementary figure
8 5B) and CD8⁺ T cells (median suppression 40.2%, n=4 tumors, supplementary figure
9 5C). In addition, ELISPOT assays demonstrated that tumoral Tregs reduced effector
10 T cell interferon- γ secretion in response to PHA (median inhibition 24.2%, p=0.0186,
11 n=11 tumors, figure 4F). These results indicate that tumoral Tregs from cSCCs can
12 suppress tumoral effector T cell function, and may therefore contribute to an
13 immunosuppressive milieu that prevents immune-mediated destruction of the tumor.

14

15 **OX40 is expressed by cSCC Tregs and OX40 agonism enhances tumoral CD4⁺** 16 **T cell function**

17 As the costimulatory receptor OX40 is expressed on effector and regulatory T cells
18 and can augment T cell receptor signaling (15-19), we next investigated whether
19 OX40 was present on tumoral lymphocytes in cSCC. Immunofluorescence
20 microscopy demonstrated the presence of OX40 predominantly on tumoral FOXP3⁺
21 Tregs (figure 5A). Flow cytometry confirmed FOXP3⁺ Tregs in cSCC expressed
22 OX40 (39.3% \pm 13.6% of FOXP3⁺ Tregs), with significantly more tumoral Tregs
23 expressing OX40 than CD4⁺FOXP3⁻ T cells and CD8⁺ T cells in cSCCs, and
24 FOXP3⁺ Tregs, CD4⁺FOXP3⁻ T cells and CD8⁺ T cells in peripheral blood (p<0.0001

1 for all comparisons, n=15 tumors, figure 5B, C and supplementary figure 5D). To
2 assess if OX40 agonism attenuates the suppressive effects of Tregs in cSCC, we
3 assessed the proliferation of tumoral CD4⁺ T cells from cSCCs in the presence of an
4 agonistic anti-OX40 mAb. The addition of anti-OX40, but not an isotype control
5 mAb, led to enhancement of PHA-induced CD4⁺ T cell proliferation (median increase
6 in proliferation 45%, p=0.0098, n=10 tumors, figure 5D); proliferation of
7 CD4⁺CD25^{high}CD127^{low} Tregs was not increased by anti-OX40 when cultured with
8 PHA in the presence of accessory cells alone (isotype control = 108.5 cpm (IQR
9 68.0-129.5 cpm), anti-OX40 = 107 cpm (IQR 73.3-135.5 cpm), n=4 tumors,
10 supplementary figure 5D). Subsequently, tumoral CD4⁺CD25^{low} effector T cell
11 proliferation was measured following culture with PHA ± anti-OX40 in the absence or
12 presence of tumoral CD4⁺CD25^{high}CD127^{low} Tregs. In cultures containing tumoral
13 CD4⁺CD25^{low} T cells without Tregs, median cell proliferation increased by 5.3% with
14 the addition of anti-OX40 compared with isotype control, whereas in cultures
15 containing tumoral CD4⁺CD25^{low} T cells and Tregs, the improvement in effector T
16 cell function with the addition of anti-OX40 was more apparent (median increase in
17 cell proliferation = 252.4% compared with isotype control, p=0.0313, n=5 tumors,
18 figure 5E and supplementary figure 5E). Similar results were also observed when
19 anti-CD3 was used as a stimulus instead of PHA, with anti-OX40 increasing tumoral
20 effector CD4⁺ T cell proliferation from 5474 to 6572 cpm (20.1%) when Tregs were
21 absent, and from 2906 to 5263 cpm (81.1%), when Tregs were present, n=4 tumors
22 (supplementary figure 5F). Furthermore, the increase in interferon-γ spot production
23 by tumoral CD4⁺ effector T cells with the addition of anti-OX40 was more apparent in
24 the presence of tumoral Tregs (53.5 with isotype versus 93.7 with anti-OX40) than in
25 the absence of tumoral Tregs (114.2 with isotype to 131.5 with anti-OX40, figure 5F).

1 Taken in combination with the fact that OX40 was found mainly on Tregs in cSCCs,
2 these results suggest that OX40 agonism reduces Treg-mediated suppression of
3 tumoral CD4⁺ T cell responses.

4

5 **Increased Treg frequencies are associated with primary cSCCs which**
6 **metastasize**

7 In order to determine whether Tregs in cSCCs are associated with poorer clinical
8 outcome, Tregs were quantified in archived FFPE primary cSCCs that had
9 metastasized and in cSCCs that were known not to have metastasized after 5 years.
10 As expected, histological data demonstrated differences in known prognostic factors
11 between the metastatic and non-metastatic groups, with primary metastatic cSCCs
12 being larger, invading deeper and being more poorly differentiated than non-
13 metastatic cSCCs (figure 6A). Nevertheless, there were significantly higher
14 frequencies of FOXP3⁺ Tregs in primary cSCCs that metastasized than those that
15 did not metastasize (49.3% ± 13.8% versus 23.5% ± 11.0% of immune infiltrate
16 respectively, p<0.0001, n=29 and 26 tumors respectively) despite similar frequencies
17 of CD3⁺ T cells in both groups (figure 6B-D), suggesting that increased Treg
18 numbers in primary cSCCs may influence the development of subsequent
19 metastasis. OX40 expressing cells were also quantified in FFPE primary cSCCs,
20 with increased percentages of tumoral immunocytes expressing OX40 observed in
21 primary metastatic cSCCs (17.0% ± 10.7% of immune infiltrate, n=48 tumors) than
22 primary non-metastatic cSCCs (11.7% ± 6.9% of immune infiltrate, n=49 tumors,
23 p=0.0041, figure 6B, E).

