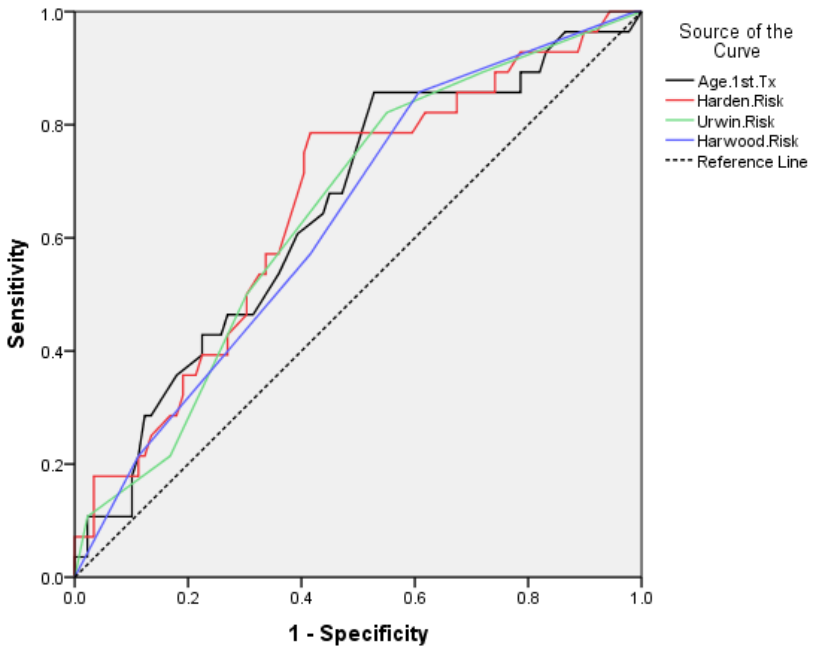
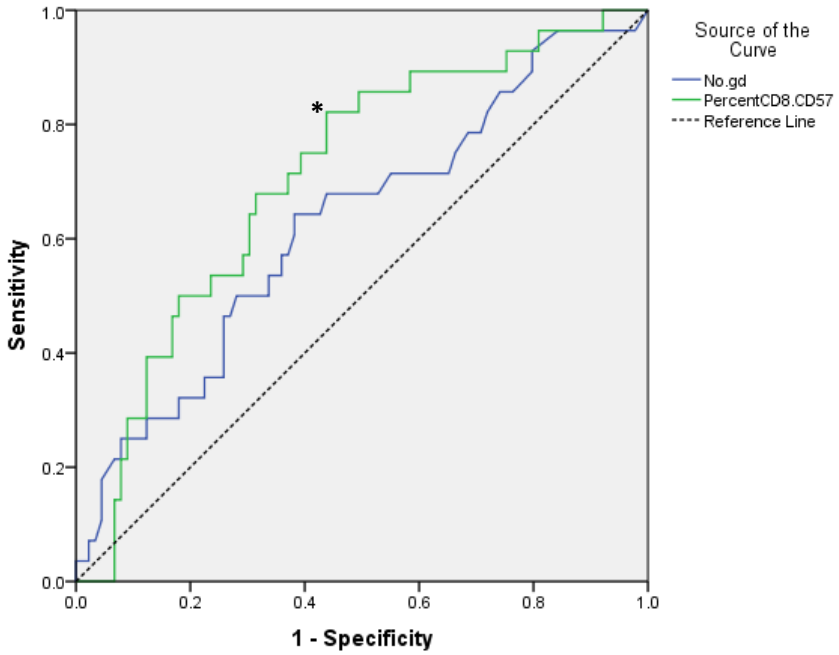
Supplemental information

A



Variable	c-statistic (95% CI)	p
Age at first transplant (increasing)	0.65 (0.53-0.76)	0.02
Harden risk score (increasing)	0.66 (0.54-0.77)	0.01
Urwin risk score (increasing)	0.65 (0.54-0.76)	0.02
Harwood risk score (increasing)	0.63 (0.52-0.75)	0.03

