Supplementary Materials

Immunological characterization of a recipient of a cross-match positive face transplant

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1. Histological examination

Skin (4mm) punch biopsies were taken from the facial allograft with any clinical signs of rejection. Additionally, non-rejection biopsies were taken at prescribed time-points following transplant. Paraffin sections were examined and graded according to the Banff 2007 classification (1) by a pathologist without the knowledge of the molecular findings.

2. Evaluation of donor-specific antibodies

Serum samples were tested for presence of circulating donor-specific anti-HLA-A, -B, -Cw, -DR, -DQ, and -DP antibodies using the single antigen bead based assay (One Lambda) on a Luminex platform.

3. Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from peripheral blood samples by density gradient centrifugation using Ficoll-Paque solution (GE Healthcare) and were cryopreserved in heat-inactivated Human AB serum (Gemini) with 10% dimethylsulfoxide (DMSO) in liquid nitrogen.

4. Flow cytometric analysis

Recipient PBMCs collected prior to transplant, and at 1 week, 22 months and 24 months after transplant were thawed and incubated overnight in 5% CO_2 at 37 degrees in RPMI-1640 medium (Lonza) supplemented with 10% Human AB serum, followed by antibody staining and flow cytometric analyses on the

following day. Cells were stained on ice for 30 minutes with anti-human CD4 (OKT4), CXCR5 (J252D4), PD-1 (EH12.2H7) and CD27 (O323) from Biolegend, CD45RA (HI100), CCR7 (3D12), IgD (IA6-2) and CD19 (SJ25-C1) from BD Biosciences. Stained PBMCs were analyzed on a FACS Canto II flow cytometer with FACSDiva software (BD Biosciences) and data analyzed with FlowJo software (TreeStar).

5. Molecular analysis

To investigate intragraft gene expression changes during antibody-mediated rejection (AMR), T cell mediated rejection (TCMR), and at non-rejection time-points, nine facial allograft biopsies taken between March 2013 and March 2015 were retrieved from the pathology archive at Brigham and Women's Hospital, Boston. These included biopsies with diagnoses of AMR (Banff grade 3) (n=1), TCMR (Banff grade 3) (n=3), and non-rejection (Banff grade 0) (n=5).

5.1. RNA Isolation

Six consecutive 10 μm sections were obtained from each formalin-fixed paraffin-embedded (FFPE) skin biopsy using a microtome. Sections were immediately transferred to sterile 1.5mL microcentrifuge tubes and stored at room temperature. Xylene deparaffinization and RNA extraction were performed with RNAeasy FFPE Kit (Qiagen Valencia, CA, USA). Total RNA concentration and purity were measured with a Nanodrop 2000 spectrophotometer (ThermoFisher

Scientific, Waltham, MA). The quality of the RNA was determined using the absorbance ratio at 260/280 nm.

5.2. mRNA NanoString Assay

The NanoString nCounter PanCancer Immunology Gene Expression (GX) Codeset with probes representing 730 candidate genes (and 40 additional housekeeping genes) was used and the sample was processed on the NanoString nCounter Analysis System according to the manufacturer's protocol (NanoString Technologies, Seattle, WA). Probe sequences of the genes tested are provided in Table S2.

Briefly, 100 ng of total RNA was hybridized to the reporter and capture probes in 30µl reaction volume for 20 hours at 65ºC. The captured targets were then immobilized on the nCounter cartridge on the Nanostring PrepStation. The cartridge was scanned at 555 Fields of View (FOV) on the nCounter Digital Analyzer. All samples were analyzed in the same batch, using identical lot of reagents.

5.3. Quality Control and Normalization

Quality control assessment and normalization of raw NanoString gene expression counts were performed with nSolver Analysis Software version 2.5 and the default settings (NanoString Technologies, Seattle, WA). The normalized log2 mRNA expression values were used for subsequent data analysis.

5.4. Statistics

The mean values were calculated from the normalized log2 mRNA expression values from biopsies during rejection (n=4) and non-rejection (n=5). Differentially expressed (DE) genes (Table S1) were evaluated by unpaired 2-tailed *t* test, using the *R* statistical package (*R* version 3.2.3). Adjusted p values were calculated using *fdrtool* (2). Genes with less than 20 counts in 50% or more of the samples were filtered to remove low abundance transcripts. Genes with adjusted *p* value ≤ 0.1 were considered as differentially expressed.