24

1 **Discussion**

2 Factors which predispose to skin cancer development in humans include light skin
3 pigmentation secondary to genetic polymorphisms in genes such as *MC1R*,
4 ultraviolet radiation (UV) exposure which causes DNA damage/gene mutation in
5 relevant skin cells and altered/ suppressed immunity (6-8, 21, 31). cSCCs have one
6 of the highest mutational burdens compared with other tumor types (6-8) and high
7 frequencies of UV-induced mutations could result in neoantigens that can be
8 recognized by T cells (32). Indeed, the importance of the immune system in cSCC
9 development is exemplified by the markedly increased prevalence of this tumor type
10 in immunosuppressed individuals (9). However, the fact that cSCCs are frequently
11 accompanied by a substantial immune infiltrate in most individuals (including non-
12 immunosuppressed subjects who constitute the majority of cSCC cases) highlighted
13 a need to characterize this infiltrate from a functional perspective. Based on the
14 viewpoint that local cutaneous immunity plays a key role in preventing cSCC
15 development and that dysregulation of this local immunity may allow the expansion
16 of neoplastic keratinocytes to go unchecked, we investigated the Treg population
17 within cSCCs. Our findings demonstrate that the cSCC tumoral immune infiltrate
18 contains higher Treg frequencies than peripheral blood, which is in keeping with
19 studies indicating that Tregs may accumulate in various tumors via several
20 mechanisms and with reports that exposure of skin to UV is conducive to the
21 generation of Tregs (33). Functionally immunosuppressive Tregs have been reported
22 in human cancer previously (10, 34-36) and this current study confirms that cSCC
23 tumoral Tregs have the capacity to suppress tumoral effector T cell function *in vitro*,
24 therefore highlighting Tregs as a mechanism by which cSCCs can avoid destruction
25 by the skin immune system. In addition, higher Treg frequencies were found to be

1 associated with primary cSCCs which had metastasized rather than primary cSCCs
2 which had not metastasized, indicating that Tregs may play a role in allowing cSCC
3 to metastasize and thus are of potential prognostic importance.

4 This study showed no difference in Treg numbers between actinic keratoses and
5 cSCC, as reported previously (37), suggesting that Tregs accumulate during a
6 precursor lesion stage, i.e. actinic keratoses, rather than being a determinant of
7 progression from actinic keratosis to cSCC. However, we found an association
8 between higher Treg frequencies in primary cSCCs and subsequent metastasis, in
9 keeping with studies in other cancer types where Tregs have been shown to promote
10 metastasis and influence clinical outcome (10-13, 38-40). Related to this, although
11 we found an association between the histological differentiation status of cSCC and
12 Treg infiltration, which was similar to a recent study (41), the association did not
13 remain after excluding cSCCs with known clinical outcome (i.e. had metastasized or
14 not metastasized at five years) (supplementary figure 6), suggesting a more robust
15 association was present between Treg infiltration and metastasis than that with
16 cSCC differentiation status in our study.

17 Although Gelb et al reported that lymphocytes infiltrating cSCC express CLA (42),
18 a more recent investigation recorded that T cells infiltrating cSCCs are non-
19 cutaneous central memory T cells which lack expression of CLA and CCR4 (29). Our
20 phenotypic characterization of tumoral Tregs in cSCC showed that most expressed
21 CD45RO and CLA, indicating a skin resident memory phenotype (which is the
22 predominant T cell phenotype in skin) in cSCCs. We confirmed the expression of
23 CLA on cSCC Tregs using both flow cytometry and immunofluorescence microscopy
24 with two different anti-CLA antibodies (from BD Biosciences and Biolegend).
25 However, our results demonstrate a high degree of variability in CLA and CCR4

1 expression between tumors, suggesting heterogeneous T cell responses in cSCC,
2 possibly accounting for the differences between previous studies. In the context of
3 the E-selectin expression by the majority of the peritumoral blood vessels, our
4 findings signify that Tregs are generally recruited to cSCCs via CLA on the Treg
5 surface interacting with E-selectin on the tumoral vasculature. This is akin to T cell
6 recruitment in inflammatory skin conditions including psoriasis (43), atopic dermatitis
7 (44) and contact dermatitis (45) as well as in other cutaneous neoplasms such as
8 melanoma (42).

9 There has been considerable interest in developing cancer treatments that use
10 monoclonal antibodies which act on T cell costimulatory pathways to augment
11 antitumor immunity (15-20). Indeed, effector T cell responses can be modulated
12 through engaging costimulatory receptors which can attenuate Treg suppressive
13 ability (15-19, 46). *In vitro* studies of human peripheral blood T cells have
14 demonstrated that anti-OX40 mAbs can be agonistic which can enhance the
15 resistance of effector T cells to suppression and reduce the suppressive activity of
16 Tregs (19). In addition, anti-OX40 mAbs could stimulate anti-tumor immunity by
17 preferential depletion of Tregs, as shown in pre-clinical studies (17, 18). Furthermore,
18 a phase I clinical trial using an anti-OX40 agonistic monoclonal antibody has shown
19 an acceptable safety/toxicity profile and some evidence of tumor regression in cases
20 of melanoma, renal cancer, urethral squamous cell carcinoma, prostate cancer and
21 cholangiocarcinoma (20). In our study, the tumoral Tregs were the T cell subset that
22 expressed OX40 at the highest frequencies, consistent with observations from
23 studies on murine tumors which used anti-OX40 antibodies to deplete intratumoral
24 Tregs and enhance anti-tumor immunity (16-18), suggesting a similar strategy could
25 be beneficial in cSCC. OX40 has been demonstrated on CD4⁺ tumor-infiltrating