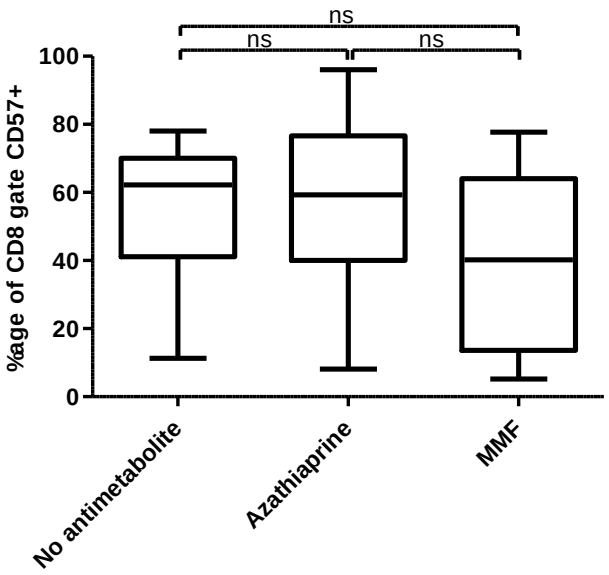
B



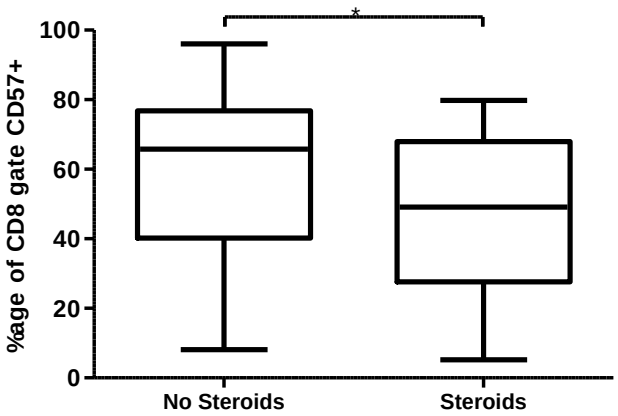
Variable	c-statistic (95% CI)	p
Percentage CD8+CD57+ (increasing)	0.71 (0.60 – 0.82)	0.001
Number of gamma-delta T cells (increasing)	0.63 (0.51 – 0.75)	0.04

Figure S1: ROC curve and c-statistic (area under the curve) for A) age at enrolment and the three clinical risk scores; B) number of gamma-delta T cells and percentage of $CD57^{+}$ $CD8^{+}$ T cells in predicting SCC during study follow-up.

A


ns
ns
ns
100
80
60
40
20
0
%age of CD8 gate CD57+
No antimetabolite
Azathiaprine
MMF

