Appendix S1.

MATERIALS AND METHODS

The study was approved by the Danish Regional Ethics Committee, protocol number: H-4-2013-197 and the Danish Data Protection Agency, journal number: BBH-2014-008, I-Suite: 02675. All participants provided signed informed consent.

Material consisted of BCC and peritumoural skin from the face obtained during Mohs surgery and 4-mm punch biopsies from the buttocks collected at the Department of Dermatology, Bispebjerg University Hospital, Denmark. The peritumoural skin was taken after complete removal of cancerous tissue by the use of Mohs technique. For qRT-PCR, we included skin from 18 patients from the 3 different skin sites: BCC, peritumoural skin and buttock biopsies with a varying number of buttock biopsies for gene analysis: CD3 $n=18$, CCL17 $n=16$, CCL18 $n=14$, CCL22 $n=12$, Perforin $n=10$, IL12, IL13 $n=9$, *FOXP3*, RORc, Granzyme-B, TNF-α, TGF-β, T-bet, IFN-γ, IL10 $n=8$, IL17 $n=6$. Material for immunohistochemical (IHC) single-staining was taken from the same samples as those used for qRT-PCR by splitting the sample material in 2 and embedding half for IHC in Tissue-Tek (Sakura, Leiden, the Netherlands) while immediately freezing the other half for qRT-PCR at –80°C until analysis. BCC material for *FOXP3*/CD4 double staining was retrospectively collected as paraffin-embedded formalin-fixed skin biopsies (2–5 mm, $n=19$) from the Department of Pathology, Rigshospitalet, Copenhagen, Denmark.

Quantitative real-time PCR (qRT-PCR)

Frozen skin material was thawed in RNA later (Sigma, St Louis MO, USA) and minced into 1-mm pieces and transferred into Trizol (Ambion; Thermo Fisher Scientific, Waltham, MA, USA) and homogenised using a TissueLyser (Qiagen, Hilden, Germany) for 2 × 15 min at 4°C. Chloroform (300 µl) (Merck Millipore, Darmstadt, Germany) was added and the samples were centrifuged at 14,000×g for 15 min at 4°C. The aqueous phases were transferred into fresh tubes and 70% ethanol was added. Samples were loaded onto RNeasy Mini spin columns (Qiagen) and RNA was purified according to the manufacturer's protocol, either manually or via the QIAcube system (Qiagen). The RNA concentration was determined by Nanodrop ND-1000 (Saveen & Werner, Limnham, Sweden).

cDNA was produced using the AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, CA, USA) and up to 3 µg of total RNA, according to the manufacturer's protocol. The cDNA was diluted to 10 ng/µl. qRT-PCR was performed on a Stratagene Mx3005P cycler (Agilent Technologies) using 40 ng of RNA per reaction, Taqman Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, CA, USA) and FAM-TAMRA labelled primer-probes (Applied Biosystems): glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (HS02758991_g1), CD3 (HS00174158_m1), CCL17 (HS00171074_m1), CCL18 (HS00268113_m1), CCL22 (HS01574247_m1), Perforin (HS00169473_m1), Granzyme-B (HS00188054_m1, TNF-α (HS01113624_g1), TGF-β (HS00234257_m1), T-bet (HS00203436_m1), IFN-γ (HS99999041_m1), RORc (HS01076112_m1), IL10 (HS00961622_m1), IL17 (HS00174383_m1), IL12 (HS01073447_m1), IL13 (HS00174379_m1). Data obtained were analysed by the ΔΔCT method (12, 14), with GAPDH as housekeeping reference gene with all analysis being performed as triplicates.

Immunohistochemistry

BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) conducted *FOXP3*/CD4 double-staining by an indirect sequential immunoenzymatic technique. Sections of 4 µm were cut and mounted on Superfrost Plus slides (Thermo Fisher Scientific). Benchmark XT reagents kits and standard setting were used for deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase blocking. Foxp3 monoclonal mouse antibody (236A/E7, 1:50, Abcam, Cambridge, UK), was incubated for 16 min at room temperature (RT) followed by horseradish peroxidase-conjugation and application of DAB (Ventana). Monoclonal rabbit CD4 (SP35, ready-to-use, Ventana) was incubated for 32 min at RT, after which Universal Alkaline Phosphatase Ready-to-Use Red Detection Kit (Ventana) was applied. Counterstaining with Mayer's haematoxylin and bluing reagent was performed. Sections from human tonsil were used as positive and negative controls.

Immunofluorescent staining

10-µm sections were cut using a Microm HM560 cryostat and mounted on slides. The sections were fixed in acetone at –20°C for 10 min. and then blocked in 10% goat serum (Dako A/S, Glostrup, Denmark) + 1% bovine serum albumin (BSA) + phosphate-buffered saline (PBS) for 1 h at RT followed by incubation with primary antibodies mouse monoclonal anti-CCL17 (1:80, LS-C198166, LSBio, Seattle, USA) diluted in blocking solution overnight at 4°C. Incubation was performed with secondary goat anti-mouse IgG Alexa Flour 488 and –565 coupled antibodies (goat anti-rabbit 1:500) (Life Technologies, Thermo Fisher Scientific) diluted in 1% BSA + PBS for 1h at RT in the dark. The slides were incubated with 0.5 µl/ml 4',6-diamino-2-phenylindole (DAPI) (Sigma) diluted in PBS for 10 min. at RT in the dark and cover-slipped with Glycergel mounting medium.

Automated image analysis

CD4/*FOXP3* slides were photographed with Nanozoomer 2.0 (Hamamatsu Phototonics KK, Hamamatsu City, Japan) at original magnification × 20. The slides were computed individually using Visiomorph DP module in Visiopharm Integrator System 4.3.1.0 (Visiopharm A/S, Hørsholm, Denmark).

T-regs were defined as double-positive cells (brown nuclear staining with red membrane staining); positive for both *FOXP3* and CD4, whereas non-regulatory T cells were defined as single CD4 positive (red membrane staining). The fraction between T-regs and non-regulatory T cells (double $FOXP3^+CD4^+$ cells relative to all $CD4^+$ cells (double and single positive) was calculated as a number index; a measure of the total number of double $FOXP3^+/CD4^+$ nuclei compared with the number of all $CD4^+$ cells:

$$\frac{FOXP3^+/CD4^+}{(FOXP3^+/CD4^+) + CD4^+}$$