1 lymphocytes in melanoma and head and neck cancer (47), and whereas a recent
2 study has documented the presence of OX40 expressing lymphocytes in cSCC (48),
3 our work highlights that this molecule is predominantly expressed by Tregs in this
4 cancer. Furthermore, our study shows that higher percentages of tumor infiltrating
5 lymphocytes express OX40 in primary metastasizing cSCCs compared with primary
6 tumors which had not metastasized. While this contrasts with some other cancers in
7 which OX40 expression correlates with improved prognosis (49-51), much higher
8 proportions of tumoral Tregs expressed OX40 than the tumoral CD4⁺FOXP3⁻ and
9 CD8⁺ T cells in our cSCC population. Furthermore, using an *in vitro* culture model,
10 enhanced tumoral CD4⁺ T cell responses were observed with addition of an OX40
11 agonist, suggesting that the use of an OX40 agonist approach *in vivo* may have
12 potential benefits in patients with cSCCs at high risk of metastasizing after surgical
13 excision. Admittedly, although OX40 agonism had little effect on enhancing
14 proliferation of tumoral CD4⁺ effector T cells and interferon- γ production in the
15 absence of Tregs, it is unclear whether part of the effect of OX40 agonism could be
16 mediated by the presence of OX40⁺FOXP3⁻ cells in the sorted
17 CD3⁺CD4⁺CD25^{high}CD127^{low} population (supplementary figure 5D). This is because,
18 due to FOXP3 being an intracellular stain, it was not possible to isolate the FOXP3⁻
19 fraction of the CD3⁺CD4⁺CD25^{high}CD127^{low} population from human cSCCs and
20 perform functional studies using this subset alone. Neither is it clear whether the
21 effect of OX40 agonism is mediated by OX40⁺CD4⁺ effector T cells in overcoming or
22 influencing Treg suppression, however, our results indicate that the effect of OX40
23 agonism in boosting the effector T cell response is only seen in the presence of the
24 CD3⁺CD4⁺CD25^{high}CD127^{low} Treg population.

1 In conclusion, this study demonstrates that cSCCs contain an abundance of Tregs
2 which can suppress tumoral effector T cell function and that activation of the
3 costimulatory receptor OX40 enhances tumoral T cell responses. Primary cSCCs
4 that metastasize are associated with higher Treg frequencies, therefore providing
5 evidence that Tregs play a key role in the pathogenesis of cSCC.

6

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19

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- 10

1 **Figure legends**

2

3 **Figure 1:** Tregs accumulate in cSCC and cluster with CD4⁺ and CD8⁺ T cells
4 predominantly in the peritumoral stroma. Immunohistochemistry of cSCC
5 demonstrating CD3⁺ T cells in (A) intratumoral and (B) peritumoral areas. (C)
6 Immunohistochemical quantification of T cell subsets in 24 cSCCs. (D) No
7 differences in frequencies of infiltrating FOXP3⁺ cells were observed between actinic
8 keratoses (AKs, n=44) and cSCCs (n=79). Immunofluorescence staining
9 demonstrating (E) CD3⁺ T cells and (F) FOXP3⁺ Tregs in cSCC. Cytokeratin (CK) 16
10 staining highlights tumor keratinocytes. (G) Confocal microscopy of CD4⁺ T cells,
11 CD8⁺ T cells and CD4⁺FOXP3⁺ T cells in cSCC, boxes highlight examples of
12 CD4⁺FOXP3⁺ Tregs (red membrane, green nucleus) in close contact with CD8⁺ T
13 cells (blue membrane, grey nucleus) and CD4⁺FOXP3⁻ T cells (red membrane, grey
14 nucleus). (H) Flow cytometry plots of CD3⁺ gated lymphocytes isolated from
15 peripheral blood and matched cSCC demonstrating CD4 and FOXP3 expression. (I)
16 Aggregate data showing fewer CD4⁺ T cells as a proportion of the CD3⁺ population
17 in cSCCs compared with blood (n=17). (J) CD3⁺CD4⁺ gated lymphocyte populations
18 in blood and corresponding cSCC plotted for FOXP3 and CD25 expression. (K) Data
19 from 32 cSCCs demonstrating higher FOXP3⁺ Treg frequencies as a percentage of
20 the CD4⁺ T cell population in cSCC than blood. (L) FOXP3⁺ Treg frequencies in
21 keratoacanthoma (KA), basal cell carcinoma (BCC) and corresponding blood. In
22 images A, B, E, F & G, scale bars = 50 μm, dashed lines indicate tumor outlines. In
23 graphs C & D, dots = mean cell frequencies in 5 separate 40x fields for each lesion,
24 horizontal bars = mean values for all lesions. In graphs I - L, horizontal bars = means.

1

2 **Figure 2:** Phenotypic characterization of cSCC Tregs. (A) FACS plots showing
3 CD45RO expression on CD3⁺CD4⁺ gated lymphocyte populations from peripheral
4 blood and cSCC. CD45RO positivity as a percentage of (B) CD4⁺FOXP3⁺ Treg and
5 (C) CD4⁺FOXP3⁻ T cell populations from blood and cSCC (n=8), showing that these
6 populations in cSCCs are mainly CD45RO⁺ memory T cells. (D) Representative
7 histograms demonstrating Helios expression in CD3⁺CD4⁺FOXP3⁺ gated
8 lymphocytes from peripheral blood and corresponding cSCC. Grey shaded areas
9 represent isotype control. (E) Percentage of tumoral CD4⁺FOXP3⁺ Tregs, tumoral
10 CD4⁺FOXP3⁻ T cells and peripheral blood CD4⁺FOXP3⁺ Tregs that are Helios⁺ (n=9).
11 (F) Representative plots demonstrating CLA and CCR4 expression in CD3⁺ and
12 CD3⁺CD4⁺FOXP3⁺ gated lymphocytes from peripheral blood and cSCC from the
13 same subject. Graphs showing aggregate data for CLA expression in peripheral
14 blood and tumoral (G) CD3⁺CD4⁺FOXP3⁺ Tregs and (H) CD3⁺CD4⁺FOXP3⁻ T cells
15 (n=19), and CCR4 expression in peripheral blood and tumoral (I) CD3⁺CD4⁺FOXP3⁺
16 Tregs and (J) CD3⁺CD4⁺FOXP3⁻ T cells (n=19). Horizontal bars on graphs B, C, E,
17 G, H, I & J = means.

