



Autoreactive T cell profiles are altered following allogeneic islet transplantation with alemtuzumab induction and re-emerging phenotype is associated with graft function

Shereen Sabbah^{1,2} | Aaron Liew³ | Augustin M. Brooks³ | Rhiannon Kundu^{1,2} | James L. Reading^{1,2} | Anneliese Flatt³ | Claire Counter⁴ | Pratik Choudhary⁵ | Shareen Forbes⁶  | Miranda J. Rosenthal⁷ | Martin K. Rutter^{8,9} | Stephanie Cairns¹⁰ | Paul Johnson¹¹ | John Casey⁶ | Mark Peakman^{1,2} | James A. Shaw^{3,12}  | Timothy I. M. Tree^{1,2}

¹Department of Immunobiology, Faculty of Life Sciences & Medicine, King's College London, London, UK

²NIHR Biomedical Research Centre, Guy's and St Thomas' NHS Foundation Trust and King's College London, London, UK

³Newcastle University Translational and Clinical Research Institute, Newcastle, UK

⁴Organ Donation and Transplantation, NHS Blood and Transplant, Bristol, UK

⁵Diabetes Research Group, Guy's, King's and St. Thomas' School of Medicine, King's College London, London, UK

⁶Edinburgh Transplant Centre and Endocrinology Unit, University of Edinburgh, Edinburgh, UK

⁷Diabetes Service, Royal Free London NHS Foundation Trust, London, UK

⁸Division of Diabetes, Endocrinology and Gastroenterology, Faculty of Biology, Medicine and Health, School of Medical Sciences, University of Manchester, Manchester, UK

⁹Diabetes, Endocrinology and Metabolism Centre, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK

¹⁰Clinical Immunology Department, Scottish National Blood Transfusion Service, Royal Infirmary of Edinburgh, Edinburgh, UK

¹¹Oxford Centre for Diabetes Endocrinology and Metabolism, University of Oxford, Oxford, UK

¹²Institute of Transplantation, Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK

Correspondence

James A. Shaw

Email: jim.shaw@ncl.ac.uk

Timothy I. M. Tree

Email: timothy.tree@kcl.ac.uk

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Abstract

Islet transplantation is an effective therapy for life-threatening hypoglycemia, but graft function gradually declines over time in many recipients. We characterized islet-specific T cells in recipients within an islet transplant program favoring alemtuzumab (ATZ) lymphodepleting induction and examined associations with graft function. Fifty-eight recipients were studied: 23 pretransplant and 40 posttransplant (including 5 with pretransplant phenotyping). The proportion with islet-specific T cell responses was not significantly different over time (pre-Tx: 59%; 1–6 m posttransplant: 38%; 7–12 m: 44%; 13–24 m: 47%; and >24 m: 45%). However, phenotype shifted significantly, with IFN- γ -dominated response in the pretransplant group replaced by IL-10-dominated response in

Abbreviations: ATG, antithymocyte globulin; ATZ, alemtuzumab; BAS, basiliximab; DAC, daclizumab; DMSO, dimethyl sulfoxide; DSAs, donor-specific antibodies; ETA, etanercept; GAD65, glutamic acid decarboxylase 65; IA-2, insulinoma-associated antigen 2; MMF, mycophenolate mofetil; MMTT, mixed meal tolerance test; PBMC, peripheral blood mononuclear cells; PCA, principal component analysis; PI, proinsulin; Tac, tacrolimus; UKITC, UK Islet Transplant Consortium.

James A. Shaw and Timothy I. M. Tree contributed equally.

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the 1–6 m posttransplant group, reverting to predominantly IFN- γ -oriented response in the >24 m group. Clustering analysis of posttransplant responses revealed two main agglomerations, characterized by IFN- γ and IL-10 phenotypes, respectively. IL-10-oriented posttransplant response was associated with relatively low graft function. Recipients within the IL-10⁺ cluster had a significant decline in C-peptide levels in the period preceding the IL-10 response, but stable graft function following the response. In contrast, an IFN- γ response was associated with subsequently decreased C-peptide. Islet transplantation favoring ATZ induction is associated with an initial altered islet-specific T cell phenotype but reversion toward pretransplant profiles over time. Posttransplant autoreactive T cell phenotype may be a predictor of subsequent graft function.

KEYWORDS

autoantibody, basic (laboratory) research/science, clinical research/practice, immunobiology, islet transplantation, islets of Langerhans, monitoring: immune, T cell biology

1 | INTRODUCTION

Allogeneic pancreatic islet transplantation is an established, minimally invasive intervention for restoration of insulin production in type 1 diabetes complicated by recurrent life-threatening hypoglycemia.^{1,2} Following seminal success with the Edmonton protocol,¹ incrementally improving outcomes have been achieved internationally.^{3–6} However, the duration of graft function remains variable, with most successfully transplanted individuals showing a steady decline in graft function over time.^{7–9} Numerous factors continue to limit engrafted islet mass. These include insufficient beta-cell mass/islet quality,¹⁰ islet apoptosis,¹¹ and delayed/inadequate revascularization.¹² Several immune-related factors may also contribute to graft dysfunction including the instant blood-mediated inflammatory reaction,¹³ the magnitude and phenotype of anti-graft alloimmune responses,^{14,15} immunosuppressive drug toxicity,¹⁶ and recurrent autoimmunity—both humoral and cellular.^{15,17–19} Rejection due to recurrent autoimmunity may be a gradual chronic process.¹⁵ Previous studies investigating the role of recurrent humoral autoimmunity on islet transplant outcomes have been conflicting.²⁰ It appears that while the presence of pretransplant islet autoantibodies does not predict graft failure,²¹ seroconversion to autoantibody positivity or a rise in autoantibody titer posttransplant is a negative predictor of islet graft survival within the first year following transplant.¹⁷ Studies assessing autoimmune T cell responses have demonstrated that the presence of these responses before or following transplantation is negatively associated with graft survival.^{15,18,21–24} Whereas previously immunophenotyped cohorts have received anti-IL-2 receptor antibody or antithymocyte globulin (ATG) induction, alemtuzumab (ATZ) is the first-line induction agent within the UK national islet transplant program with the hypothesis that this would reduce the risk of recurrent autoimmunity through peritransplant lymphodepletion.

In this study, we set out to examine the relationship between the frequency and functional phenotype of islet-specific T cells and graft function through a cross-sectional analysis of recipients within the UK Islet Transplant Consortium (UKITC).

