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ORIGINAL ARTICLE

Peripheral blood immune cell profiling of acute corneal transplant rejection

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Acute rejection (AR) of corneal transplants (CT) has a profound effect on subsequent graft survival but detailed immunological studies in human CT recipients are lacking. In this multi-site, cross-sectional study, clinical details and blood samples were collected from adults with clinically diagnosed AR of full-thickness (FT)-CT (n = 35) and posterior lamellar (PL)-CT (n = 21) along with Stable CT recipients (n = 177) and adults with non-transplanted corneal disease (n = 40). For those with AR, additional samples were collected 3 months later. Immune cell analysis was performed by whole-genome microarrays (whole blood) and high-dimensional multi-color flow cytometry (peripheral blood mononuclear cells). For

Abbreviations: AR, acute rejection; CM, central memory; EM, effector memory; FT-CT, full-thickness corneal transplant; PL-CT, posterior lamellar corneal transplant; Treg, regulatory T cell.

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both, no activation signature was identified within the B cell and T cell repertoire at the time of AR diagnosis. Nonetheless, in FT- but not PL-CT recipients, AR was associated with differences in B cell maturity and regulatory $CD4^+$ T cell frequency compared to stable allografts. These data suggest that circulating B cell and T cell subpopulations may provide insights into the regulation of anti-donor immune response in human CT recipients with differing AR risk. Our results suggest that, in contrast to solid organ transplants, genetic or cellular assays of peripheral blood are unlikely to be clinically exploitable for prediction or diagnosis of AR.

KEYWORDS
biomarker, corneal transplantation/ophthalmology, flow cytometry, rejection: acute, translational research/science

1 | INTRODUCTION

Allogeneic corneal transplantation (CT) is a long-established, highly successful procedure for restoring sight to people with partial or total loss of vision due to corneal opacification.[1] Globally, corneal diseases, ranging from hereditary and acquired dystrophies to inflammatory infections, autoimmunity and chemical injuries, constitute a common cause of disability that is frequently irreversible without CT.[2–4] The clinical impact of CT has advanced substantially through technical innovations—particularly, the development of posterior and anterior partial thickness (lamellar) transplant procedures for diverse indications—and is being extended further through clinical translation of advanced therapies and tissue engineering solutions.[1,5,6] Although posterior lamellar CT (PL-CT) procedures predominate over full-thickness CT (FT-CT/penetrating keratoplasty) in many countries, FT-CT remains the procedure of choice for re-transplantation and for corneal disease involving all tissue layers.[1,7–10]

Immunosuppressive therapy for CT is typically limited to topical corticosteroid for 12–24 months and short-term outcomes are highly favorable with 1–3 year graft survival >90% and acute rejection (AR) documented in 15%–20% of FT-CT and 2%–8% of PL-CT.[1,8,11–15] This generally low requirement for immunosuppressive therapy in CT recipients likely reflects the small overall size and cellular content of the transplanted tissue, the limited number of graft-derived antigen presenting cells (APCs), the lack of vascular channels in healthy cornea and the presence of natural immune tolerance mechanisms within the intact anterior eye.[1,16,17] Nonetheless, when AR occurs, it has a profound effect on subsequent graft survival, indicating that immunological damage is often not fully reversible.[8,11] Furthermore, some CT procedures are associated with higher (>30%) AR risk. These include re-transplants, transplants for infectious/inflammatory diseases, and those with extensive neovascularization.[1,8] Systemic immunosuppression with oral corticosteroid, calcineurin inhibitor, mycophenolate mofetil or mTOR inhibitors may reduce AR risk, there are few clinical trials to guide the optimal selection, dosing and duration of these agents in high-risk CT.[1,18] Registries and longitudinal cohorts continue to indicate poor overall long-term survival of FT- and PL-CTs with risk factors for or history of AR.[1,8,11,16] There is a significant need, therefore, for clinical investigations of post-transplant immunosuppression regimens and of emerging therapies to limit the impact of AR and improve the long-term CT survival.[1,13,18]

Unlike solid organ transplants, monitoring of local or systemic immunological events remains little used for the diagnosis and prediction of AR or to guide the type or duration of immunosuppression in CT recipients.[1] As recently reviewed by Di Zazzo et al., however, novel in vivo imaging modalities and biomarkers detected in corneal tissue or aqueous humor can provide early diagnostic information about AR and other CT complications.[17] For instance, direct in vivo microscopy of the donor cornea and recipient bed can be used to track the changes in the density of inflammatory cell population post-transplant.[19] In contrast, despite experimental evidence that AR and immune tolerance of corneal allografts involve systemic components,[16,17,20] little research has been conducted into the potential peripheral immunological signatures of human CT complications and long-term stability.[21] Specifically, no comprehensive peripheral blood immune profiling studies of human CT recipients have been conducted to determine whether a distinct cellular signature of AR is detectable. In the current study, we aimed to compare the circulating lymphocyte repertoires of prevalent human CT recipients attending five academic Ophthalmology centers with clinically diagnosed AR of FT- and PL-CTs to those of stable non-rejecting CT recipients as well as patients with corneal diseases in the absence of transplantation.

2 | MATERIALS AND METHODS

Detailed information related to Biological sampling, cryopreservation and transport, flow cytometric and transcriptomic analysis and statistical analysis are provided in the supporting information.