18

19 **Figure 3:** cSCC Tregs express the skin homing marker CLA. (A)
20 Immunofluorescence microscopy of cSCC cryosections showing CLA⁺ tumoral
21 immunocytes, cytokeratin (CK) 16 highlights tumor keratinocytes. (B)
22 Immunofluorescence microscopy demonstrating CLA⁺ FOXP3⁺ Tregs in cSCC. (C)
23 CD31⁺ staining highlighting peritumoral blood vessels in cSCC stroma, cytokeratin
24 (CK) 17 positivity indicates tumor keratinocytes. (D) Sequential cSCC sections

1 showing e-selectin expression in the peritumoral vasculature (highlighted by CD31
2 staining) between cytokeratin (CK) 16 positive tumor islands. Arrows in the right
3 hand boxes, which depict higher power images of the merged images, indicate the
4 same blood vessels in sequential sections. Dashed lines represent the outlines of
5 the tumor in A - D, scale bars = 50 μ m.

6

7 **Figure 4:** cSCC Tregs suppress PHA-induced tumoral effector T cell responses *in*
8 *vitro*. (A) Representative flow cytometry gating strategy for isolating cSCC Tregs and
9 effector T cells. Highlighted box in the left FACS plot shows the CD3⁺CD4⁺
10 population, and sub-gating of this population is displayed in the right FACS plot,
11 where the sorted CD25^{high}CD127^{low} Treg population is shown in the highlighted area.
12 Effector T cells were CD3⁺CD4⁺CD25^{low} or CD3⁺CD8⁺. (B) The majority of sorted
13 CD3⁺CD4⁺CD25^{high}CD127^{low} cells expressed FOXP3, consistent with their Treg
14 status. (C) Sorted CD3⁺CD4⁺CD25^{high}CD127^{low} cells were stimulated with PMA and
15 ionomycin for 5 hours and intracellular flow cytometry was performed for FOXP3 and
16 interferon- γ . (D-F) Tumoral effector T cells were co-cultured in the presence of
17 autologous irradiated PBMCs and 1 μ g/ml PHA with/without the addition of tumoral
18 Tregs. Tritiated thymidine uptake was used to assess (C) tumoral CD4⁺ T cell
19 proliferation, n=10 tumors, (D) tumoral CD8⁺ T cell proliferation, n=9 tumors and
20 ELISPOT assays to determine (E) tumoral effector T cell interferon- γ secretion, n=11
21 tumors. In C-E, dots = median values for each tumor from triplicate well experiments,
22 horizontal bars = median values for all tumors.