B


*
100
80
60
40
20
0
%age of CD8 gate CD57+
No Steroids
Steroids

C

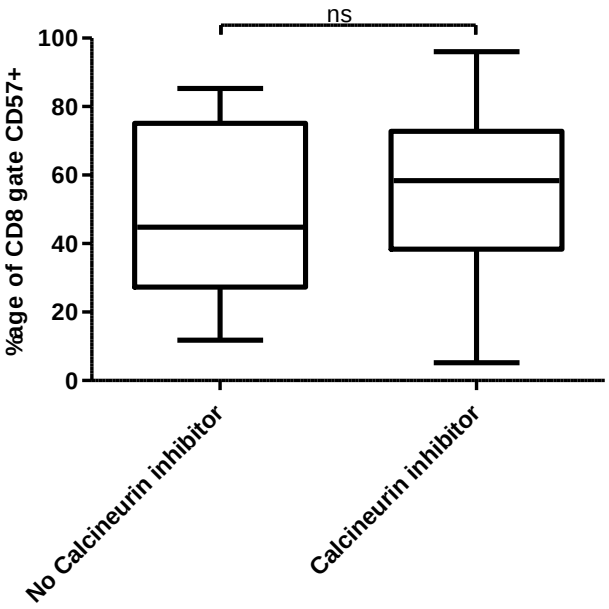
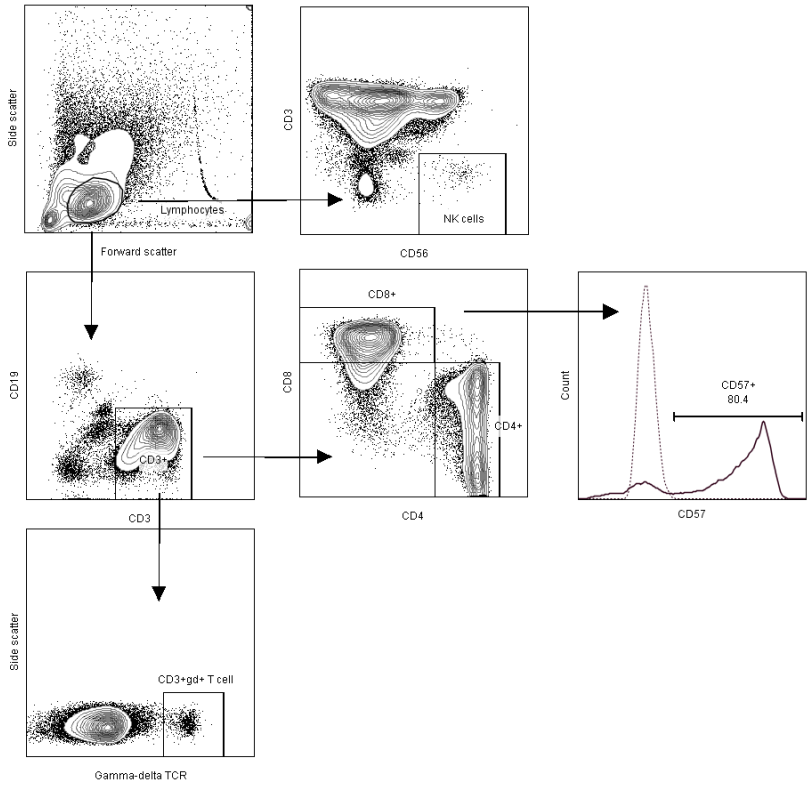
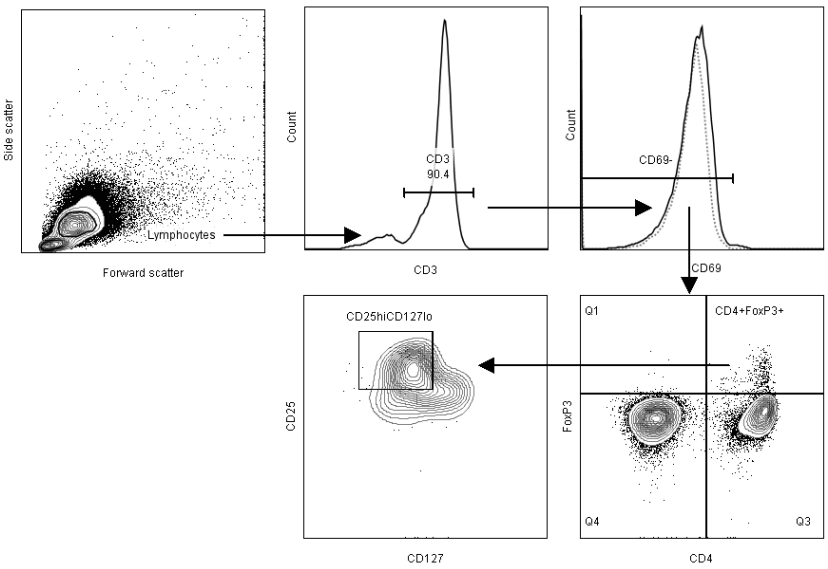

ns
100
80
60
40
20
0
%age of CD8 gate CD57+
No Calcineurin inhibitor
Calcineurin inhibitor

Figure S2: Distribution of percentage of CD57-expressing CD8+ T cells by immunosuppression use. A) Stratified by azathioprine use. B) Stratified by steroid use. C) Stratified by calcineurin inhibitor use. *indicates specificity and sensitivity offered by use of the 50% cut-off.