2 | METHODS

2.1 | Participants

Following ethical approval and informed consent, participants on the active islet waiting list and posttransplant within the nationally commissioned UK service were studied between April 2012 and April 2015. Participants were recruited from five centers: Freeman Hospital, Newcastle; Edinburgh Royal Infirmary; Manchester Royal Infirmary; King's College Hospital, London; and the Royal Free, London. Inclusion criteria included C-peptide-negative type 1 diabetes complicated by impaired awareness of hypoglycemia and recurrent severe hypoglycemia requiring assistance in management despite optimized conventional diabetes self-management.²⁵ Clinical-grade islets were isolated at dedicated facilities in Oxford, King's, and Edinburgh. Details on islet procurement, assessment, transport, and transplantation have been previously reported.^{2,26–28}

2.2 | Metabolic assessment

Participants were assessed at 1, 3, 6, and 12 months posttransplant and every 3–6 months thereafter. HbA1c, total daily insulin dose, body weight (kg), and standardized mixed meal tolerance test (MMTT) were performed as previously described.¹⁴ Graft failure was defined as stimulated serum C-peptide <50 pmol/L.

2.3 | Measurement of autoantibodies

Antibodies to glutamic acid decarboxylase 65 (GAD65) were measured at each visit as previously described,^{14,29–31} by an enzyme-linked immunosorbent assay (ELISA) using kits from Euroimmun (Newcastle and Royal Free) or RSR (Edinburgh and Manchester) and at King's College Hospital by a radioimmunoassay using a kit from

RSR. However, as two different assays were used with different cut-off levels for determining a positive response, only the presence and absence of antibody and not absolute levels were included in the present analysis.

HLA antibody testing was performed by flow cytometry as previously reported.¹⁴ Samples were initially screened for the presence or absence of HLA antibodies using Labscreen mixed HLA antibody screening kits (One Lambda, Inc). For all positive and reactive results, HLA antibody specificities were determined using Labscreen single antigen kits (One Lambda, Inc) with a mean fluorescence intensity >1000 considered positive.

2.4 | Detection of β -cell-specific IFN- γ and IL-10-secreting T cells

Fresh heparinized blood samples were obtained from a total of 58 participants, over a period of 3 years ($n = 110$ samples). Samples received overnight were treated with T cell Xtend (Oxford Immunotec) as recommended. Peripheral blood mononuclear cells (PBMC) were isolated with cytokine ELISpot performed and analyzed as previously described.³² Stimuli are detailed in Table S1 and include pools of peptides previously identified as representing naturally processed and presented epitopes of insulinoma-associated antigen 2 (IA-2), GAD65 and proinsulin (PI) and whole-recombinant islet antigens GAD65 and PI. Positive control stimuli included Pediacel (a pentavaccine) and CytoStim (a polyclonal positive rapid T cell receptor stimulator). Cell medium (RPMI-1640, 10% human serum) alone and cell medium with the peptide diluent dimethyl sulfoxide (DMSO) at 1 μ l/ml (Sigma) were used as negative controls. Data were expressed as the mean number of spots per triplicate and compared with the mean spot number in the presence of the negative control (Stimulation index; SI) and SI ≥ 2 was considered positive.

2.5 | Statistical analysis

T cell response data were aggregated for all autoantigens (GAD65, PI, and IA-2) and considered positive if any of the peptide pools or recombinant antigens elicited a response (SI ≥ 2). Clustering was analyzed by agglomerative hierarchical testing, based on Pearson's correlation distance metric between autoantigens/patients using MeV_4_8 version 10. Principal component analyses were performed on centered and scaled Z-scores for ELISpot stimulation indices using the prcomp function from the in-built stats package in R 3.2.1. Biplots with superimposed meta-information were generated using the ggbiplot package (VQ Vu, <https://github.com/vqv/ggbiplot>), also in R 3.2.1. Patient cohorts stratified for time since first transplant were tested for differences in C-peptide values and percentages assessed by one-way ANOVA with Bonferroni correction and differences in the frequency and phenotype of autoimmune responses by chi-square analysis. A comparison of the percentage C-peptide and time since first transplant between clusters was assessed by

unpaired *t*-test. Data were analyzed using GraphPad Prism 8 software. A value of $P < .05$ was considered statistically significant.

3 | RESULTS

Fifty-eight UK islet transplant recipients were studied with autoreactive T cell phenotyping undertaken in 23 participants pretransplant and 40 participants posttransplant samples (including 5 providing pre- and posttransplant samples). Pretransplant demographics and distribution of HLA genotypes associated with high risk of type 1 diabetes (HLA DRB1*0301 and/or *0401-05) were comparable in both groups (Table 1).

All had recurrent severe hypoglycemia and confirmed C-peptide-negative type 1 diabetes pretransplant with >50% using continuous subcutaneous insulin infusion pumps (Table 1). In the group immunophenotyped posttransplant, 32 (80%) received islet transplantation alone (ITA) with 7 (17.5%) received islet after kidney transplants (IAK) and 1 (2.5%) received islet after failed vascularized pancreas transplant. Median number of mismatches per recipient (when considering all transplants for an individual) was 7 (range 0–16).

Twelve (30%) participants received a single islet transplant and 28 (70%) participants received two grafts from separate donors, with one of these receiving a third transplant. Median time between first and second transplant was 6 (range 1–34) months (Table 2). Median viability of transplanted islets was 90% (range 88%–95%) with purity 75% (66%–88%).

Thirty-six (90%) recipients received ATZ induction including all single islet transplants. Remaining transplants were preceded by anti-IL-2 receptor induction with the exception of one recipient who received ATG for the first and ATZ for the second transplant

TABLE 1 Baseline characteristics in immunophenotyped cohorts pre- and posttransplant

Characteristic	Pretransplant cohort ($n = 23$)	Posttransplant cohort ($n = 40$)
Age (y)	50 (45–54)	49 (43–54)
Female (%)	16 (70%)	25 (62.5%)
Duration of Type 1 diabetes (y)	35 (31–37)	32 (26–38)
Weight (kg)	67 (62–73)	64 (59–75)
Total daily insulin dose (units)	31 (22–38)	34 (22–42)
Insulin (unit/kg)	0.47 (0.38–0.64)	0.53 (0.44–0.68)
Continuous subcutaneous insulin infusion	13 (56.5%)	23 (57.5%)
HbA1c (mmol/mol)	58 (50–80)	66 (55–79)
HLA DRB1*0301	8 (35%)	14 (35%)
HLA DRB1 *0401-05	5 (22%)	7 (18%)
DRB1*0301 and *0401-05	8 (35%)	14 (35%)

Note: Data are n (%) or median (interquartile range).