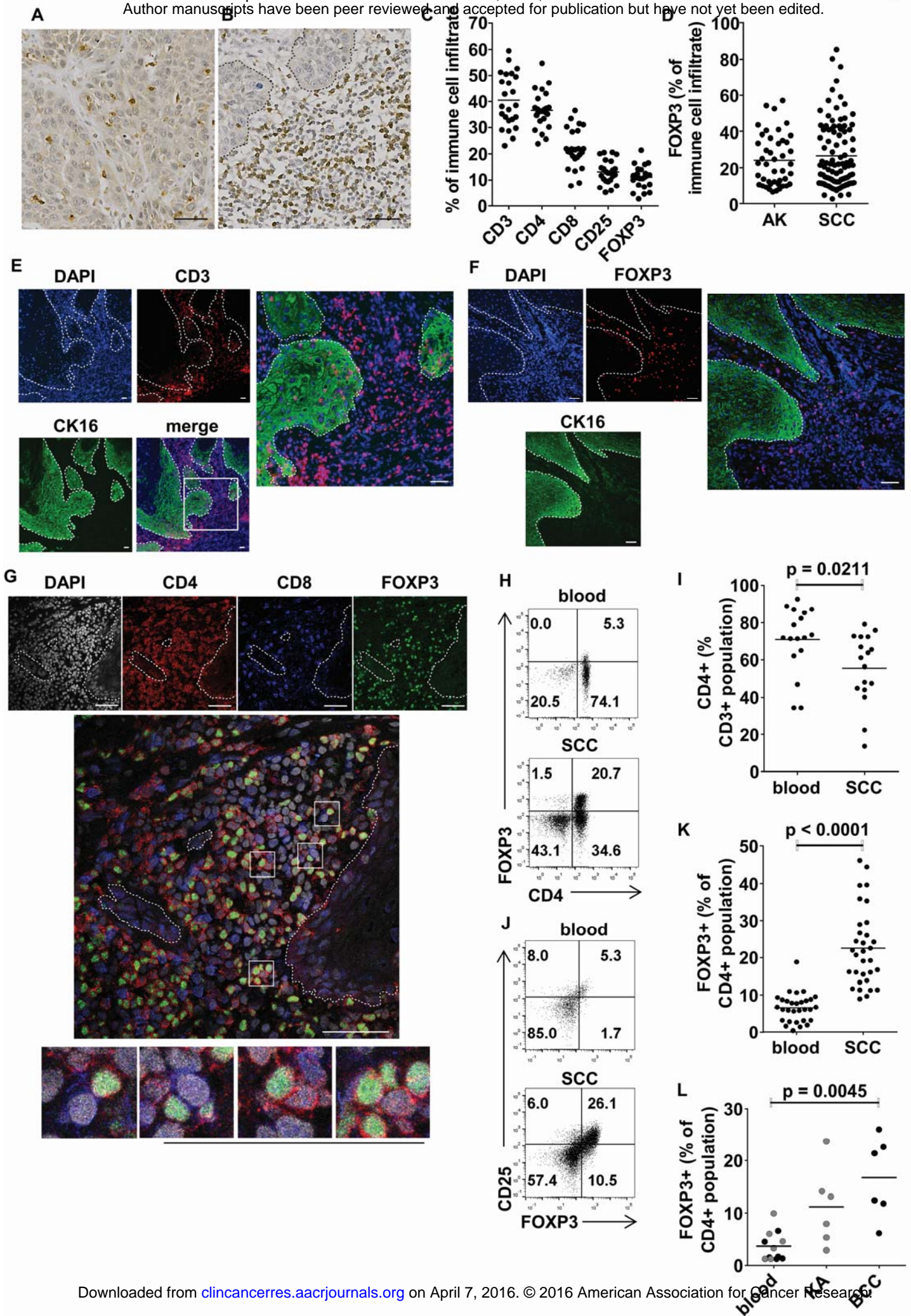
23

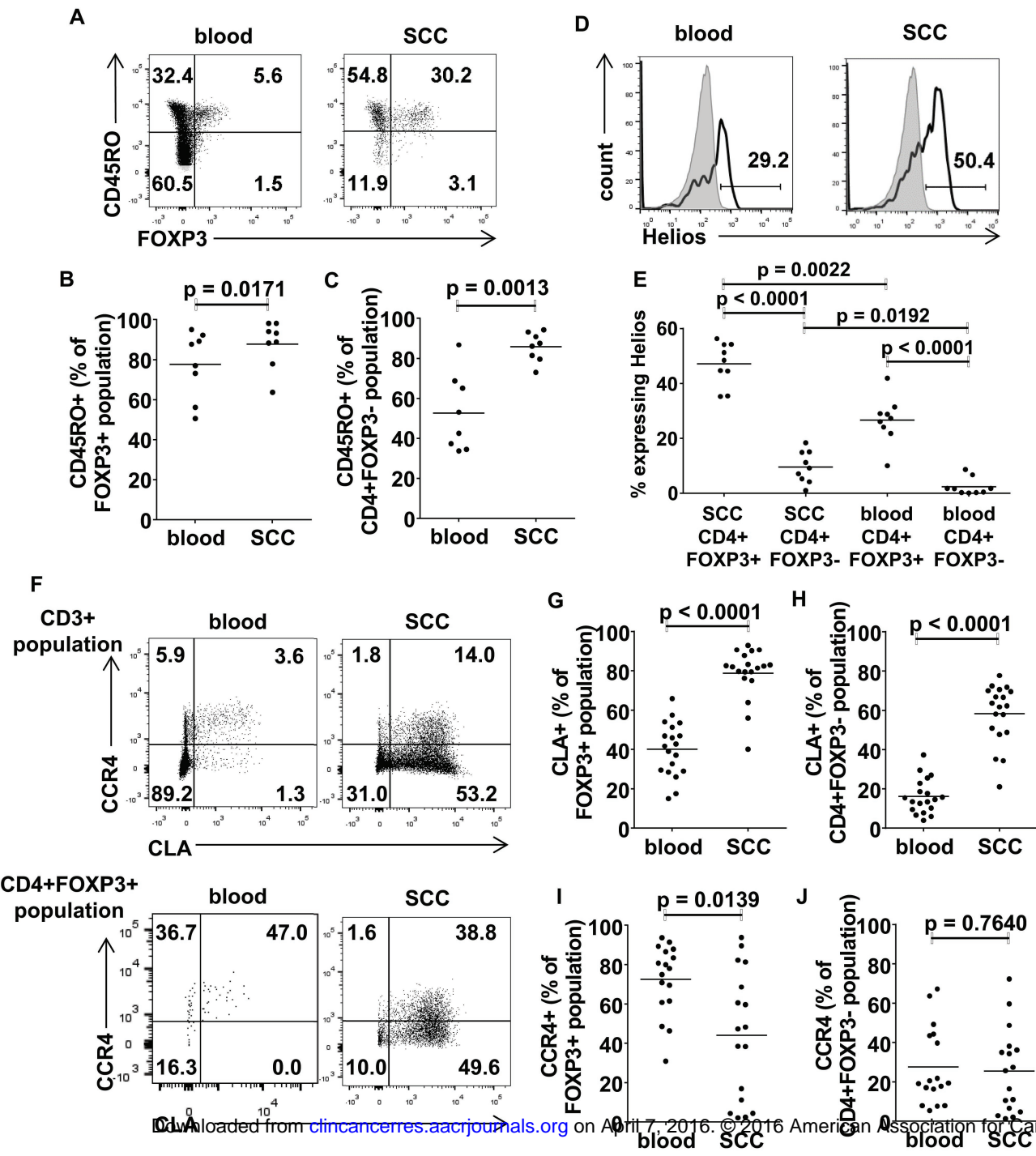
1 **Figure 5:** OX40 is expressed by Tregs in cSCC and *in vitro* OX40 activation
2 enhances tumoral CD4⁺ T cell proliferation. (A) Immunofluorescence microscopy
3 showing OX40 on tumoral FOXP3⁺ Tregs. Dashed lines indicate the outline of the
4 cSCC tumor islands, scale bars = 50 μm. (B) Flow cytometry plots of CD3⁺CD4⁺
5 gated lymphocytes from cSCC and corresponding peripheral blood showing OX40
6 and FOXP3 expression. (C) FACS quantification of OX40 expression in tumoral
7 Tregs, tumoral effector T cells and peripheral blood Tregs, n=15 tumors, horizontal
8 bars=means. (D) Tumoral CD4⁺ T cells isolated by flow cytometry from 10 fresh
9 cSCCs were cultured with 1μg/ml PHA + autologous irradiated CD3⁺ cell depleted
10 irradiated PBMCs ± agonistic anti-OX40 antibody (5μg/ml). Proliferation was
11 assessed by tritiated thymidine uptake, dots = median values from triplicate wells for
12 each tumor, horizontal bar = median value from all tumors. (E) Tritiated thymidine
13 uptake assays were performed where tumoral CD4⁺ effector T cells were cultured ±
14 tumoral Tregs ± agonistic anti-OX40 antibody. Median values from triplicate wells are
15 shown and represented as normalized to 100% of CD4 Teff + Treg + iso, n=5 tumors.
16 (F) interferon-γ ELISPOT assays, showing that the effect of the agonistic OX40
17 antibody is more apparent when tumoral Tregs are present in culture. Dots/circles =
18 median values for each tumor with circles for isotype control, squares for anti-OX40,
19 horizontal bar = median for all tumors, error bars = IQR, n=4 tumors.