A


Side scatter
Lymphocytes
Forward scatter
CD3
NK cells
CD56
CD19
CD3+
CD3
CD8+
CD8
CD4+
CD4
Count
CD57+
80.4
CD57
Side scatter
CD3+gd+ T cell
Gamma-delta TCR

B


Side scatter
Lymphocytes
Forward scatter
Count
CD3
90.4
CD3
Count
CD69-
CD69
CD25hiCD127lo
CD25
CD127
Q1
CD4+FoxP3+
FoxP3
Q4
Q3
CD4

C

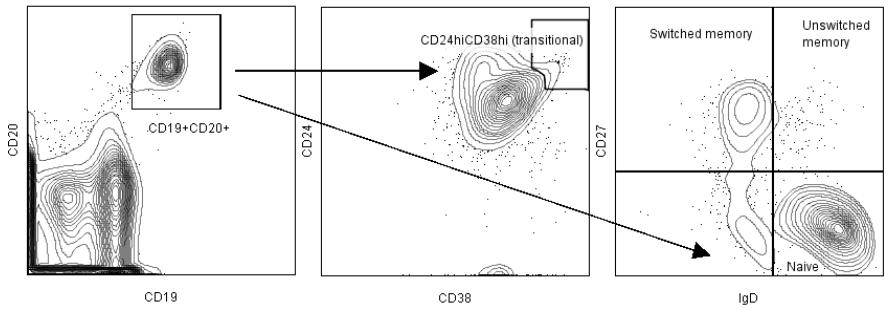

CD20
CD19+CD20+
CD19
CD24hiCD38hi (transitional)
CD24
CD38
Switched memory
Unswitched memory
CD27
Naive
IgD

Figure S4: Representative gating strategy for analysis of flow cytometry data. A) Gating strategy for $CD3^{-}CD56^{+}$ NK cells, gamma-delta, $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells. CD57 positivity on $CD8^{+}$ T cells was established using an isotype control (dotted line). B) Gating strategy for identification of $CD4^{+}CD25^{hi}CD127^{lo}FoxP3^{+}$ regulatory T cells. The lower limit of the FoxP3+ gate was set at 0.1% $CD4^{-}FoxP3^{+}$ cells. The CD69-negative gate is set using an isotype control (dotted line). C) gating strategy for identification of B cell subsets, using the lymphocyte gate detailed in A. The total proportion of memory B cells was calculating by summing the proportion of switched and unswitched memory.

Variable	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age at enrolment	1.09 (1.04-1.14)	<0.001	1.07 (1.02-1.13)	0.005
Age at first transplant	1.06 (1.02-1.10)	0.001		
Male gender	1.38 (0.63-3.04)	0.60		
Current or previous smoker	0.56 (0.26-1.20)	0.14		
Duration of immunosuppression	1.00 (0.99-1.00)	0.35		
Number of transplants	0.69 (0.32-1.49)	0.34		
Total number of HLA-ABDR mismatches	1.10 (0.86-1.39)	0.45		
Number of HLA-AB mismatches	1.07 (0.80-1.43)	0.65		
CMV IgG seropositivity	14.4 (5.48-37.8)	<0.001	12.5 (4.60-33.7)	<0.001
Post-transplant CMV seroconversion*	3.43 (0.70-16.8)	0.13		
History of previous SCC	1.57 (0.75-3.26)	0.23		
Chronic UV exposure	1.36 (0.64-2.85)	0.42		
Fitzpatrick skin type (I-II, III-IV, V-VI)	1.51 (0.72-3.15)	0.27		
Serum creatinine	1.00 (0.99-1.00)	0.45		
eGFR	1.00 (0.98-1.02)	0.73		
On calcineurin inhibitor	1.48 (0.57-3.80)	0.42		
Trough level (ciclosporin)	0.99 (0.98-1.01)	0.27		
On azathioprine	1.26 (0.56-2.82)	0.58		
On mycophenolate	0.46 (0.14-1.50)	0.20		
On steroids	0.60 (0.28-1.26)	0.18		
Previous treatment for rejection	1.00 (0.47-2.14)	1.00		
Pre-transplant dialysis	0.69 (0.27-1.74)	0.43		
Duration of pre-transplant dialysis	1.00 (0.99-1.01)	0.45		
Previous non-NMSC malignancy	0.58 (0.20-1.67)	0.31		

Table S1: Clinical predictors of CD57hi phenotype. Continuous variables are expressed as an odds ratio per unit (as defined in Table 1) increase. The multivariate (bivariate) regression model included those variables significant on univariate analysis. *compared to pre-transplant seroconversion (only in those RTR who were seropositive at enrolment)

Variable	Multivariate	
	HR (95% CI)	p
Age at first transplant (years)	1.02 (0.99 – 1.06)	0.19
Duration of dialysis pre-transplant (months)	1.00 (1.00 – 1.01)	0.24
History of previous SCC	3.58 (1.44 – 8.90)	0.006
Number of gamma-delta T cells (cell/µl)	1.01 (1.001 – 1.010)	0.015
>50% $CD57^+$ $CD8^+$ T cells	3.06 (1.13 – 8.32)	0.028

Table S2: Predictive value of clinical and immunological factors for SCC development during study using age at first transplant as covariate. Variables are expressed as a hazard ratio, per unit increase for continuous variables (units in parentheses where appropriate).