2.1 | Study subjects, enrollment, and clinical data collection

Adult patients with prevalent FT- or PL-CTs were enrolled by informed consent in a cross-sectional observational study at 5 academic Ophthalmology Departments between 2015 and 2018. The study protocol was approved by each institutional ethics committee (see Table S1 for site-specific dates and details of ethical approvals). Inclusion criteria were: (1) age ≥ 18 years, (2) current FT- or PL-CT, and (3) willingness and capacity to provide informed consent. There were no specific exclusion criteria. Patients were enrolled at the outpatient facilities by an experienced clinical optometrist, clinical research nurse or clinical ophthalmology fellow and were categorized into groups on the basis of a detailed clinical history and ophthalmological examination. Group definitions were as follows: Acute rejection: Clinical examination findings of precipitates on the corneal graft but not on the peripheral recipient cornea, either scattered or in the form of a Khodadoust line, with increased central corneal thickness. Stable (≤3 years post-transplant): Clinical examination documenting clear graft more than 1 and less than 3 years post-transplantation with no prior history of AR. Long-term rejection-free (>3 years post-transplant): Clinical examination documenting clear graft >3 years post-transplantation with no prior history of AR. Non-transplanted corneal disease: Non-operated outpatients with Fuchs endothelial dystrophy, keratoconus, or stromal dystrophy. For the first three groups, enrolled patients were subdivided into FT and PL subgroups based on type of transplant. Each CT recipient was also categorized on the basis of transplant indication as High Risk (Corneal ulceration threatening or actual perforation, Herpes simplex keratitis [Scar Ulceration Perforation], Regraft and Secondary bullous keratopathy), Low Risk (Corneal scar without vessel ingrowth, Fuch's dystrophy, and Stromal dystrophy), or Very Low Risk (Iatrogenic ectasia and Keratoconus).[22,23] Other relevant variables including patient age, sex, original diagnosis, time since transplantation, whether the other eye had been transplanted, general health, specific eye and corneal related descriptors (corneal thickness, endothelial cell count, intraocular pressure), and immunosuppressive treatment were recorded in a bespoke, web-based clinical database and sample tracking system (VISICORT Information Management System [VIMS], www.visicort.eu). Enrolled patients with AR were scheduled for a follow-up visit 3 months later at which clinical examination and biological sampling were repeated. Successful treatment of a graft rejection episode was defined as normalization of corneal thickness and recovery of a clear graft based on clinical examination.

3 | RESULTS

3.1 | Demographic and clinical characteristics of the cohort

In order to define whether clinically diagnosed AR was accompanied by an alteration of the immune response in the peripheral blood of CT recipients, PBMC profiles were compared among three patient groups: Acute Rejection: 56 CT patients with AR (35 with FT-CT, 21 with PL-CT), Stable CT: 177 CT patients with clinically stable grafts (105 with FT-PL, 72 with PL-FT), and Non-CT Corneal Disease (Patient Controls): 40 patients with corneal diseases that had not been subjected to CT. The demographic and clinical characteristics of the AR and Stable CT patients are presented in Table 1. Of note all CT patients with AR were enrolled at the time of presentation to Ophthalmology clinic with clinical symptoms and signs. Gender and age were equally distributed among the AR and Stable CT groups. As expected, CT patients with AR had a shorter time since transplantation (median [IQR_{25-75}] 19.1[10.0–37.6] vs. 31.3 [18.7–37.6] months respectively; $p < .001$), a thicker cornea (median [IQR_{25-75}] 693 [573–780] vs. 568 [519–622] µm respectively; $p < .001$) and a higher frequency of topical immunosuppressive drugs (55.4% vs. 35.4%; $p = .012$) as compared to Stable CT patients. Representative images of pre-transplant eyes, grafts from the different surgical procedures, and successful or acute rejection grafts are shown in Figure S1.

3.2 | Stability of immune signature of CT patients with a stable graft

As described in Methods, stable CT patients were initially enrolled in the cross-sectional study under separate group definitions based on shorter (<3 years) and longer (>3 years) time after surgery. In planning the final analysis strategy, we first assessed whether the time post-transplantation impacted the immune profiling of clinically stable CT patients. As shown in Figure 1, the main immune cell populations (B cell, CD4 T cell, CD8 T cell, and CD4/CD8 T cell ratio) were similarly distributed between the Stable CT groups enrolled at < and >3 years post-transplant. Furthermore, the frequencies of B cell subsets (Naïve, $CD27^-IgD^-$; Non-switched memory, $CD27^+IgD^+$; Switched memory, $CD27^+IgD^-$; Other, $CD27^-IgD^-$; Transitional, $CD24^{hi}CD38^{hi}$), CD4 and CD8 T subsets (TEMRA, $CD45RA^+CCR7^-$; Naïve, $CD45RA^+CCR7^+$; Effector memory (EM), $CD45RA^-CCR7^-CD28^-$; Central memory (CM), $CD45RA^-CCR7^+$); CD4 regulatory T cells (Treg) ($CD3^+CD4^+CD127^{low}Foxp3^+$) were similarly distributed in the two Stable CT groups (Figure 1; Figure S2). Finally, the expression of transcription factors regulating the memory and effector program of T cells (EOMES and Tbet), of cytotoxic molecules (GZMb and PERF1), and of CD57 were not different between Stable CT sampled before or after 3 years post-transplant (Figure 1). Collectively, our data showed that the immune profiles of clinically stable CT recipients were highly similar regardless of the post-transplant sampling time. Subsequently, therefore, the

