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ORIGINAL ARTICLE

The use of NGAL and IP-10 in the prediction of early acute rejection in highly sensitized patients following HLA-incompatible renal transplantation

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Conflicts of interest
The results of this study have not been published elsewhere in whole or in part.

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Summary

Acute rejection is a significant problem for patients undergoing HLA-incompatible renal transplantation, affecting between 12 and 53% of patients. Any mechanism of detecting rejection in advance of current methods would offer significant benefit. This study aimed to evaluate whether serum biomarkers could predict rejection in HLAi transplants recipients. Sera from 94 HLAi transplant recipients from a single centre were analysed for a panel of biomarkers including: NGAL, KIM-1, IP-10, cystatin C, cathepsin L and VEGF. Biomarker levels pre-operatively, day 1 and at day 30 post-transplant were correlated with the development of early rejection. Significantly higher levels of IP-10 and NGAL were seen on day 1 following transplant in those patients who developed acute rejection ($P < 0.001$ and 0.005) and generated AUC of 0.73 and 0.67, respectively. No differences were seen for the other biomarkers or at the other time points. In this study cohort, IP-10 and NGAL have demonstrated good predictive ability for the development of acute rejection at a very early time point. They may have a role in identifying patients at higher risk for rejection and stratifying immunosuppression or surveillance.

Introduction

In the general renal transplant population, acute rejection is relatively uncommon, rates of antibody-mediated rejection (AMR) being between 5 and 7% [1] and overall rejection below 15% [2]. Patients undergoing HLA-incompatible (HLAi) transplantation, defined as transplantation in the face of detectable preformed donor-specific antibody (DSA), continue to experience a significant rate of acute rejection 12–53% [3–7], which limits the broader application of the technique.

In such patients, a mechanism of predicting acute rejection earlier and by less invasive methods than currently used would offer significant benefit.

Peripheral biomarkers now present attractive potential mechanisms by which acute rejection may be identified in advance of clinical features, possibly avoiding the need for invasive biopsies [8].

This retrospective study aimed to determine whether peripheral biomarkers had the ability to inform on acute rejection in a group of HLAi transplant recipients.

Materials and methods

Patient selection

Retrospective analysis was performed on a library of sera from 94 HLA-sensitized patients who underwent HLAi renal transplantation between 2003 and 2012. Patients were

selected for inclusion in the University Hospital Coventry and Warwickshire programme if they had current reactivity with donor-specific HLA mismatches as measured by complement-dependent cytotoxic crossmatch (CDC), flow cytometric crossmatch (FC) or single antigen bead (SAB) assay. Patients who were assessed as transplantable and with donor antibodies of MFI >500 were included. The patient cohort details are outlined in Table 1. Samples were stored in the NHSBT Histocompatibility and Immunogenetics laboratory in Birmingham, following collection from patients at various time points pre- and post-transplantation. The study had ethical approval and all patients consented to serum samples being taken and stored as part of the ongoing research study.

All sera were separated from clotted whole blood and stored at −40 °C prior to testing.

CDC crossmatch

A total of 2 μl of patient serum and 1 μl donor cells (standardized to 2×10^6/ml) were mixed and incubated at 22 °C for 60 min, with and without DTT. This was followed by addition of 5 μl rabbit serum as a source of complement and then incubated for a further 60 min at 22 °C. Cellular cytotoxicity was visualized using acridine orange/ethidium bromide cocktail under UV light microscopy. Anti-human globulin (AHG) augmentation (sometimes used as a method of enhancing the CDC match) was not used.

Flow cytometry crossmatch

A total of 25 μl patient serum was incubated with 25 μl donor cells (10×10^6/ml) at 22 °C for 30 min. Cells were washed, and then, 100 μl of goat anti-human IgG-FITC (Sigma-Aldrich, UK) was added, and samples were incubated at 4 °C in the dark for 15 min. After washing with 100 μl of mouse anti-human IgG-FITC, CD3-PE (Dako) was added for the T-cell crossmatch and CD19-PE (Dako) for the B-cell crossmatch and incubated for a further 15 min at 4 °C in the dark. Samples were then resuspended in PBS containing 2% BSA and analysed on a Becton Dickinson FacsCanto II flow cytometer. For each test sample, the median channel shift for IgG-FITC was divided by that of the negative control serum. The cut-off for positivity in HLAi transplant cases was defined as >2.5× the negative channel fluorescence.

SAB analysis

HLA class I- and class II-specific antibodies were detected using SAB manufactured by One Lambda (Canoga Park, USA). All assays were performed according to manufacturer's instructions. For each case, the antibody specificities were determined prior to antibody removal therapy. Donor-specific and third-party antibody (antibodies directed against HLA, but crucially recognizing epitopes not carried on those HLA which constitute mismatched donor antigens) specificities were defined using a positive cut-off of 1000MFI, although knowledge of HLA epitopes was considered in each case to more accurately assign antidonor reactivity.

Desensitization protocol

Live donor transplant recipients with a DSA MFI <1000 and for all deceased donor transplants, no pre-operative desensitization treatment was used. For higher levels of

Table 1. Table demonstrating characteristics of cohort between those who subsequently did or did not develop rejection.