A: All RTR (SCC during follow-up)

	No SCC during follow-up	SCC during follow-up
CD57hi phenotype	42	23
CD57lo phenotype	47	5

B: RTR_{No} (SCC during follow-up)

	No SCC during follow-up	SCC during follow-up
CD57hi phenotype	24	5
CD57lo phenotype	27	2

C: RTR_{SCC} (SCC during follow-up)

	No SCC during follow-up	SCC during follow-up
CD57hi phenotype	18	18
CD57lo phenotype	20	3

D: RTR with SCC during study or preceding year (further SCC)

	No further **SCC during follow-up**	Further **SCC during follow-up**
CD57hi phenotype	15	13
CD57lo phenotype	11	2

Figure S4: 2 x 2 tables for the development of SCC

Full methods

The conduct of the study was approved by NHS research ethical committee prior to commencement (reference 12/WS/0288) and was conducted according to the principles of the Declaration of Helsinki. Informed consent was given prior to enrolment. The study is reported according to Strengthening The Reporting of Observation Studies in Epidemiology (STROBE) guidelines.

Participants

Renal transplant recipients with stable graft function (defined as a serum creatinine that had not risen greater than 30% compared to baseline in the last 12 weeks) were identified and recruited during routine transplant outpatient follow-up at the Oxford Transplant Unit between March 2013 and November 2014. RTR_{No} were recruited to match RTR_{SCC} for age, sex and duration of immunosuppression at a 1:1 ratio – however, due to a lack of elderly RTR_{No} it was not possible to completely match for age.

Potential participants were eligible if they were greater than 18 years old, had received their most recent transplant greater than one year prior to recruitment, were HIV seronegative, free of malignancy (with the exception of SCC and basal cell carcinoma) for at least five years prior to enrolment, and were without intercurrent infection at time of sampling (defined as systemic upset due to infective symptoms or the use of antibiotics within a week of sampling). RTR developing SCC prior to or within one year of transplant were excluded, in order to only include RTR with post-transplant SCC.

An enrolment questionnaire was completed to assess lifetime sun exposure, family history of malignancy and smoking history. Skin type was assessed by a single member of the research team (MB), using a combination of participant-reported susceptibility to tanning or burning upon sun exposure, as well as physician-based assessment of participant phenotype (skin colour, eye colour, hair colour). Clinical data was collected from medical and transplant records and pathology databases. eGFR was calculated using the four-variable MDRD equation. Treatment for suspected rejection was defined as a short course of high-dose steroids or intravenous immune therapy undertaken after transplantation in response to deterioration in renal function, irrespective of whether a biopsy showed rejection.

Sera taken at time of enrolment was tested for immunoglobulin G (IgG) to CMV by combined immunoassay-chemiluminescence platform (Diasorin, Dartford, UK); a titre of greater than 14IU/mL was considered positive. A randomly selected subset of CMV seropositive participants was tested for CMV viral load by PCR of whole blood during follow-up sampling during the study – the lower limit of detection was 164 copies per millilitre of blood. Data was available regarding recipient CMV serostatus at time of transplant for 108 RTR.

Immune phenotyping

Blood was taken at trough levels of immunosuppression, by vacuum aspiration into EDTA-containing blood tubes (BD Biosciences, Oxford, UK) and stored on ice prior to extraction of peripheral blood mononuclear cells (PBMC), which occurred within 4 hours of venepuncture. PBMC were isolated by density-gradient centrifugation using Lymphocyte Separation Medium (GE Healthcare, Amersham, UK). Remaining erythrocytes were lysed (Pharm-Lyse, BD Biosciences) then aliquots of $4x10^5$ PBMC were stained for 45 minutes at 4^oC with a combination of antibodies. PBMC were fixed and permeabilised for at least 30 min