TABLE 1 Demographic and clinical characteristics of the cohort

	Acute rejection ($n = 56$)		Stable ($n = 177$)		p (AR vs. Stable)
	FT ($n = 35$)	PL ($n = 21$)	FT ($n = 105$)	PL ($n = 72$)	
Gender male (%)	28 (50%)		81 (45.76%)		.7
	17 (48.6%)	11 (52.4%)	62 (59%)	19 (26.4%)	
Age mean ± SD	56.8 ± 17.9		60.1 ± 17.0		.2
	50.6 ± 19.3	67.1 ± 8.01	53.6 ± 17.9	69.6 ± 9.62	
PL-CT (%)	21 (37.5%)		72 (40.7%)		.8
Other eye grafted (%)	16 (28.6%)		70 (39.8%)		.15
Diagnostic *n* (%)					.13
High risk[a]	23 (41.1%)		47 (26.7%)		
	18 (51.4%)	5 (23.8%)	35 (33.3%)	12 (16.7%)	
Low risk	20 (35.7%)		74 (42.0%)		
	5 (14.3%)	15 (71.4%)	15 (14.3%)	60 (83.3%)	
Very low risk	13 (23.2%)		55 (31.2%)		
	12 (34.3%)	1 (4.8%)	55 (52.4%)	0	
Time since CT, *n* (%)					.004
<18 m	27 (48.2%)		41 (23.2%)		
	15 (42.9%)	12 (57.1%)	24 (22.9%)	17 (23.6%)	
>18 m <36 m	14 (25.0%)		44 (24.9%)		
	11 (31.4%)	3 (14.3%)	25 (23.8%)	19 (26.4%)	
>36 m	15 (26.8%)		92 (52.0%)		
	9 (25.7%)	6 (28.6%)	56 (53.3%)	36 (50.0%)	
Topical IS (%)	31 (55.4%)		62 (35.4%)		.012
	23 (65.7%)	8 (38.1%)	45 (42.9%)	17 (23.6%)	
Systemic IS (%)	3 (5.36%)		6 (3.45%)		.5
	2 (5.71%)	1 (4.76%)	5 (4.76%)	1 (1.39%)	
Months since CT, median [IQR]	19.1 [10.0–37.6]		31.3 [18.7–37.6]		<.001
	19.6 [10.7–37.6]	14.7 [9.6–40.0]	36.7 [18.4–99.8]	40.8 [19.0–40.8]	
Corneal thickness µm, median [IQR]	693 [573–780]		568 [519–622]		<.001
	662 [558–734]	755 [644–803]	553 [516–596]	600 [543–656]	

Abbreviations: AR, acute rejection; CT, corneal transplant; FT, full thickness; IQR, interquartile range; IS, immunosuppression; PT, partial thickness; SD, standard deviation.