TABLE 2 Transplant and T cell sampling timelines

Recipient	Pre	Tx	Time Since Transplant (Months)																	Immunosuppression					Category	Cluster	HLA mismatches (HLA-A, B, & DR)		
																				Induction		Maintenance					1st Tx	2nd Tx	3rd Tx
			1 to 6	7 to 12	13 to 24	>24	1st Tx	2nd Tx	3rd Tx	1st Tx	2nd Tx	3rd Tx																	
NE004		T																	ATZ			Tac + MMF			ITA	n/a	1,2,2		
RF009		T																	ATZ			Tac + MMF			IAK	2	1,2,1		
ED010		T																	ATZ			Tac + MMF			ITA	2	2,2,2		
NE007		T																	ATZ			Tac + MMF			ITA	1	1,1,1		
NE003		T																	ATZ			Tac + MMF			ITA	2	2,2,1		
RF007		T																	ATZ			Tac + MMF			ITA	1	1,1,1		
RF005		T																	ATZ			Tac + MMF			ITA	2	2,2,0		
RF015		T																	ATZ			Tac + MMF			ITA	n/a	1,1,2		
MA065		T																	ATZ			Tac + Pred			IAK	n/a	1,1,1		
ED017		T																	ATZ			Tac + MMF + Pred			ITA	1	0,0,0		
RF006		T																	ATZ			Tac + MMF + Pred			ITA	n/a	0,2,1		
ED005		T																	ATZ			Tac + MMF	Tac + MMF		ITA	n/a	1,1,1	2,2,2	
ED001		T																	ATZ			Tac + MMF	Tac + MMF		ITA	n/a	2,2,1	2,2,1	
ED015		T																	ATZ			Tac + MMF	Tac + MMF		ITA	n/a	1,2,1	0,2,2	
ED013		T																	ATZ			Tac + MMF	Tac + MMF		ITA	2	1,1,2	2,1,1	
RF004		T																	ATZ			Tac + MMF	Tac + MMF		IAK	1+2	1,1,1	1,1,2	
ED003		T																	ATZ			Tac + MMF	Tac + MMF		ITA	1	1,2,1	1,2,1	
ED007		T																	ATZ			Tac + MMF	Tac		ITA	n/a	1,2,1	2,2,2	
ED008		T																	ATZ			Tac + MMF	Tac + MMF		ITA	1	0,1,1	0,1,0	
ED004		T																	ATZ			Tac + MMF	Tac + MMF		ITA	1	1,2,2	1,2,1	
ED006		T																	ATZ			Tac + MMF	Tac + MMF		ITA	2	1,2,1	1,2,1	
ED002		T																	ATZ			Tac + MMF	Tac + MMF		IAP	1	1,2,1	1,1,1	
ED011		T																	ATZ			Tac + MMF	Tac + MMF		ITA	n/a	2,1,2	0,1,1	
RF011		T																	ATZ			Tac + MMF	Tac		ITA	n/a	1,2,1	1,1,2	
NE019		T																	ATZ			Tac	Tac		IAK	2	0,1,1	1,2,1	
RF008		T																	ATZ			Tac + MMF	MMF		ITA	1+2	2,2,2	0,2,2	
NE024		T																	ATZ			Tac + MMF	Tac + MMF		ITA	1+2	1,2,1	0,0,1	
NE010		T																	ATZ			Tac + MMF	Tac + MMF	Tac	ITA	1	2,2,2	2,2,2	2,1,1
NE011		T																	ATZ			Tac + MMF	Tac + MMF		ITA	n/a	1,2,1	1,2,1	
NE013		T																	ATZ + ETA			Tac + MMF			ITA	1	2,1,1		
KC005		T																	ATZ + ETA			Tac + MMF	Tac + MMF		ITA	1	0,1,0	1,1,2	
KC006		T																	ATZ + ETA			Tac + MMF	Tac + MMF		IAK	1	2,2,1	1,1,1	
MA002		T																	ATG			Tac+ MMF	Tac		ITA	n/a	2,2,2	1,2,1	
NE001		T																	DAC			Tac + MMF	Tac + MMF		ITA	2	1,1,0	1,2,0	
NE014		T																	BAS			Tac	Tac + MMF		ITA	n/a	1,1,2	1,2,1	
NE021		T																	BAS			Tac + MMF	Tac + MMF		ITA	n/a	1,2,2	0,1,1	
RF012		T																	BAS			Tac + MMF	Tac + MMF		ITA	1	0,1,1	2,1,1	
KC004		T																	BAS			Siro + MMF	Tac + MMF		ITA	2	1,2,2	1,1,2	
RF001		T																	BAS			CSA + MMF	CSA + MMF		IAK	n/a	2,2,2	1,1,2	
KC003		T																	DAC			Tac + MMF	Tac + MMF		IAK	1+2	n/a	n/a	

Note: Islet transplant and autoreactive T cell sampling/meline, immunosuppression and number of HLA mismatches in each participant studied posttransplant. Time of each transplant is indicated by (T). Highlighted boxes indicate when samples were tested by ELISpot for autoimmune T cells. Black X: no response detected; white box: IL-10 response; black box: IFN- γ response; grey box: both an IL-10 and IFN- γ response. Recipients who tested positive for donor-specific antibodies (DSAs) are highlighted within the boxes. Transplant category: islet transplant alone (ITA), islet after kidney (IAK), and islet after pancreas (IAP). Hierarchical cluster analysis grouping for each recipient is outlined, as Cluster 1, Cluster 2, or not applicable (n/a). Recipients received pretransplant induction with alemtuzumab (ATZ), basiliximab (BAS), or daclizumab (DAC) with/without etanercept (ETA) 50 mg intravenous infusion followed by 25 mg subcutaneously on posttransplant day 3, 7, and 10. Maintenance immunosuppression was tacrolimus (Tac) (trough 8–12 ng/ml) and mycophenolate mofetil (MMF) (500 mg–2 g daily), unless white blood cell count was too low to enable MMF addition. A single recipient received sirolimus (Siro) in combination with MMF. Prednisolone (Pred) was continued over 12 months posttransplant at (5 mg on alternate days) in three IAK recipients with a fourth IAK recipient continuing ciclosporin (CSA) and MMF post-islet transplant. HLA mismatches between recipient and donor were calculated by comparing the mismatches at HLA-A, B, and DR loci for each transplant.

(Table 2). Additional peritransplant 'anti-TNF- α therapy was used in only four transplants. All recipients received tacrolimus (Tac)/mycophenolate mofetil (MMF) maintenance immunosuppression with a small percentage additionally receiving prednisolone (7.5%), sirolimus (2.5%), or ciclosporin A (2.5%). No adverse events related to induction or immunosuppression therapy were reported to the central NHS Blood and Transplant registry in any of the study participants.

Posttransplant autoreactive T cell sample time-points and details of recipients sampled are shown in Table 2. Eleven (85%) of those immunophenotyped at 1–6 months after first transplant had graft function; 18 (100%) at 7–12 months; 29 (97%) at 12–24 months; and 19 (86%) at >24 months. The 90-min mixed meal tolerance test stimulated C-peptide values at each time-point are shown in Figure 1A. Plotting stimulated C-peptide values for each recipient as a percentage of their C-peptide attained at 1 month after final transplant³³ (as a putative marker of individualized peak graft function) revealed a significant decline in within-person function in the group studied >24 months posttransplant in comparison to the group assessed

7–12 months posttransplant (Figure 1B). Stimulated C-peptide values over time in all recipients are plotted in Figure S1.