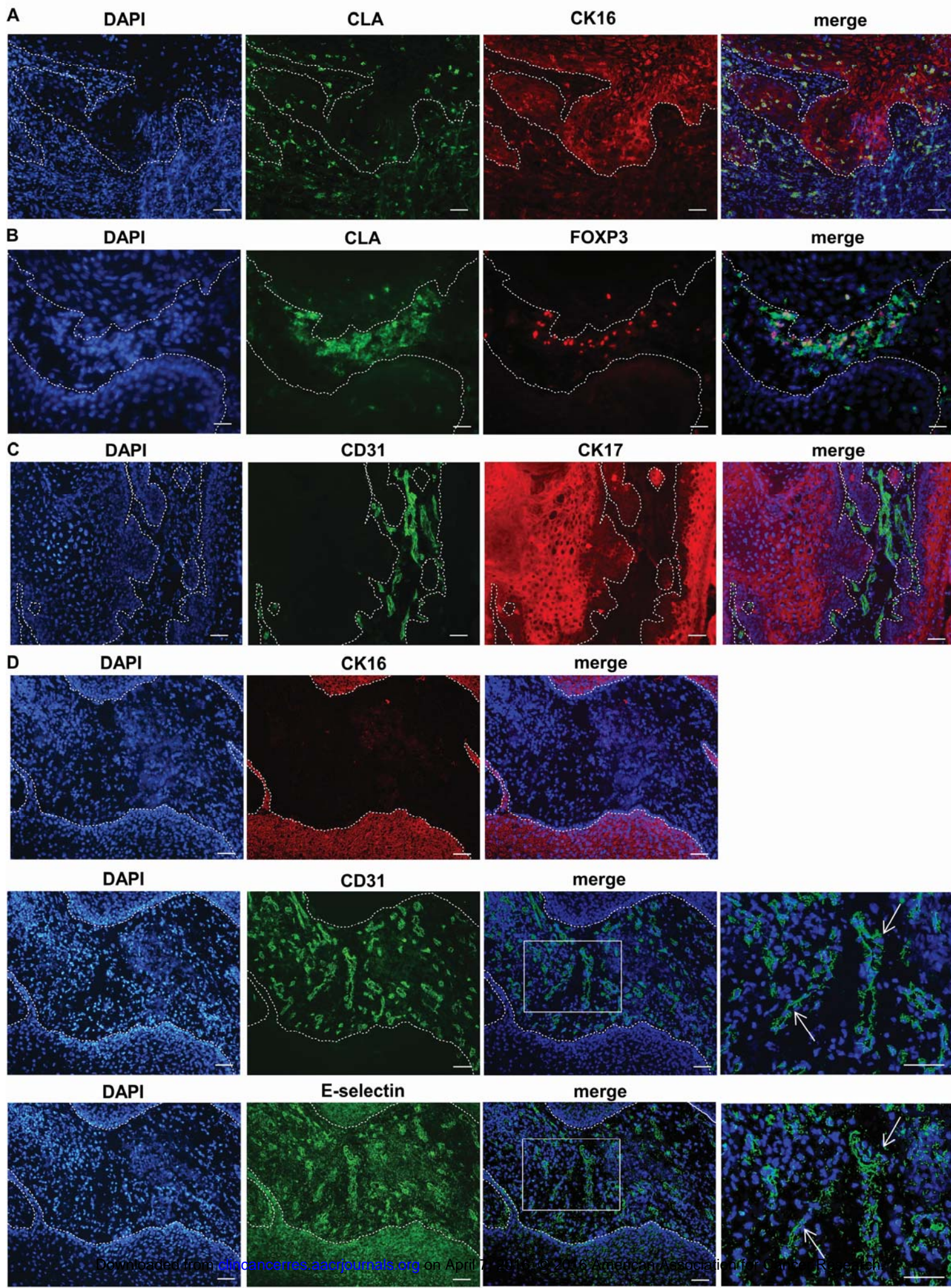
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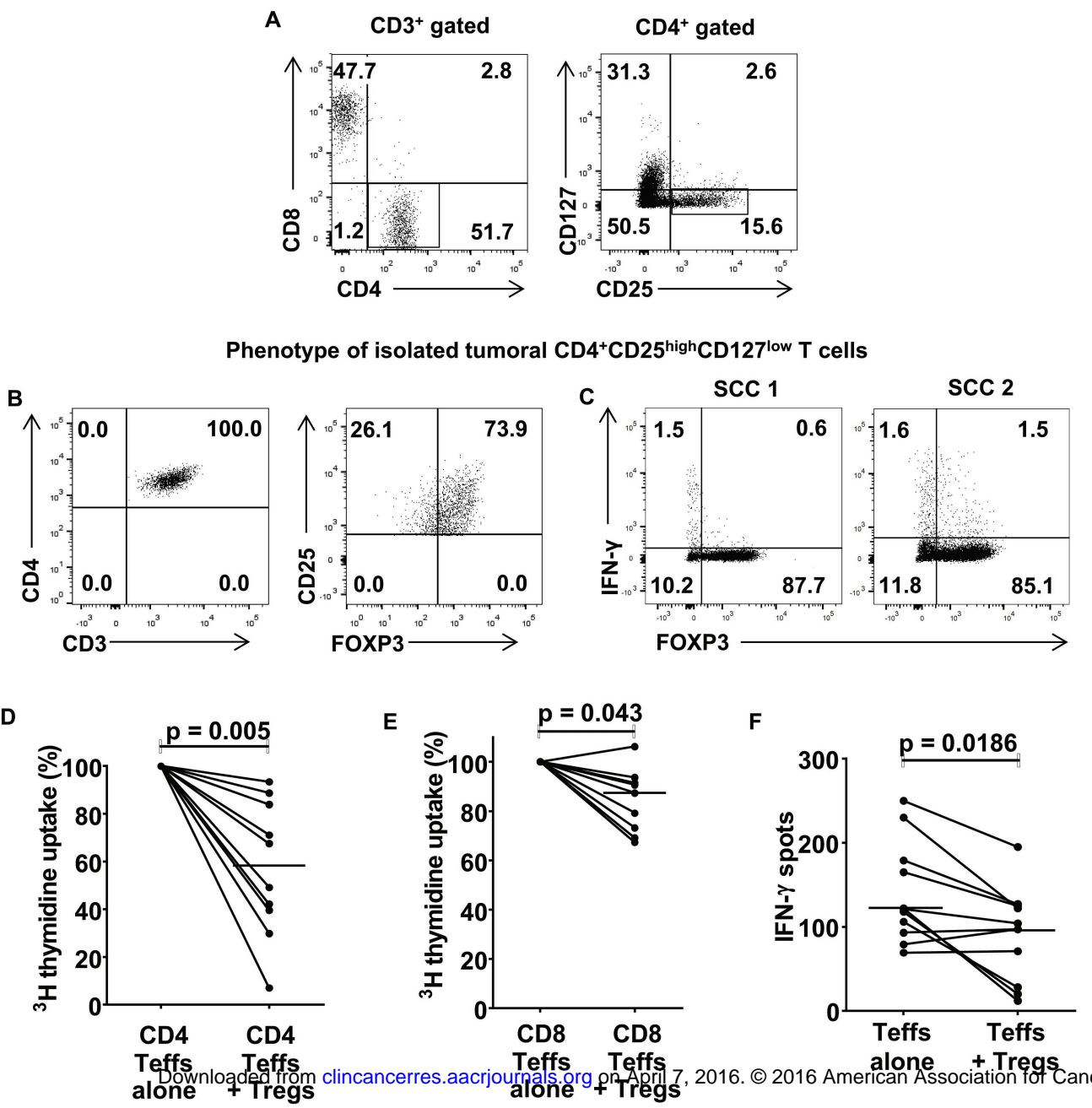
21 **Figure 6:** Higher numbers of tumoral Tregs are associated with subsequent
22 development of metastasis from primary cSCCs. Immunohistochemistry was
23 conducted on archived FFPE sections of primary cSCCs that had not metastasized
24 at 5 years post-excision (primary non-metastatic) and primary cSCCs which had
25 metastasized (primary metastatic) post-excision. (A) Table showing histological