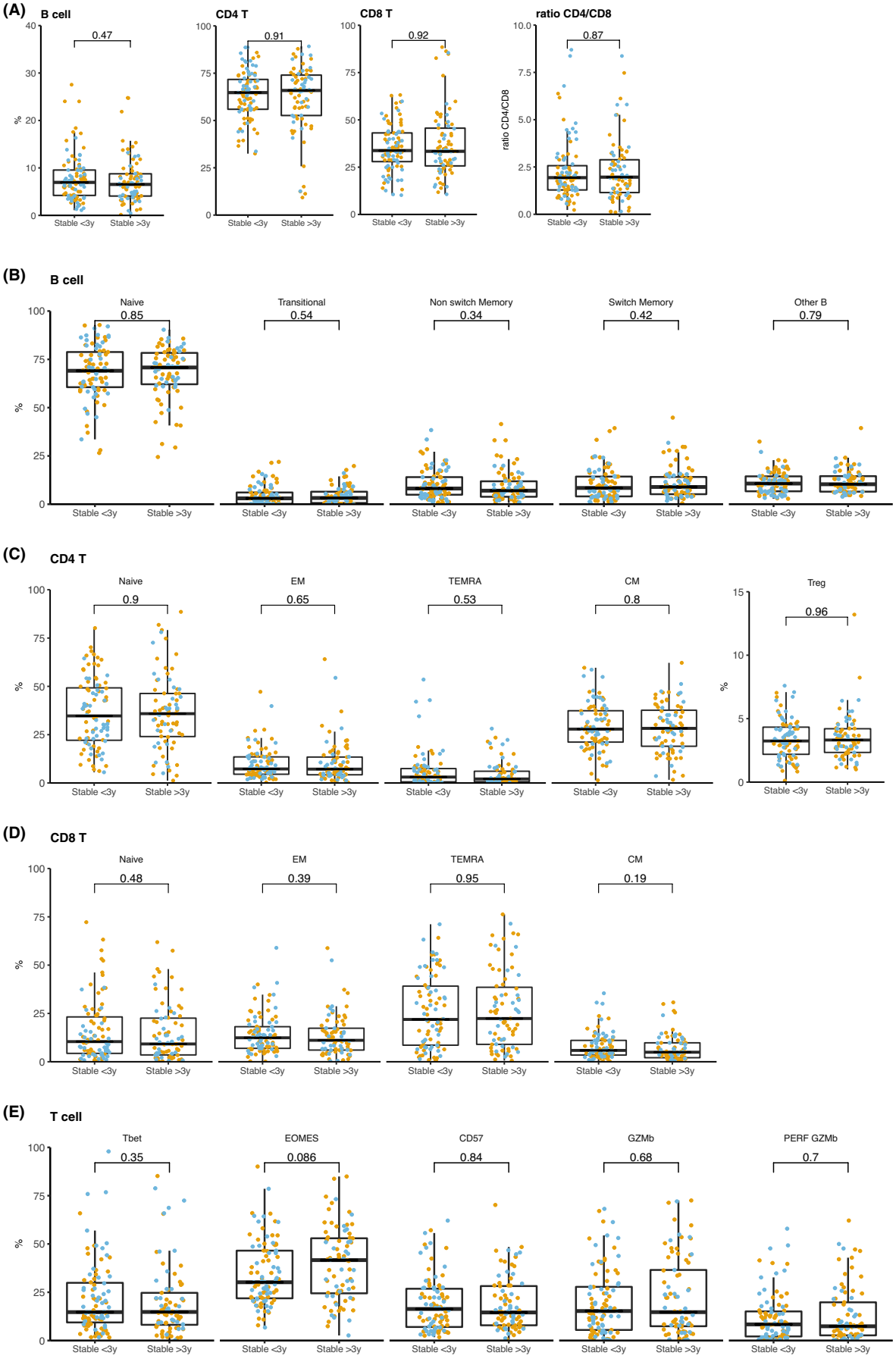
[a]High risk: Corneal ulceration threatening or actual perforation, Herpes simplex keratitis (Scar Ulceration Perforation), Regraft and Secondary bullous keratopathy; Low risk: Corneal scar without vessel ingrowth, Fuchs dystrophy, and Stromal dystrophy; Very low risk: Iatrogenic ectasia and Keratoconus.

data obtained from the 2 clinically stable CT groups were merged into a single Stable CT group.

3.3 | Immune signature of acute rejection of CT

We next sought to determine, in PBMC, the immunological profile of acute CT rejection. The transcriptomic analysis of whole blood samples using whole-genome microarrays revealed only minor modifications in patients with AR. The analysis of differentially expressed genes (DEGs) indicated small numbers of genes with higher ($n = 21$) and lower ($n = 1$) expression in AR compared to Stable CT patients when using less stringent cut-offs with an uncorrected p-value < .05 and absolute fold-change ≥1.3 (Figure S3). Importantly, none of the DEGs identified on the basis of uncorrected p values retained significance after false discovery rate (FDR) p-value

FIGURE 1 Stability of the immune profiles of patients with CT with stable graft function. Immune profiling of main immune populations (B cell, CD4 T, CD8 T; A), B cell subsets (Naïve, Switched, Non-switched, Transitional, other; B), CD4 and CD8 T cell subsets (NAÏVE, EM, CM, TEMRA, and CD4 T_{REG}; C and D) and markers associated with T cell differentiation and immune activation (E) were analyses in CT with a stable graft function and sampled less than 3 years (yellow; $n = 95$) or more than 3 years (blue; $n = 82$). Each point represents a single patient and the boxplot represent median, IQR_{25-75}, and IQR_{10-90}. [Color figure can be viewed at wileyonlinelibrary.com]


(A)
B cell
0.47
% 0 10 20 30 40
Stable <3y
Stable >3y
CD4 T
0.91
0 25 50 75 100
CD8 T
0.92
ratio CD4/CD8
0.87
ratio CD4/CD8 0.0 2.5 5.0 7.5 10.0
(B)
B cell
Naive
0.85
Transitional
0.54
Non switch Memory
0.34
Switch Memory
0.42
Other B
0.79
(C)
CD4 T
Naive
0.9
EM
0.65
TEMRA
0.53
CM
0.8
Treg
0.96
(D)
CD8 T
Naive
0.48
EM
0.39
TEMRA
0.95
CM
0.19
(E)
T cell
Tbet
0.35
EOMES
0.086
CD57
0.84
GZMb
0.68
PERF GZMb
0.7

adjustment. The absence of major alteration in the frequency of immune cells was confirmed by flow cytometry and we observed that the main immune cell populations (B, CD4, CD8, and CD4/CD8 T cell ratio) were distributed similarly between CT with AR, Stable CT and non-transplanted patients (Figure 2A). We then focused on T and B cell detailed subsets. Interestingly, CT recipients with AR exhibited a modest but significant increase of naïve B cells (69.0 ± 13.0 vs. 67.8 ± 14.1% respectively; $p = .034$) and, to a more striking extent, transitional B cells (5.52 ± 3.59 vs. 4.23 ± 4.79% respectively; $p = .0025$) compared to Stable CT (Figure 2B). The frequency of switched memory B cells was also lower in AR compared to Stable CT patients (8.57 ± 9.43 vs. 10.6 ± 7.26% respectively; $p = .012$, Figure 2B). Similar findings were obtained when CT patients with AR were compared to the Patients Control group, whereas no difference was observed between Stable CT and Patient Controls—indicating that the modulation of this B cell compartment was restricted to patients with AR.