3.1 | Frequency and phenotype of antigen-specific T cell responses

Immune response was assessed cross-sectionally in a total of 110 samples from the 58 participants using sensitive IFN- γ and IL-10 cytokine ELISpot assays (representative example shown in Figure S2). Lymphodepletion (lymphocyte count $<0.1 \times 10^9/L$) over the first 7 days was confirmed after all transplants preceded by ATZ induction. No significant impact of induction therapy and maintenance immunosuppression on T cell response to polyclonal stimulation with CytoStim was seen, with the majority mounting a combined IFN- γ and IL-10 response pre- and posttransplant and no significant differences in polarization of the responses at any posttransplant time-point (Figure 2A). In contrast, a significant reduction in the proportion

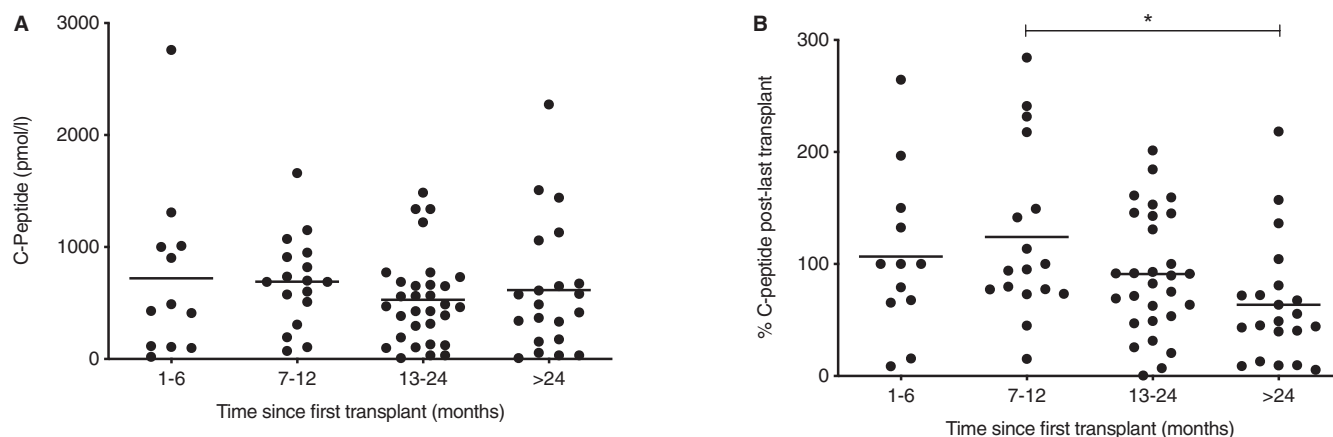


FIGURE 1 Graft function at times of T cell samples (A) 90-min MMTT-stimulated C-peptide values at each posttransplant time-point. Horizontal lines represent mean C-peptide value within the group. (B) Stimulated C-peptide values calculated as a percentage of each recipient's stimulated C-peptide at first MMTT post-last transplant. Significance tested using a one-way ANOVA test corrected using Bonferroni's multiple test comparison, * $P < .05$

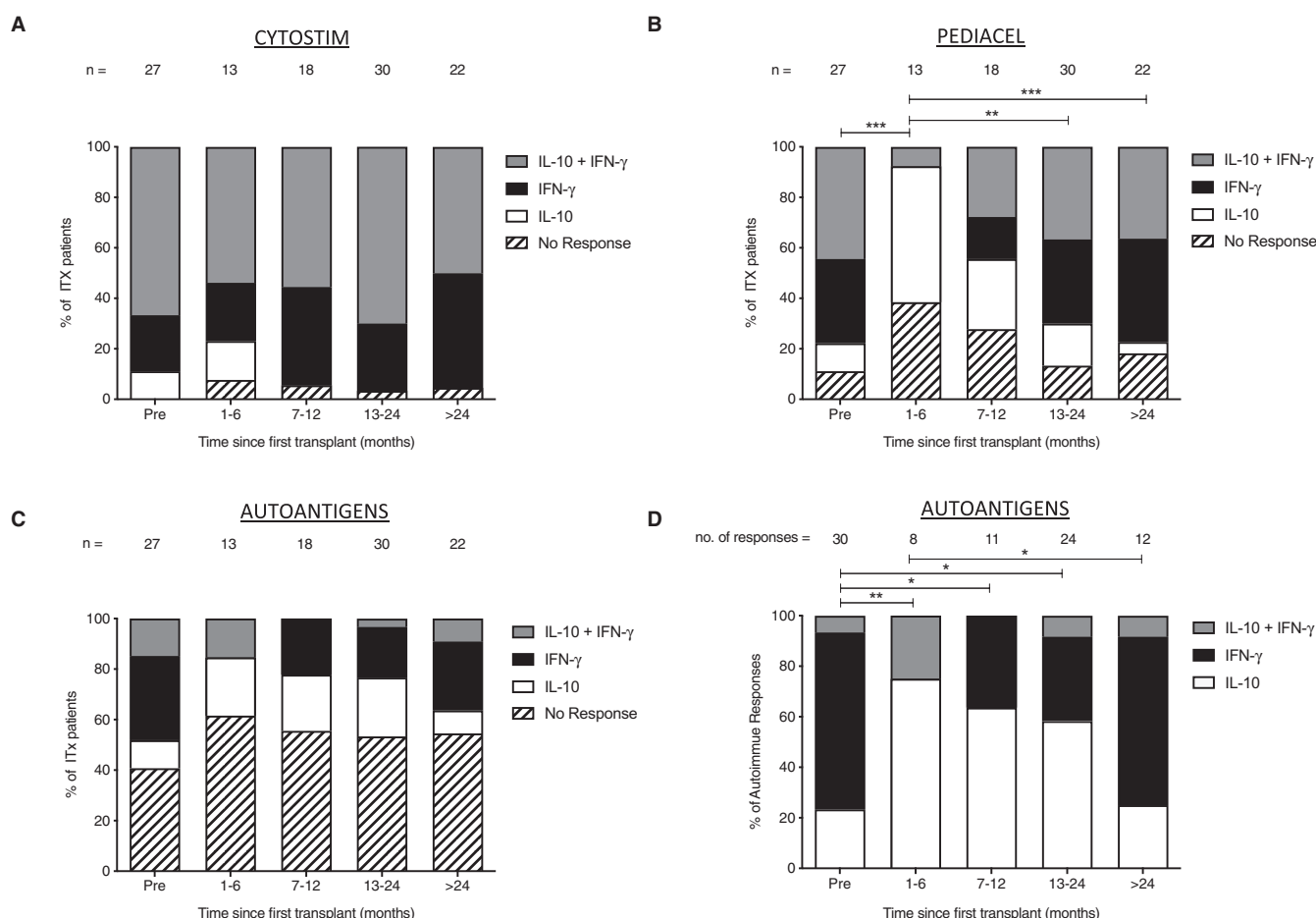


FIGURE 2 Frequency and phenotype of T cell responses in groups at each time-point. N indicates the number of individuals or responses within each group. (A–C) Frequency of individuals mounting a particular response to (A) CytoStim, (B) PediaCiel, and (C) islet antigens/peptide pools. (D) Cumulative frequency and phenotype of all islet-specific responses detected. Samples with no detectable response were excluded from this analysis. Hatched lines: no response detected; white bars: IL-10 response; black bars: IFN- γ response; gray bars: both IL-10 and IFN- γ responses. Chi-squared tests performed on all conditions tested, * $P < .05$, ** $P < .01$, *** $P < .001$