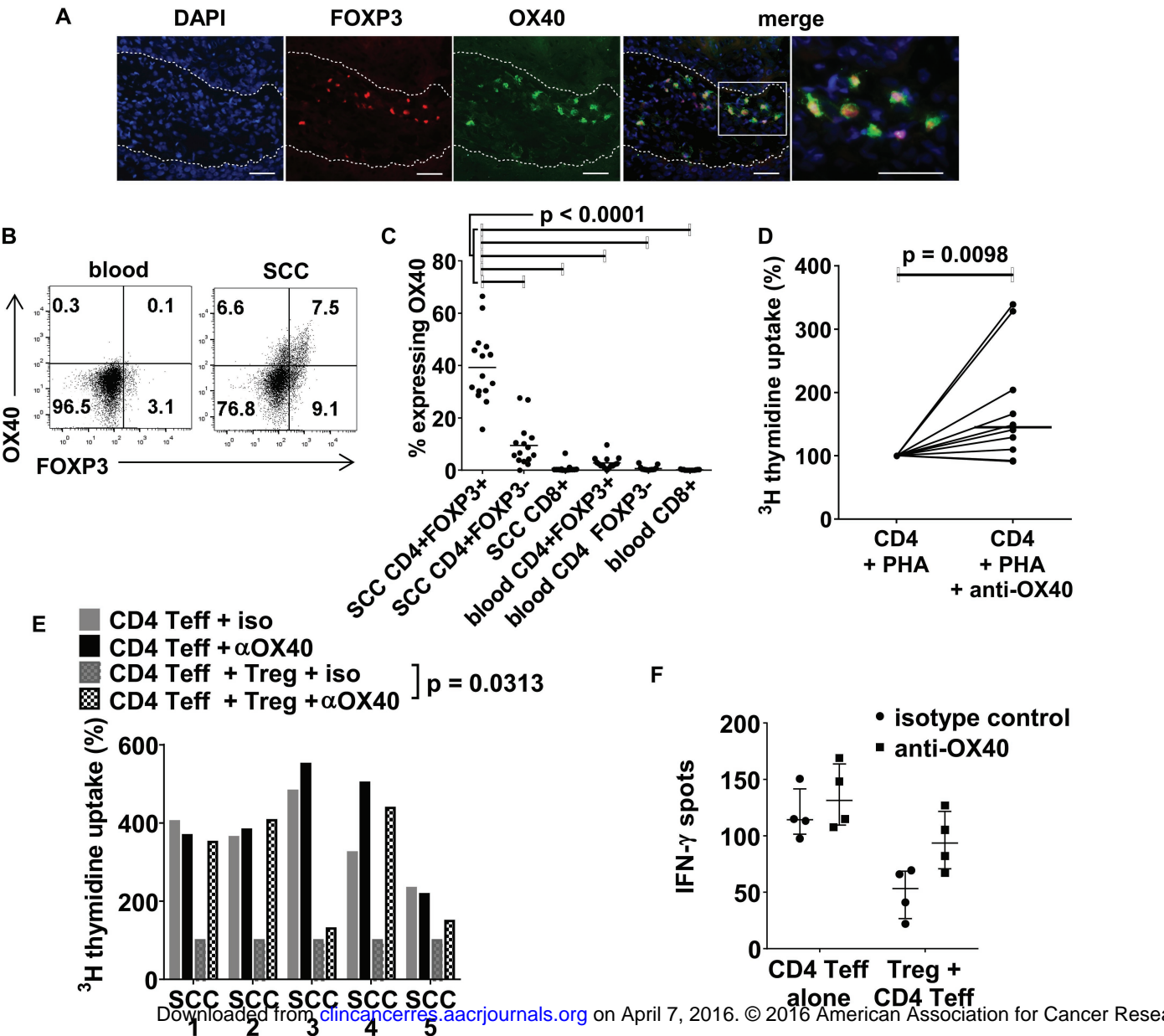
1 details of primary non-metastatic and primary metastatic cSCCs. (B) Representative
2 images of tumoral CD3⁺ T cells, FOXP3⁺ Tregs and OX40⁺ immunocytes in primary
3 non-metastatic and primary metastatic cSCCs, scale bars = 50 μ m. Quantification of
4 tumoral (C) CD3⁺ T cells, (D) FOXP3⁺ Tregs in 26 primary non-metastatic and 29
5 primary metastatic cSCCs and (E) OX40⁺ cells in 49 primary non-metastatic and 48
6 primary metastatic cSCCs. Dots on the graphs indicate mean values for each tumor
7 from 5 high power fields, horizontal bars represent mean values for each group.