We then investigated the impact of AR on the CD4 and CD8 T cell compartment. The frequencies of CD4 and CD8 T cell subsets (Naïve, EM, CM, TEMRA) were equally distributed across the different clinical groups apart from a higher proportion of early intermediate memory CD4 T cells in AR compared to Stable CT (Figure 2C,E). In addition, CT patients with AR had higher proportions of CD4 Treg as compared to Stable CT patients (4.22 ± 2.46 vs. 3.43 ± 1.67% respectively; $p = .03$, Figure 2D). The frequency of expression of the Ikaros family member Helios by $Foxp3^+$ CD4 Treg was not different between the groups suggesting that the stability and regulatory properties of CD4 Treg were similar in CT patients undergoing or not undergoing acute rejection (Figure 2D). Finally, the frequencies of activation, differentiation, and cytotoxic functional markers (Tbet, EOMES, CD57, PERF1, GZMB, CD38, and HLA-DR) by T cells were no different for CT patients with AR compared to the other groups (Figure 2E; Figure S4), indicating that the overall impact of AR results in minor modification of T cell compartment.

As the immune mechanisms leading to AR may differ according to the nature of the CT procedure (FT-CT vs. PL-CT), additional analyses were performed. When the analyses of B cell and T cell subpopulations were carried out for recipients of FT-CTs, the observed differences between AR and Stable CT groups for naïve, transitional and switched memory B cells (all higher in acute rejection), for early intermediate CD4 T cells and for CD4 Treg (both higher in AR) were preserved. (Figure S5). In contrast, an analysis confined to PL-CT recipients showed no differences (Figure S6), suggesting that presence of an immune signature within the circulation during AR may be dependent on transplantation of all layers of the cornea.