at 4^{o}C (FoxP3 staining buffer kit, eBioscience, Hatfield, UK or Cytofix/Cytoperm intracellular staining kit, BD Biosciences) prior to intracellular staining for FoxP3, Granzyme B and Ki67. Antibodies (and clones) used were: CD3-eFluor 450 (UCHT1), gamma-delta TCR-FITC (B1.1), CD28-APC- eFluor 780 (CD28.2), CD57-PE (NK1.1), CD38-APC (HIT2), Ki67-FITC (20Raj1) and CD20-APC-eFluor 780 (2H7) from eBioscience; CD4-ECD (SFCI12T4D11), HLA-DR-Krome Orange (Immu-357) and CD19-Krome Orange (J3-119) from Beckman Coulter, Wycombe, UK; CD8-PerCP-Cy5.5 (SK1), CD16-PE-Cy7 (3G8), CD56-APC (B159), CD27-FITC (M-T271), CD127-PE (HIL-7R-M21), CD25-PE-Cy7 (M-A251), CD69-Alexa Fluor 700 (FN50), CCR7-PE-Cy7 (3D12), CD24-PE (ML5), Granzyme-B-Alexa Fluor 700 (GB11) and IgD-PE-Cy7 (IA6-2) from BD Biosciences; CD45RA-APC (MEM-56) from Invitrogen (Life Technologies, Paisley, UK); FoxP3-Alexa Fluor 647 (259D) from Biolegend, London, UK. Where appropriate, isotype controls were used for gating.

At least 50,000 events were acquired using a Navios flow cytometer and analysed using Kaluza version 1.4 (both Beckman Coulter) and FlowJo version X.0.7 (Tree Star Inc, OR, US) – the gating strategy is illustrated in the Supplementary information (Figure S4). Absolute counts for cell subsets were calculated by applying the proportion of each cell type in the lymphocyte gate from flow cytometry data to the total lymphocyte count from routine haematology laboratory testing undertaken simultaneously at time of sampling. Two participants did not have lymphocyte quantification performed at time of sampling and were excluded from all analyses involving cell number. For analyses of follow-up samples, analysis was performed blinded to the enrolment phenotyping results.

Follow-up

The primary outcome was time from study enrolment to first (or further) new diagnosis of SCC. Secondary outcomes were total number of SCC during follow up and occurrence of metastatic SCC. All diagnoses of SCC, or metastases, were made on histological grounds: where diagnostic uncertainty was present (such as with keratoacanthoma), the histologist's decision was considered final. If the histologist was unable to provide a favoured diagnosis, a non-diagnosis of SCC was presumed. SCC arising within scar tissue, or clinical described as recurrence, within one year of previous excision were not count as a new SCC event. No patients were lost to follow-up except for death. Participants without an event were censored at the time of their latest follow-up data, graft loss, or death, whichever occurred first. In the event of graft loss, participants continued to be observed for SCC occurrence but this was not included in the follow up data. No patient developed SCC after graft loss.

For the analysis of disease recurrence, time zero was taken as the date of SCC excision. The first SCC to occur during the study period or year preceding was used as time zero.

Repeat sampling for immune phenotyping occurred at the first outpatient appointment occurring after a six month period and twelve month period from enrolment.

Statistical analysis

All analyses were performed on Graphpad Prism for Windows 5.03 (Graphpad, San Diego, CA) or IBM SPSS Statistics for Windows 20.0 (IBM Corp., New York, NY). Continuous variables are shown as median (interquartile range) unless specified otherwise, except hazard ratios which are reported as hazard ratio (95% confidence interval). Categorical variables are reported as number (percentage of group). Comparison between groups was performed using the non-parametric two-tailed Mann-Whitney (two

groups) or Kruskal-Wallis (multiple groups) test, with continuous variables. For categorical variables the chi-squared test or Fisher's exact test were used. Where the Kruskal-Wallis test was significant, a subsequent post-hoc Dunn test was applied, and these are the results shown on figures. Correlations were performed using Pearson's test. Survival analyses were performed using Cox regression, as described in the results. Odds ratios were calculated by logistic regression using variables significant on univariate analysis. ROC curves were generated and tested using a non-parametric assumption of distribution. Throughout the study a p-value of less than 0.05 was considered significant.