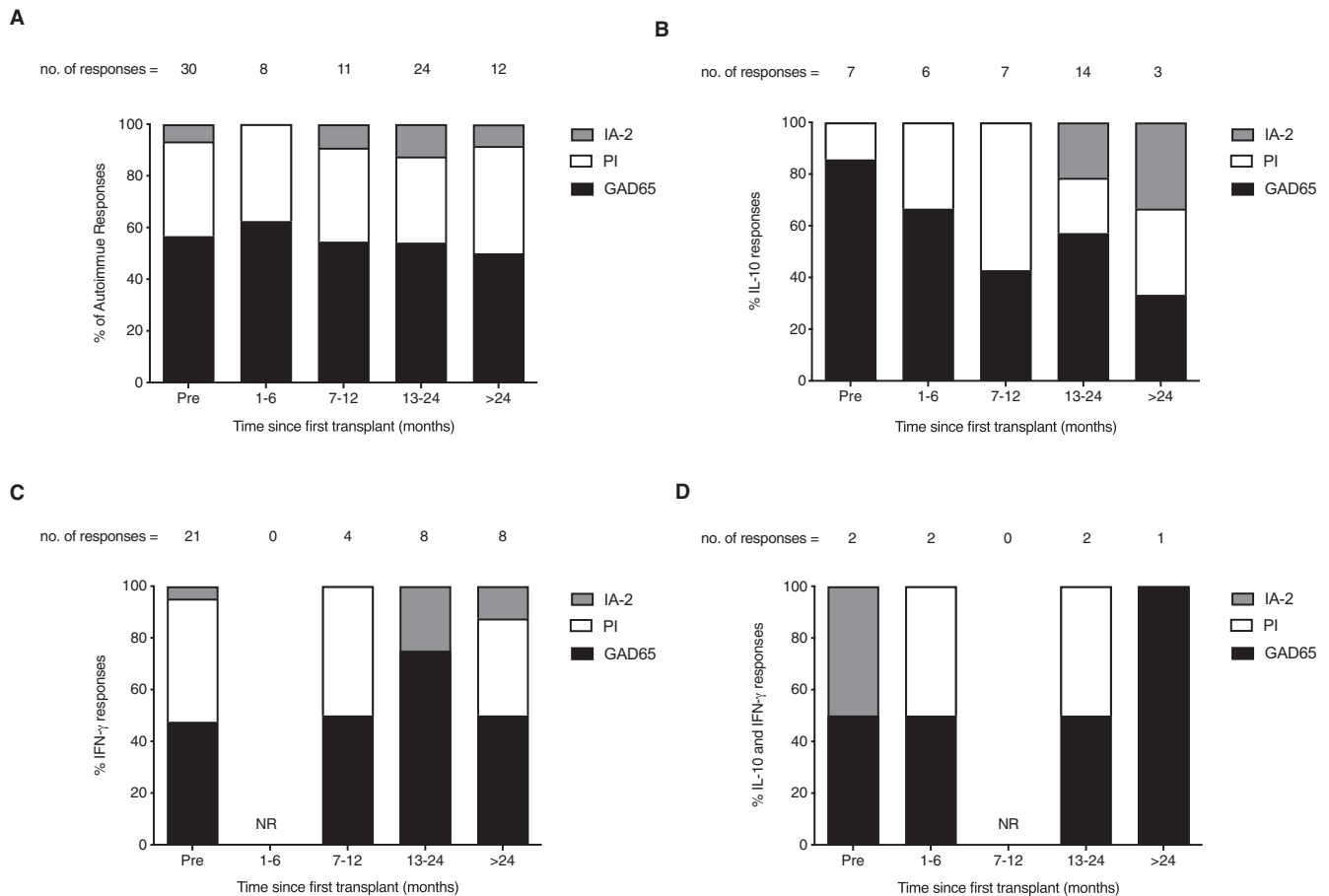


FIGURE 3 Frequency, phenotype, and autoimmune targets of islet-specific T cell responses at each time-point. (A) Frequency of responses to autoantigens GAD65 (black), PI (white), and IA-2 (gray). Division of the autoimmune response by phenotype (B) IL-10 only, (C) IFN- γ only, and (D) IFN- γ and IL-10-targeting autoantigens GAD65, PI, and IA-2. Proportions with each phenotype at each time-point were compared by chi-squared tests and showed no significant differences. NR, no response

of recipients responding to (Pediace) vaccine stimulation was seen (Figure 2B) over the first 6 months posttransplant (pretransplant: 89% vs. 1–6 months posttransplant: 62%; $P < .05$). This was associated with an increased proportion showing an IL-10-only response (pretransplant: 11% vs 1–6 months posttransplant: 54%; $P < .005$) and none showing an IFN- γ alone response. At later posttransplant time-points, this predominantly IL-10 phenotype response incrementally reverted to an IFN- γ alone or combined IFN- γ /IL-10 combined response, comparable to that seen in pretransplant participants.

A significant response to at least one islet autoantigen was observed in 60% of pretransplant samples and this response was dominated by an IFN- γ or combined IFN- γ /IL-10 phenotype (Figure 2C). At 1–6 months posttransplant, none of the immunophenotyped recipients showed an IFN- γ alone T cell response to islet autoantigens, mirroring the responses to vaccine stimulation. At later time-points, the phenotype of autoreactive T cell responses incrementally reverted to include IFN- γ secretion (with phenotypes at >24 months comparable to those pretransplant). As responses to multiple islet autoantigens (GAD65, IA-2, and PI) were assayed at each time-point and an individual may respond to more than one antigen or epitope, the total number and phenotype of all islet-specific responses in responding participants assessed at each time-point

were also assessed (Figure 2D). Significant differences in the polarization of the total number of islet autoantigen-specific responses per individual before and after first transplant were seen—from an IFN- γ -dominated response in the pretransplant cohort, to an IL-10-dominated response immediately posttransplant (1–6 months) replaced by predominantly IFN- γ responses at >24 months posttransplant.

3.2 | Molecular targets of islet-specific T cell responses

Besides defining overall autoreactive T cell response, we investigated responses to individual antigens from the same samples tested in Figure 2 that showed an autoimmune T cell response. GAD65 was the most frequently targeted autoantigen before and after islet transplantation constituting $\geq 50\%$ of responses at all time-points, while PI and IA-2 constituted $\geq 33\%$ and $\leq 12.5\%$ of responses, respectively (Figure 3A). Polarization of responses to each autoantigen was comparable to overall response at each time-point.