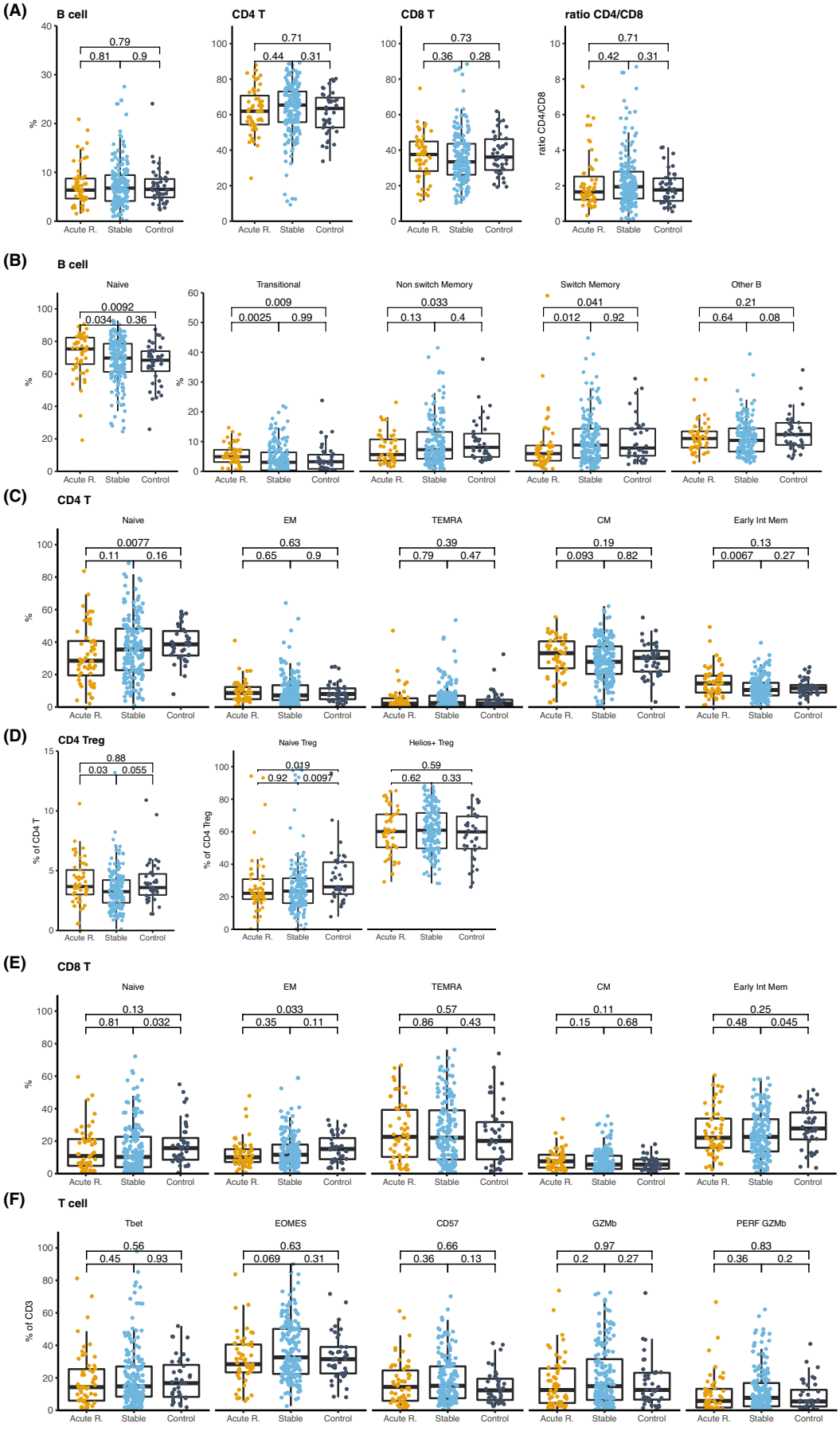
3.4 | Post-rejection follow-up

Finally, we tested the impact of therapeutic intervention for AR (typically topical dexamethasone drops hourly for 1–2 weeks then tapered) on the immune cell profiles. In 28 FT-CT patients with AR, blood samples were collected 3 months after the diagnosis of acute rejection and the same immune profiling assays were performed on PBMCs. As summarized in Table 2, 23 (82%) of these patients were responsive to treatment for AR exhibited clear CT. Comparing immune cell profiles at the time of AR diagnosis and following treatment, the frequencies of Naïve B cells, switched memory B cells and CD4 Treg remained stable, whereas a decrease in transitional B cells was observed (median [IQR_{25-75}] 4.91 [3.56–7.79] vs. 3.77 [2.24–4.61] % respectively; $p = .0068$; Figure 3). Analysis of DEGs from whole-genome microarrays of blood samples taken at the time of AR and 3 months later indicated moderate numbers of genes with higher ($n = 60$) and lower ($n = 38$) expression when using less stringent cut-offs with an uncorrected p-value < .05 and absolute fold-change ≥1.3. Pathway enrichment analysis did not reveal any strongly significant pathways among the differentially expressed genes. Again, the fold changes were small (Figure S1B) and did not retain significance following FDR adjustment. Collectively, the stability of the immune signature of AR suggests that therapy did not strongly impact the lymphocyte repertoire and immune cell-associated transcriptional profiles detected in the periphery.

4 | DISCUSSION

In this multi-site study, we report the first comprehensive profile of the peripheral blood lymphocyte repertoire at the time of AR diagnosis in FT- and PL-CT recipients compared to stable CT and native corneal disease. Despite abundant pre-clinical evidence of a systemic component to the immunological processes that cause AR following allogeneic CT,[16,20,21] the most striking observation from our analyses is that there was no major distortion of the circulating B cell and T cell effector repertoire at the time of AR diagnosis. In keeping with the results of well-established multi-color flow cytometry assays of PBMCs, whole blood transcriptomic analyses from the same subjects also failed to reveal a robust signature of immunological activation at the time of AR. Of interest, however, we found evidence for differences in circulating B cell maturity and regulatory $CD4^+$ T cell frequency in recipients of FT-CT with AR compared to their stable counterparts, which, as discussed below, raise questions about how sensitization and tolerance to corneal allo-antigens may be reflected in the circulation of human recipients.

FIGURE 2 Immune signature of acute rejection of CT. Immune profiling of CT patients with acute rejection (yellow; $n = 56$) or stable graft (blue; $n = 177$) and patients with corneal diseases that have not been subjected to CT (gray; $n = 40$). The frequencies of the main immune populations (B cell, CD4 T, CD8 T; A), B cell subsets (Naïve, Switched, Non-switched, Transitional, other; B), CD4 (C, D) and CD8 (E) T cell subsets (NAÏVE, EM, CM, TEMRA and CD4 T_{REG}) and markers associated with T cell differentiation and immune activation (F) are shown for each patient (point) and summarize using boxplot (median, IQR_{25-75}, and IQR_{10-90}). [Color figure can be viewed at wileyonlinelibrary.com]


(A)
B cell
0.79
0.81
0.9
CD4 T
0.71
0.44
0.31
CD8 T
0.73
0.36
0.28
ratio CD4/CD8
0.71
0.42
0.31
ratio CD4/CD8
%
Acute R.
Stable
Control
(B)
B cell
Naive
0.0092
0.034
0.36
Transitional
0.009
0.0025
0.99
Non switch Memory
0.033
0.13
0.4
Switch Memory
0.041
0.012
0.92
Other B
0.21
0.64
0.08
(C)
CD4 T
Naive
0.0077
0.11
0.16
EM
0.63
0.65
0.9
TEMRA
0.39
0.79
0.47
CM
0.19
0.093
0.82
Early Int Mem
0.13
0.0067
0.27
(D)
CD4 Treg
0.88
0.03
0.055
% of CD4 T
Naive Treg
0.019
0.92
0.0097
% of CD4 Treg
Helios+ Treg
0.59
0.62
0.33
(E)
CD8 T
Naive
0.13
0.81
0.032
EM
0.033
0.35
0.11
TEMRA
0.57
0.86
0.43
CM
0.11
0.15
0.68
Early Int Mem
0.25
0.48
0.045
(F)
T cell
Tbet
0.56
0.45
0.93
EOMES
0.63
0.069
0.31
CD57
0.66
0.36
0.13
GZMb
0.97
0.2
0.27
PERF GZMb
0.83
0.36
0.2
% of CD3