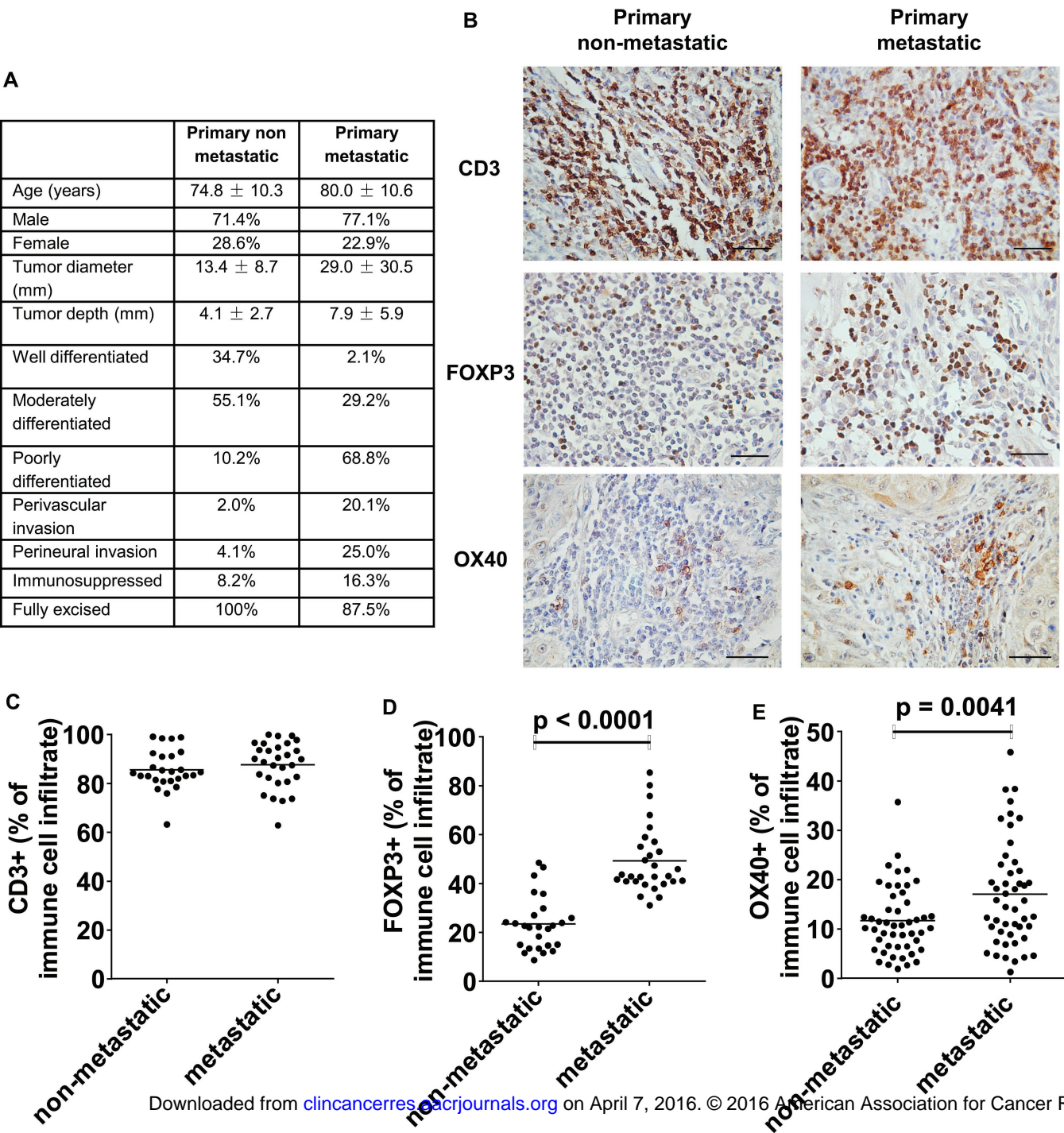












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