As no whole-recombinant IA-2 autoantigen with high enough purity was available for use at the time of these studies, only IA-2

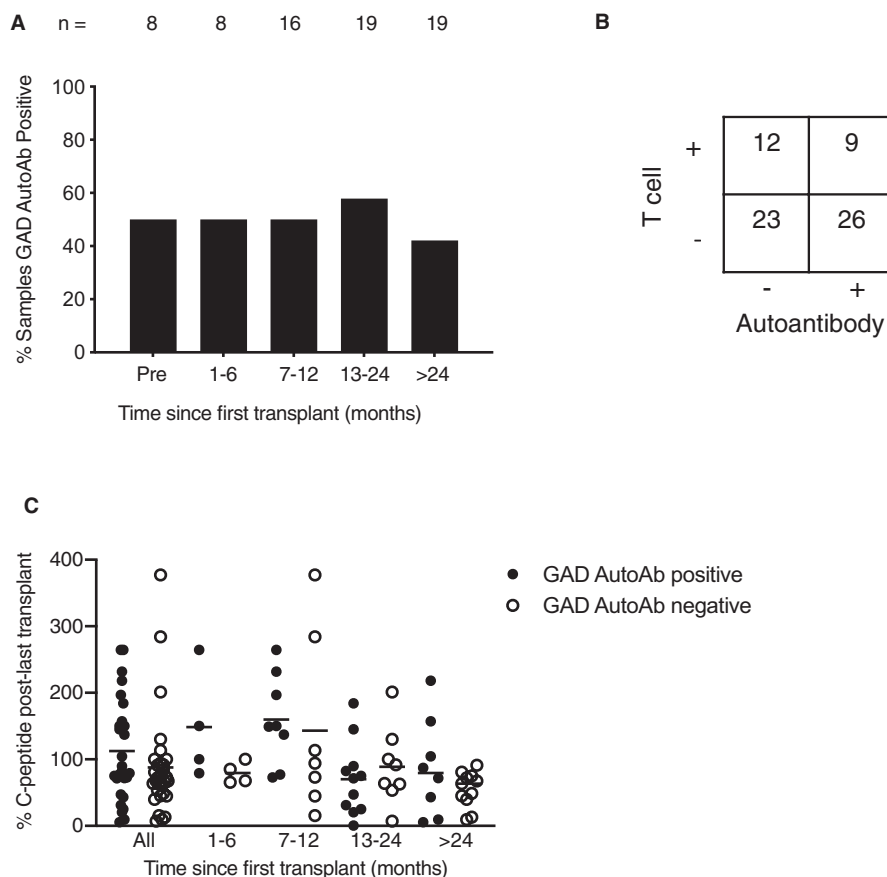


FIGURE 4 GAD autoantibody status and associations with T cell responses and graft function. (A) Bar graph representing the percentage of participant samples at each time-point tested which were GAD65 seropositive. (B) Contingency table showing relationship between autoantibody and T cell responses to GAD65 in individuals where both variables were tested simultaneously. Fisher's exact test showed no significant association between GAD autoantibody status and GAD-specific T cell response. (C) Stimulated C-peptide as a percentage of each recipient's stimulated C-peptide at first MMTT post-last transplant in GAD65 seropositive and seronegative recipients at each sampling time-point. Means were compared by unpaired *t*-test and were not significantly different ($P = .2246$)

peptide pool was used. For GAD65 and PI, whole autoantigens and peptide pools were used. Combining responses to both stimuli removed any HLA or peptide bias, increasing the likelihood of detecting a T cell response. Subdividing the phenotype of islet-specific responses by individual autoantigen targets in participant cohorts at each time-point after first transplant revealed no statistically evident dominance of any response by a particular phenotype of a single islet antigen (Figure 3B–D). This was unsurprising given the low number of responses in each group.

3.3 | Islet autoantibody responses

Assessment of GAD antibody status was undertaken in parallel with T cell phenotyping ($n = 70$; 64% of T cell samples) with a comparable proportion of participants (40%–60%) seropositive at all time-points (Figure 4A). There was no association between GAD antibody status and GAD-targeted autoreactive T cell response (Figure 4B) or cross-sectional stimulated C-peptide value (Figure 4C).

3.4 | Alloantibody responses

Despite maintenance immunosuppression, *de novo* donor-specific antibodies (DSAs) were detected posttransplant in three participants. This was associated with rapid loss of graft function in two recipients. No associations between alloantibody and autoreactive T cell responses were seen.

3.5 | Investigating the relationship between islet-specific T cell responses and graft function using unbiased hierarchical clustering analysis

Combined analysis of autoimmune T cell responses (IFN- γ and IL-10 to five islet antigen preparations) including all participant samples in which a response was detected using unbiased hierarchical clustering in a heat map illustrated the division of patients into two main clusters, with the major discriminating factor being the phenotype of the responses detected. This was detected in both pre- and post-transplant sample responses, with two main clusters formed for each