Given that CT is the oldest and most frequently performed human allogeneic transplant, with close to 200 000 procedures performed worldwide each year[24] and a distinguished history of innovative basic research on the immunological mechanisms underlying corneal rejection and tolerance,[1,16] it is perhaps surprising that there have been very few profiling studies of cellular immunity carried out in human CT recipients. This stands in contrast to kidney and other commonly performed solid organ transplants for which substantial efforts have been made to understand and clinically exploit the immunological signatures of rejection and tolerance that are detectable in readily accessible blood samples using immune cell profiling and/or genomic approaches.[25–29] In particular, the presence, absence, or risk for biopsy-proven AR have been associated with the discovery and validation of blood biomarker signatures with links to pro-inflammatory lymphocyte activity, some of which have recently progressed toward commercialization and clinical application in kidney transplantation.[30–33]

The lack of overt AR-associated changes in the circulating lymphocyte repertoire that we observed in this cross-sectional study of CT recipients might be considered unexpected to the extent that several decades of research in standard and high-risk experimental models has proven a role for systemic elements in the priming and effector mechanisms of corneal allo-antigen-specific adaptive immune responses.[16,34] Specifically, once the inherent immune privilege of the anterior chamber is disrupted by surgery, inflammation, and neoangiogenesis/lymphangiogenesis, the activation of IFNγ-producing (Th1) allo-antigen-specific $CD4^+$ T cells is triggered by the egress of donor and/or recipient dendritic cells (DCs) to draining lymph nodes (LN).[35] Following activation in the LN, Th1 cells enter the bloodstream from which they infiltrate the allograft to mediate rejection.[16] Allo-antigen effector T cells are also detectable in the spleen and other distant sites in the context of experimental CT rejection, consistent with a systemic adaptive immune response.[16,36] Similarly, it should be noted that immunological tolerance associated with the healthy anterior chamber [referred to as anterior chamber-associated immune deviation (ACAID)] and experimental corneal allograft tolerance are also associated with systemic modulations to the repertoire of effector and regulatory lymphocytes.[37–39] Thus, although the abundant literature on systemic immunological responses in experimental models of CT rejection has focused predominantly on secondary lymphoid tissues rather than on blood, our initial hypothesis that AR is associated with increased proportions of activated, pro-inflammatory T cells and, potentially, other effector lymphocytes in the circulation was plausible. Our finding that this was not the case may reflect relatively small proportions of allo-antigen-specific T cells and other effector lymphocytes among PBMCs in AR of CT compared to solid organ transplants. However, it also highlights the fact that immunological mechanisms of CT rejection and tolerance that have been extensively characterized in small and, to some degree, large animal models remain less well understood in human CT recipients.[16] By comparison with solid organ, vascularized transplantation, our findings suggest that the nature of the graft and its vascularization has distinct influence on the extent to which rejection-related immune responses are detectable in the peripheral blood. Decades of research in the field of solid organ transplantation have highlighted the balance in various proportion of humoral and cellular response in the multiple steps of rejection. The vascularization of solid organs facilitates the capture of large number of allo-antigens that can be presented in draining lymph nodes. By contrast, the limited vascularization of CT likely impairs the magnitude of antigen capture resulting in minor modification to the composition of the circulating immune cell repertoire, as highlighted in our study. It would be interesting to investigate the contribution of tissue resident memory T cells to the process of CT rejection. These

TABLE 2 Clinical outcome of corneal transplant recipients 3 months after diagnosis of acute rejection

Status after rejection (Clear/Failure *n* [%])	23/5 (82%/18%)
CCT (μm) median [IQR]	591 [539–616]
Topical treatment	
None	3 (11%)
Low dose	10 (36%)
High dose	15 (54%)
Systemic IS treatment (*n* [%])	1 (4%)

Abbreviations: CCT, central corneal thickness; IS, immunosuppression; IQR, interquartile range.

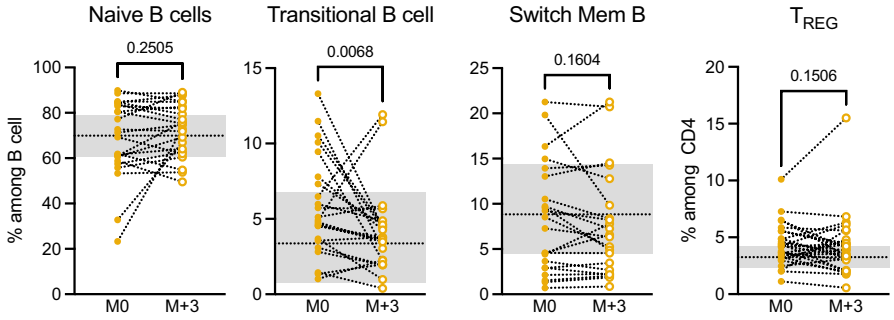


FIGURE 3 Stability of the immune signature of acute rejection of CT after therapeutic adjustment. Immune signature of acute rejection was measured at the time of acute rejection diagnosis and 3-month after in 28 FT-CT patients. The frequency of each subset is shown for each patient (point) and gray box summarizes the value of stable CT patients (median, dot line; IQR_{25-75}, gray box). [Color figure can be viewed at wileyonlinelibrary.com]

non-circulating T cell populations have been identified in human ocular surface and in all conjunctival layers.[40,41]