5.5. Unsupervised principal component analysis (PCA) of AMR versus TCMR

Unsupervised principal component analysis (Figure 6A) was performed on 80 genes differentially expressed between rejecting and non-rejecting biopsies, using the normalized log2 mRNA expression values from the AMR (n=1) and TCMR (n=3) biopsy samples. The loading for each gene in the PCA was used as a measure of its contribution to the sample clustering. Using a cutoff of 0.1 for the absolute value of the loadings, we selected the top 31 ranked genes (Table 3). The PCA analysis was performed using the R package prcomp (R version 3.3.1). The gene expression values for these 31 genes were clustered using hierarchical clustering and visualized in a heatmap (Figure 6B), using the R package pheatmap (R version 3.3.1).

6. Immunohistochemical Staining for ICAM-1 and Granzyme B on Leica Bond III

Four μm thick sections were obtained from each FFPE skin biopsy for immunohistochemical staining. Slides were baked for 60 minutes in an oven set to 60°C. They were then loaded into the Bond III staining platform with appropriate labels. Slides were antigen retrieved in Bond Epitope Retrieval 1 for 30 minutes (for ICAM-1), or Bond Epitope Retrieval 2 for 20 minutes (for Granzyme B). They were then incubated with ICAM-1 (Rabbit polyclonal, Sigma) or with Granzyme B (clone GrB-7, DAKO) at 1:200 for 30 minutes at room temperature. Primary antibody was detected using Bond Polymer Refine Detection kit. Slides were developed in 3, 3'-Diaminobenzidine (DAB) then dehydrated and coverslipped.

Figure S1. Dynamics of T cell subsets following facial transplantation. Peripheral blood mononuclear cell (PBMC) were stained and analyzed by flow cytometry post-transplant. A, Contour plots and percentage of CD8 cells according to CD45 and CCR7 expression. $CD8^+$ TEMRA population ($CD8^+CD45RA^+CCR7^-$) significantly expanded during the TCMR time point. B, Contour plots and percentage of regulatory T cells according to $CD25^+CD127^-$ of CD4-gated cells. Treg proportion was maintained throughout the course, regardless of rejection episodes. (AMR = antibody-mediated rejection; TCMR = T cell mediated rejection; TCM = central memory T cells; TEM = effector memory T cells; TEMRA = terminal differentiated effector memory T cells)

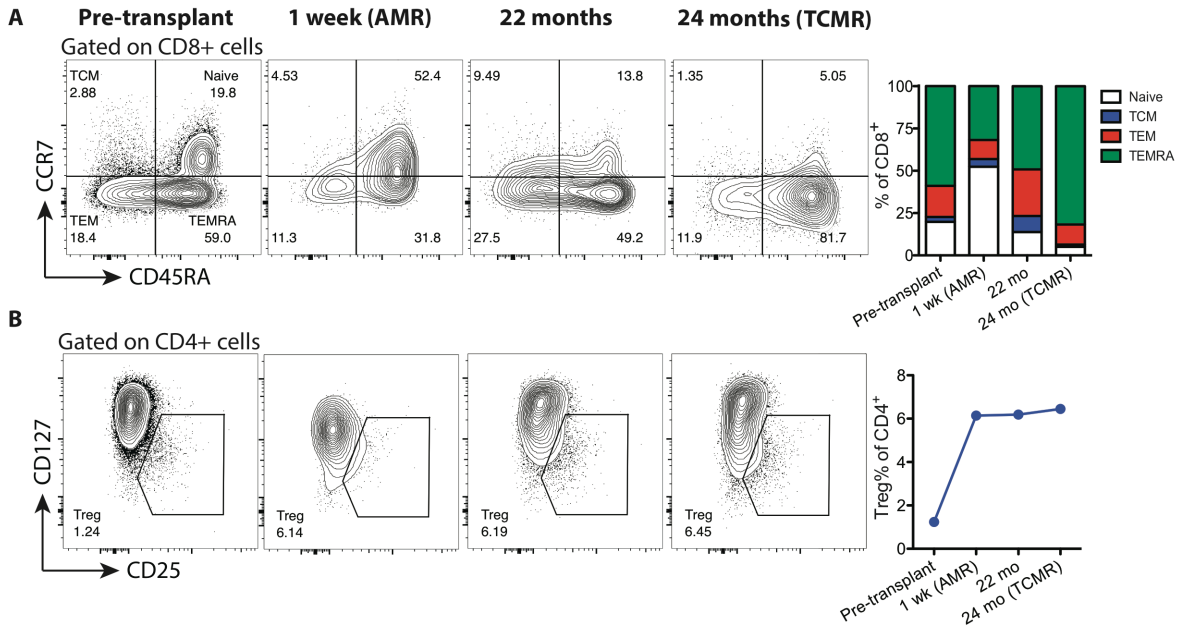

Figure S2. The clinical status of the facial allograft at four years following transplantation.