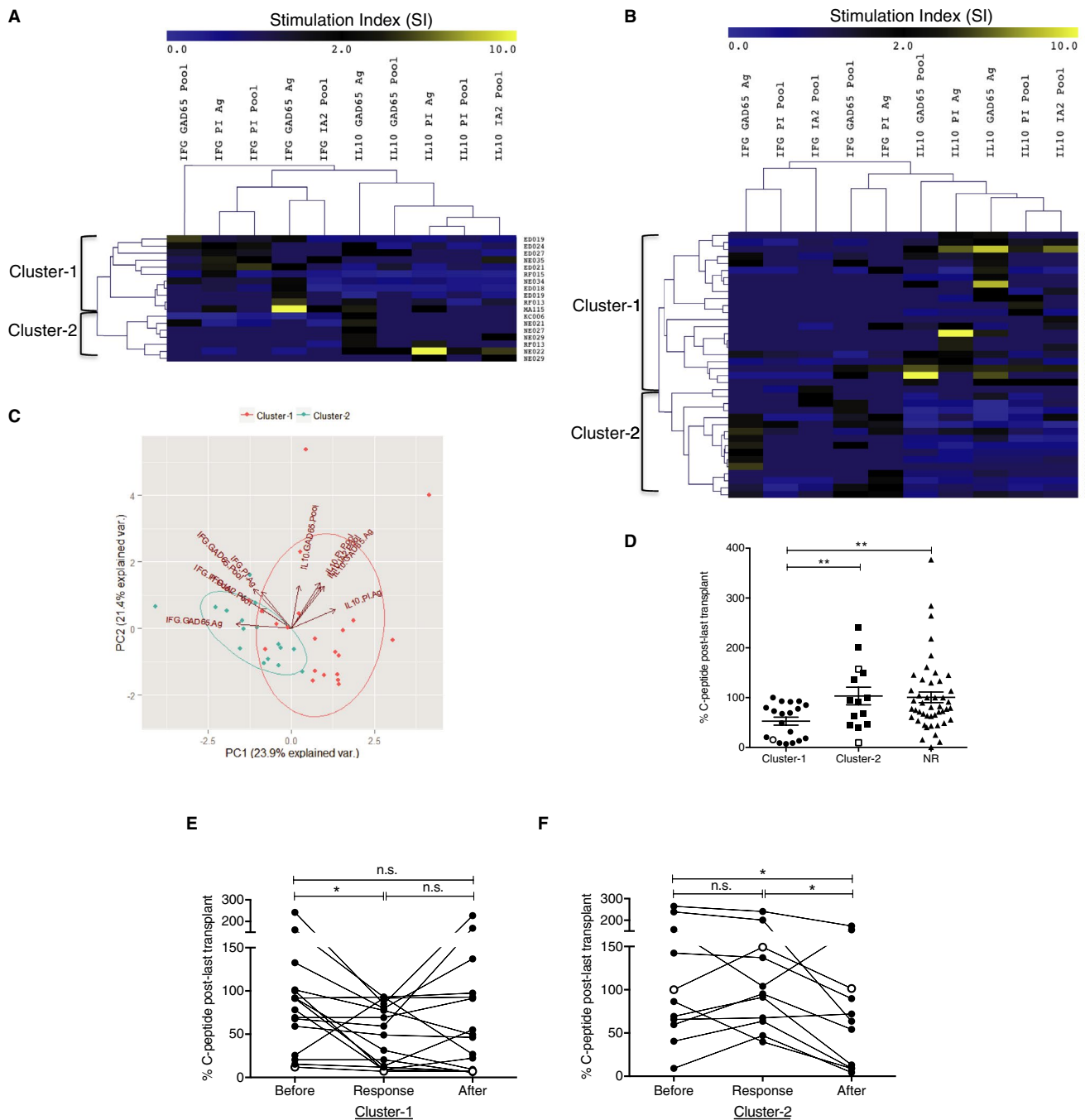


FIGURE 5 Unbiased hierarchical clustering of islet-specific T cell responses and relationship with graft function. Unbiased hierarchical clustering of IL-10 and IFN- γ autoimmune T cell responses Stimulation Index (SI) values in the (A) pretransplant and (B) posttransplant patient sample groups represented as heat maps. Patient codes shown vertically on the right side of the heat map. Participants positive for de novo DSAs are highlighted with an asterisk (*). Autoantigens and phenotypes are represented horizontally across the top. Blue indicates a negative response ($SI \leq 2$), black boxes represent a borderline positive response ($SI \geq 2$), black-yellow boxes represent a positive response ($SI \geq 3$). (C) Plots showing the two principal components (PCs) of autoimmune T cell responses in the posttransplant sample cohort. The ovals outline putative patient clusters identified from the heat map, with Cluster-1 (pink dots) predominantly reflecting IL-10 and Cluster-2 (blue dots) IFN- γ responses. (D) Graph comparing graft function (% C-peptide post-last transplant) from individuals in Cluster-1, Cluster-2, and nonresponders (NR), C-peptide values represent those measured at the same time as the detection of the autoimmune response used in clustering analysis. Data were analyzed using an unpaired *t*-test. (E,F). Longitudinal assessment of graft function in individuals in Cluster-1 (E) and Cluster-2 (F). Graphs show C-peptide assayed before, at the time, and after detection of the islet antigen-targeted immune response represented as percentage of stimulated C-peptide at 1 month after last transplant. Participants with de novo DSAs are represented by empty symbols in D–F. Data were compared by paired *t*-test; **P* < .05, ***P* < .01, n.s. not significant

group of samples. In the pretransplant responses (Figure 5A) the autoimmune T cell responses in Cluster-1 are mainly of an IFN- γ phenotype with a broader range of autoimmune targets, while Cluster-2 consists of mainly IL-10 responses predominantly to GAD65 antigen.

In the posttransplant cohort of autoimmune T cell responders, the reverse trend was seen following the analysis (Figure 5B), with one cluster (Cluster-1) showing a substantial frequency of higher magnitude IL-10 responses, targeting a broad range of autoantigens. The magnitude and diversity of the responses were smaller in the second cluster (Cluster-2) of primarily lower magnitude IFN- γ responses. We observed no significant differences in number of transplants or immunosuppression regimen between recipients in posttransplant Cluster-1 and Cluster-2. Analysis omitting recipients who did not receive ATZ induction did not alter clustering results.

To corroborate our hierarchical clustering, we performed principal component analysis (PCA) using the ELISpot stimulation indices in posttransplant participants demonstrating any positive response to the autoantigens tested (Figure 5C). PCA allows exploration of a dataset through reduction of multiple dimensions to a set of component scores generated from co-correlated variables that account for decreasing proportions of variance within the dataset. Plotting principal component 1 (PC1; accounting for the greatest proportion of variance within the dataset) versus PC2 revealed a bifurcation in the samples separated by a predominantly IFN- γ or predominantly IL-10 response to the antigens tested. We found no significant differences in PC1 scores in the sample with respect to recipient gender or age (data not shown). Overall, this analysis demonstrates clustering (polarization) of responses to autoantigens in the samples independently of any potential confounders tested.

We assessed whether graft function differed in the two participant clusters in the posttransplant cohort by comparing C-peptide levels (% of value at 1 month post-last transplant) measured at the same time as autoreactive T cell phenotyping (Figure 5D). Graft function was relatively low in the participants in the IL-10-oriented posttransplant Cluster-1 (55% of 1 month post-last transplant stimulated C-peptide) compared with those in the IFN- γ -oriented Cluster-2 being (110% of 1 month post-last transplant stimulated C-peptide; $P = .0038$). Interestingly, graft function in those with no islet-specific responses was comparable to those in Cluster-2 and significantly greater than that in Cluster-1 ($P = .0077$). These results could not be attributed to time since transplant as no significant differences between the two clusters or nonresponders were seen (data not shown).

To further explore the relationship between islet-specific immune response and graft function, we assessed C-peptide levels before and after an islet-specific immune response was detected for all individuals in each cluster with available data (Figure 5E,F and Figure S3). Longitudinal analysis in Cluster-1 recipients revealed a significant decline in graft function in the period preceding the IL-10 response ($P = .0165$), but stable graft function revealed thereafter (Figure 5E). In contrast, in Cluster-2 recipients, although stimulated C-peptide levels were higher at the time of an IFN- γ response, they declined following this response ($P = .01$), and were significantly lower than C-peptide levels preceding the IFN- γ -dominated response

($P = .02$). A single recipient within each cluster had detected de novo DSAs posttransplant. In Cluster-1, this associated with loss of graft function preceding the IL-10-dominated response. In Cluster-2, this was associated with decreased graft function after the development of IFN- γ -dominated response. Excluding recipients with DSAs did not affect the significant decline prior to "stabilizing" IL-10 response or following an IFN- γ response.

Reanalysis of T cell responses excluding IAK recipients, already on immunosuppression following their preceding renal transplant and those who did not receive ATZ induction did not alter any study findings (Figure S4).