Although the hypothesized AR-associated changes in circulating effector/memory lymphocytes were not observed in this study, there were some differences between AR and Stable or Control patient groups which merit consideration. In the B cell compartment, this consisted of higher proportions of naïve and transitional B cells with lower proportions of switched memory B cells in AR—suggesting lower rates of B cell maturation.[42] Although this might seem counterintuitive, a potential explanation for greater B cell maturation in stable CT recipients could lie in the immunological roles of IL-10 to promote both B cell class switching and pro-tolerogenic cellular immune responses.[43,44] Given the lack of focus on B cell phenotype and function to date in studies of CT immunobiology and the apparent lack of a major impact of HLA matching and allo-antibody response in human CT outcomes,[45] this observation suggests the need for more investigation of B cell roles in CT rejection and tolerance. A second, unexpected finding was the presence of higher proportions of T reg among the total circulating $CD4^+$ T cell population in CT recipients with AR compared to stable CT recipients but not to patients with non-transplanted corneal disease. With the normal proportion of $FoxP3^+/CD25^+$ T reg in human blood being approximately 5%,[46] the result, as shown in Figure 2D and Figure S5C, suggests that stable, rejection-free CT recipients have relatively fewer circulating T reg—potentially reflecting enhanced migration of T reg to the allograft and relevant secondary lymphoid organs.[35,37] Replication of these findings in additional patient cohorts (ideally in prospective studies) accompanied by functional analyses of circulating B cell and T cell subpopulations will be needed to validate their relevance to AR in human CT. Nevertheless, the observation that differences in B cell maturation and T reg numbers between AR and stable groups were confined to FT-CT recipients suggests that they reflected responses to the more immunogenic elements of corneal tissue.[16,17] Of note, the potential cofounding effect on immune responses of infection by corneal herpes simplex virus infection (3 FT-CT with acute rejection and 5 FT-CT with stable graft) was ruled out as similar differences in subsets of B cells and Treg between AR and Stable CT were observed after the exclusion of these 8 patients from the analysis (Figure S7).

Strengths of this study include its multi-center design, extensive capture of clinical details at the time of patient enrolment, inclusion of FT- and PL-CT recipients, inclusion of a post-AR follow-up timepoint, and application of state-of-the-art flow cytometry panels and analysis approaches. Of note, the clinical and demographic features of CT recipients with or without AR were similar, including recipient age, which has been shown to be inversely correlated with the risk of immunological rejection in a recent prospective clinical trial.[45,47] It is also not without limitations. Given the nature of the graft and its location, we cannot rule out that the lack of detection of overt AR-associated changes in peripheral blood lymphocytes at the time of sampling reflected that those cells had already migrated to the cornea. To address this possibility, longitudinal immune profiling studies will be required. It would also be of interest to extend the scope of the analysis of immune cells beyond our targeted analysis of T and B lymphocytes subsets and to include profiling of circulating myeloid cells such as $CD14^+$ monocytes which may mediate corneal inflammation or serve as precursors for indirect allo-antigen presenting macrophages and dendritic cells.[48] Larger studies with stratified, disease-focused, or multi-time-point design will be required to fully account for the potential confounding effects of clinical heterogeneity among CT recipients with and without AR. Integrated analyses of the immunological profiles of blood samples with those of localized biological samples such as aqueous humor, tears, or corneal surface cells would likely yield a more complete understanding of the effector mechanisms of AR and their related biomarker signatures.[18] Finally, while the PBMC flow cytometric profiles and whole blood transcriptomes that were compared at the time of AR diagnosis and 3 months later did not reveal evidence of overt changes following treatment, only one of these 28 patients was managed with systemic immunosuppression. Thus, immune profiling studies in the context of clinical trials comparing standard therapy with more novel interventions to prevent or treat AR in high-risk CT recipients will be of very high interest in the future.[1] To conclude, this study provides a first comprehensive analysis of peripheral blood immune profiles in human CT recipients with and without AR. It reveals the potential for better understanding systemic aspects of the complex immunological response to corneal allo-antigens and highlights opportunities for further application to CT of profiling technologies that are beginning to transform the diagnosis and management of complications among the recipients of solid organ transplants.[49]

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Transcriptomic data are deposited in GEO under the accession number GSE194124.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. No disclosure for N. Degauque, S. Brouard, M.Cadoux, M. Karakachoff, CC Murphy, J. Hjortdal, B. Vabres, M.D. Walkinshaw, U. Pleyer, W. John Armitage, and M. Griffin. M. Bylesjo is a paid employee of Fios Genomics Ltd. P Mac Gabhann is the founder and owner of Biostór Ireland. PA Gourraud is the founder of Methodomics (2008) (www.methodomics.com) and the co-founder of Octopize (2018) (https://octopize-md.com). He consults for major pharmaceutical companies, all of which are handled through academic pipelines (AstraZeneca, Biogen, Boston Scientific, Cook, Edimark, Ellipses, Elsevier, Methodomics, Merck, Mérieux, Sanofi-Genzyme, Octopize). PA Gourraud is board member at AXA mutual insurance company (2021). He has no prescription activity for either drugs or devices. He has no prescription activity with either drugs or devices.

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