Clinical photograph of facial allograft at routine follow up clinic at four years following transplantation, showing no signs of rejection.

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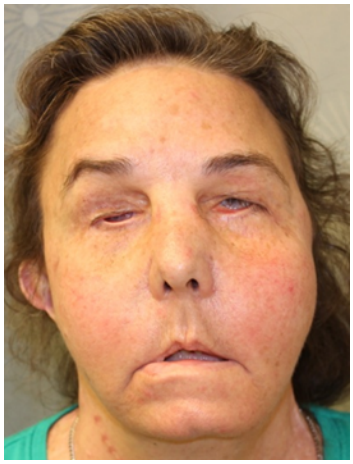

Table S1. List of the 80 genes differentially expressed in rejecting facial allograft biopsies (n=4) compared to non-rejecting (n=5) biopsies.

Differentially expressed genes were evaluated by unpaired 2-tailed *t* test, using the *R* statistical package (*R* version 3.2.3). Adjusted p values were calculated using *fdrtool.*

Gene Symbol	Log2 fold change	Adjusted p value
GZMB	3.59	0.00961824
CCL18	2.89	0.039786459
LAMP3	2.7	0.010238331
LTB	2.6	0.033330268
IL2RG	2.36	0.00678203
CD48	2.24	0.010204871
CCL19	2.21	0.056499809
CD53	2.06	0.045746601
CCR2	2.01	0.003301284
IL7R	2.01	0.015103772
SELE	1.97	0.035637943
HLA-DQA1	1.95	0.009004989
CXCL9	1.89	0.063110952
IL2RA	1.88	0.022192956
HLA-DOB	1.87	0.024385787
RELB	1.87	0.061485366
PTPRC	1.85	0.009884163
LCP1	1.84	0.008577109
HLA-DRA	1.82	0.009671942
GZMK	1.75	0.025212577
LY9	1.74	0.000890074
SELPLG	1.74	0.074966713
CD3D	1.73	0.009179509
IRF8	1.71	0.02644317
HLA-DMB	1.69	0.026602528
ITGAX	1.66	0.007366157
JAK3	1.66	0.050897716
KLRK1	1.66	0.07707173
HLA-DPA1	1.65	0.01789038

HLA-DPB1	1.65	0.010106254
CD27	1.62	0.029574181
CLEC7A	1.61	0.008217722
CTSS	1.6	0.042078168
CD83	1.59	0.023653322
CCL13	1.58	0.047018053
CD8A	1.56	0.029463132
TNFSF13B	1.53	0.009033186
KLRB1	1.51	0.010015757
TNFSF14	1.5	0.038125742
CXCL1	1.42	0.090560824
CD247	1.39	0.045322356
CD5	1.39	0.058770292
ITGAL	1.36	0.042904029
STAT1	1.35	0.03882516
ICAM2	1.33	0.034647241
CD4	1.32	0.000890074
CXCL2	1.32	0.082219993
XCL2	1.31	0.046904077
CX3CR1	1.24	0.030756422
PRF1	1.24	0.021004692
SOCS1	1.24	0.034566533
AMICA1	1.23	0.043680685
CD40	1.23	0.010097919
IRF1	1.23	0.041943241
CASP1	1.21	0.090886021
HLA-DQB1	1.2	0.079850487
HLA-G	1.2	0.021585462
CD96	1.18	0.066697525
CYBB	1.18	0.040940236
IRF4	1.18	0.095145041
GZMA	1.17	0.023307901
PSMB10	1.15	0.00923385
ZAP70	1.15	0.055090239
OAS3	1.14	0.036317539
VCAM1	1.14	0.047588467
CCL5	1.13	0.009795082
FCER1G	1.12	0.052399653
PIK3CD	1.09	0.054383316

C3	1.07	0.047420907
FCGR2A	1.07	0.067804734
RUNX3	1.07	0.04704797
TARP	1.07	0.035425077
IL10RA	1.06	0.011150894
ICAM1	1.04	0.099944719
IFI27	1.04	0.010276693
INPP5D	1.04	0.074890406
LCK	1.04	0.042864405
LYN	1.02	0.041650571
TNF	1.02	0.07873791
HSD11B1	-1.36	0.024938

Table S2. NanoString codeset list containing official gene symbol, accession number and probe sequence is available as a separate Excel file.

References

1. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. Am J Transplant. 2008; 8(7): 1396-1400.
2. Benjamini Y and Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. 1995; 1 (57): 289-300.