4 | DISCUSSION

In this study, we examined frequency and phenotype of islet-specific T cell responses in recipients within a national transplant program favoring ATZ induction. Treatment with ATZ was not associated with significant reduction in the proportion with an autoreactive T cell response, but associated with a shift toward an IL-10-oriented response, which tended to revert to an IFN- γ -oriented response over time.

To interpret MMT-stimulated C-peptide values at the time of T cell phenotyping in the context of preceding graft function, C-peptide was considered as a percentage of each recipient's stimulated value at 1 month after their last transplant. This revealed significantly lower "proportion of maximal graft function" in those phenotyped at >24 months post-first transplant compared with those assessed at 7–12 months, consistent with findings from previous studies of longitudinal follow-up of allogeneic islet transplant recipients.⁸

By assessing frequency and phenotype of islet-specific T cell responses in parallel with graft function, we were able to form an immune "snapshot" that may influence metabolic function at that time posttransplant. GAD65 was the predominant autoantigen targeted by T cells, followed by PI, irrespective of transplant status (pre/post) or the phenotype of cytokines produced. Previous studies using proliferation as a readout have detected CD4⁺- and CD8⁺ GAD65-specific T cells in islet transplant recipients and noted that the presence of these cells either before or after transplantation was associated with poorer graft outcome.^{21,34} However, these studies did not include recipients receiving ATZ lymphodepleting induction. Although the current study did not include longitudinal follow-up of the same individuals before and after transplantation, the pretransplant cohort was studied and analyzed using congruent techniques contemporaneously with the posttransplant cohort and used as a well-matched comparator group drawn from the same overall UK islet transplant population. Alemtuzumab induction followed by the immunosuppression of Tac and MMF maintenance was associated with a shift in T cell response in those with a detectable response, from IFN- γ -dominated pretransplant to IL-10-dominated immediately posttransplant, for both the autoimmune and Padiacel T cell responses, suggesting that this

regimen influences all antigen-specific T cell responses. This shift is consistent with previous findings in which induction with ATZ compared to anti-IL-2 receptor antibody or ATG led to a marked increase in the frequency of CD4⁺ Foxp3⁺ T cells within the first month following islet transplantation, with a return to baseline levels by 6 months, although regulatory function of these T cells was not tested.³⁵ Additional studies in renal transplant patients and multiple sclerosis clinical trials in which ATZ therapy was administered have also reported an increase in the proportion of regulatory T cells,³⁶⁻⁴⁰ and a decrease in IL-17 and IFN- γ -producing CD4⁺ T cells.⁴⁰ The reduction in the frequency of IFN- γ -producing cells in UK islet transplant recipients may be further influenced by Tac immunosuppression, which is capable of abrogating IFN- γ production by expanded autoreactive T cells.²³ Although the skewing of cytokine responses following induction therapy is clear, our analyses were limited in breadth, focusing on IFN- γ and IL-10 as prototypical pro- and anti-inflammatory cytokines without isolation and functional characterization of these cells. Moreover, we are not able to directly relate these findings to immune reconstitution. However, our results in combination with other studies strongly support the hypothesis that there is a "resetting" of the immune system following ATZ induction in islet transplant recipients. Why IL-10-producing memory T cells are spared depletion or may reconstitute more rapidly remains unclear.

Previous studies have investigated the influence of anti-graft immune reactivity on islet function and concluded that both cellular and humoral alloimmune responses are associated with rapid loss of graft function.^{15,41} We have previously reported an association between early posttransplant de novo DSA formation and rapid loss of graft function in one UK islet transplant center.¹⁴ In that study which informed subsequent national practice, it was concluded that ATZ induction protected against alloantibody formation. In the current analysis, DSAs were rare and were not associated with an increased frequency or particular phenotype of islet-specific autoreactive T cell response. However, as we did not assess alloimmune T cell responses, we cannot exclude the possibility that these were associated with a particular autoimmune response.

While other studies have reported associations between humoral autoimmune responses and loss of graft function,^{17,19} we observed no significant correlation between GAD autoantibody status and graft function, a difference which may be influenced both by the specificity of islet autoantibodies assessed and also differing immunosuppressive regimens used.

Detailed analysis of agglomerated autoimmune T cell responses using unbiased hierarchical clustering and PCA revealed that participants fell into two main clusters differentiated by phenotype, with a clear distinction between those with IL-10-oriented and IFN- γ -oriented responses. This segregation of responses has been described in individuals with new-onset type 1 diabetes or multi-autoantibody-positive nondiabetic siblings at high risk of disease progression in whom similar response phenotypes were observed.^{32,42} Detection of IL-10 islet-specific T cell responses was associated with

the nondiabetic state and, when seen in new-onset type 1 diabetes, correlated with older age at disease onset. This in combination with *in vitro* analysis of these cells suggests that these responses are potentially islet protective.^{32,43}

In the current study, an IL-10-oriented posttransplant response was associated with relatively low graft function. Although potential mechanisms underlying this association remain unclear, longitudinal analysis of graft function was possible in a small subset of recipients from the IL-10 and IFN- γ clusters. Graft function was significantly higher prior to an IL-10 response with subsequent stabilization. In contrast, function deteriorated following detection of an IFN- γ -oriented autoreactive T cell response, in keeping with a pathogenic role of this cytokine in reducing graft function.

An association between GAD65-specific IFN- γ responses and graft dysfunction in allogenic islet transplant recipients but no IL-10 production was reported in a small cohort receiving anti-IL-2 receptor antibody or ATG induction.³⁴ It remains to be determined how the IL-10⁺ cells detected posttransplant are related to those in a nontransplant setting. Posttransplant IL-10-secreting cells may have been generated by chronic exposure to autoantigen released from damaged islets and may be able to control further graft destruction in the posttransplant setting. Future studies combining analysis of the phenotype and functional potency of islet-specific CD4⁺ and CD8⁺ T cells will be required to fully elucidate the impact of these cell types on graft survival.

Although hypothesis-generating only at this stage, it appears that, following islet transplantation with ATZ induction, an "islet-protective" phenotype may predict maintained subsequent graft function with a more "destructive" phenotype predicting subsequent declining function. Autoreactive T cell phenotyping posttransplant, either protocolized or in response to acute decrement in graft function, may help inform subsequent management including personalized, immune-targeted rescue strategies.

This study has a number of limitations including its largely cross-sectional nature with few recipients immunophenotyped pre- and posttransplant. There was also a degree of heterogeneity in participants (predominantly ITA but including some IAK recipients) and in number of islet infusions as well as immunosuppression regimens, although core immunosuppression constituted Tac/MMF. Reanalyses excluding IAK and those who did not receive ATZ did not alter findings.

In conclusion, we have demonstrated that ATZ induction therapy leads to a phenotypic shift in islet-specific T cell responses following islet transplantation and that profile of these posttransplant T cell responses may predict subsequent graft function.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

Data are available on reasonable request due to privacy/ethical restrictions.

ORCID

Shareen Forbes  <https://orcid.org/0000-0002-9127-0641>

James A. Shaw  <https://orcid.org/0000-0002-2893-3211>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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