	No rejection (n = 50)	Rejection (n = 44)	P value
Age mean (median, range)	41.8 (42, 18–67)	44.2 (43.5, 22–68)	0.38
Sex (M:F)	18:26	21:29	1.0
Time on dialysis (mean, months)	70	79	0.37
Time on waiting list (mean, months)	53	59	0.41
Donor-specific antibody	Class I – 25 Class II – 14 Both – 11	Class I – 17 Class II – 11 Both – 16	0.30
Number of mismatches (Median)	3 (0–5)	3 (1–5)	0.24
Number of previous transplants (median)	1 (0–3)	1 (0–3)	0.80
Crossmatch			
CDC+/FC+/SAB+	11	13	0.18
CDC−/FC+/SAB+	23	24	
CDC−/FC−/SAB+	16	7	
DSA MFI			
Total pretransplant (mean, range)	6574 (221–36 360)	8594 (124–33 730)	0.123
Day 1 (mean, range)	2963 (97–17 760)	3278 (75–13 950)	0.630
Creatinine day 1 (mean and 95% CI)	331 (284–378)	369 (318–420)	0.262

DSA recipients underwent double-filtration plasmapheresis with the aim being to achieve a negative crossmatch prior to transplantation as previously described [7, 9]. Standard maintenance immunosuppression was mycophenolate mofetil 1000 mg twice a day, tacrolimus 0.15 mg/kg/day in two divided doses aiming for a target trough level of 10–15 μg/l in the first month, prednisolone 20 mg once a day, intravenous 500 mg methylprednisolone intra-operatively and basiliximab 20 mg on day 0 and day 4.

Biomarker analysis

Serum samples representing the times points of pretransplantation (and prior to antibody removal), day 1 post-transplant and day 30 post-transplant were thawed and analysed, using a Luminex™ assay [10], for a panel of candidate biomarkers.

Biomarkers selected for inclusion in the analysis panel included those previously implicated as correlates of rejection at the time of biopsy – either as markers of immunological activation or as a result of injury to the kidney. Biomarkers included were as follows: kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), cathepsin L, vascular endothelial growth factor (VEGF), interferon γ-induced protein 10 kDa (IP-10) and cystatin C.

Antibody pairs and standards were supplied as follows: NGAL, KIM-1, cystatin C, cathepsin L – R&D systems, MN; VEGF, IP-10 – Peprotech, NJ.

Clinical correlation

Serum biomarker levels were correlated with acute rejection occurring within the first 30 days after transplantation.

Antibody-mediated rejection (AMR) was suspected on decline in renal function, confirmed by the presence of DSA and a biopsy showing the cellular changes in acute antibody-mediated rejection (peritubular capillaritis and/or glomerulitis) and not necessarily positive for C4d (i.e. a biopsy 'suspicious' for AMR by Banff '07 criteria and confirmed in Banff 2013 as AMR)[11–13].

Donor-specific antibody levels prior to pretransplant antibody removal, day 1 post-transplant and at 30 days following transplant were also recorded. These were correlated with the rejection occurring during the first 30 days following transplantation and to serum biomarker findings.

Delayed graft function (DGF) was defined as the need for dialysis in the first 7 days following transplant.

Statistical analysis

Analysis was undertaken using GRAPHPAD PRISM 5™ (SD, California) using Mann–Whitney between patients with early rejection and those without. Contingency table analysis was undertaken using Fisher's or chi-square test. A receiver operator characteristic (ROC) analysis was undertaken to further assess the potential of any significant biomarkers' ability to predict rejection. Correlation statistics were undertaken using Spearman rank testing. $P < 0.05$ was considered significant.

Results

Between 2003 and 2012, 94 patients underwent HLAi transplant of whom 44 (46%) developed acute rejection in the first month. The mean time to onset of rejection was 4.2 days. The majority of organs were from live donors with only 8 from deceased organ donors. There were no significant differences between patient demographics although differences between pretransplantation antibodies were seen between the group of rejectors and nonrejectors (Table 1).

Figures 1–3 show the levels of selected biomarkers pretransplant (Fig. 1), day 1 (Fig. 2) and day 30 (Fig. 3). Overall analysis revealed no significant differences in biomarker levels either pretransplant or at 30 days following transplant.

However, significantly elevated levels of NGAL ($P = 0.005$, means of 7657 ng/ml vs. 10 080 ng/ml) and IP-10 ($P = 0.0001$, means of 54.5 pg/ml vs. 74.2 pg/ml) were seen on day 1 in recipients who subsequently rejected in the first 30 days (Fig. 2). There was no significant difference between the two groups with regard to other evaluated biomarkers. Analysis was also undertaken to determine the relationship between levels of NGAL and IP-10 and demonstrated a Spearman r of 0.25 (CI 0.45–0.44, $P = 0.015$). This suggested a weak association between the two levels.

Receiver operator characteristic (ROC) analysis was undertaken to determine the ability of day 1 NGAL and IP-10 to predict rejection within the first 30 days. This demonstrated an AUC of 0.67 and 0.73, respectively (Fig. 4). At a cut-off of 50 pg/ml, IP-10 demonstrated a specificity of 72% and sensitivity of 70% for predicting rejection. At a cut-off of 6865 ng/ml, NGAL demonstrated a specificity of 60% and a sensitivity of 73% for predicting rejection.

Patients who developed acute rejection demonstrated a trend towards higher rates of DGF than those without rejection, but this was not significant (38% vs. 25% $P = 0.47$). Analysis revealed that NGAL and IP-10 levels were not significantly different on day 1 between those who had DGF or primary function overall ($P = 0.32$ and 0.12, respectively). Similarly, no significant differences were seen between NGAL and IP-10 levels in those patients with rejection whether they developed DGF or not ($P = 0.99$ and 0.51, respectively).

Donor-specific antibody levels were compared to biomarker levels at the time points described previously. DSA levels at these time points did not correlate with levels of any of the biomarkers at the same time points. Analysis was

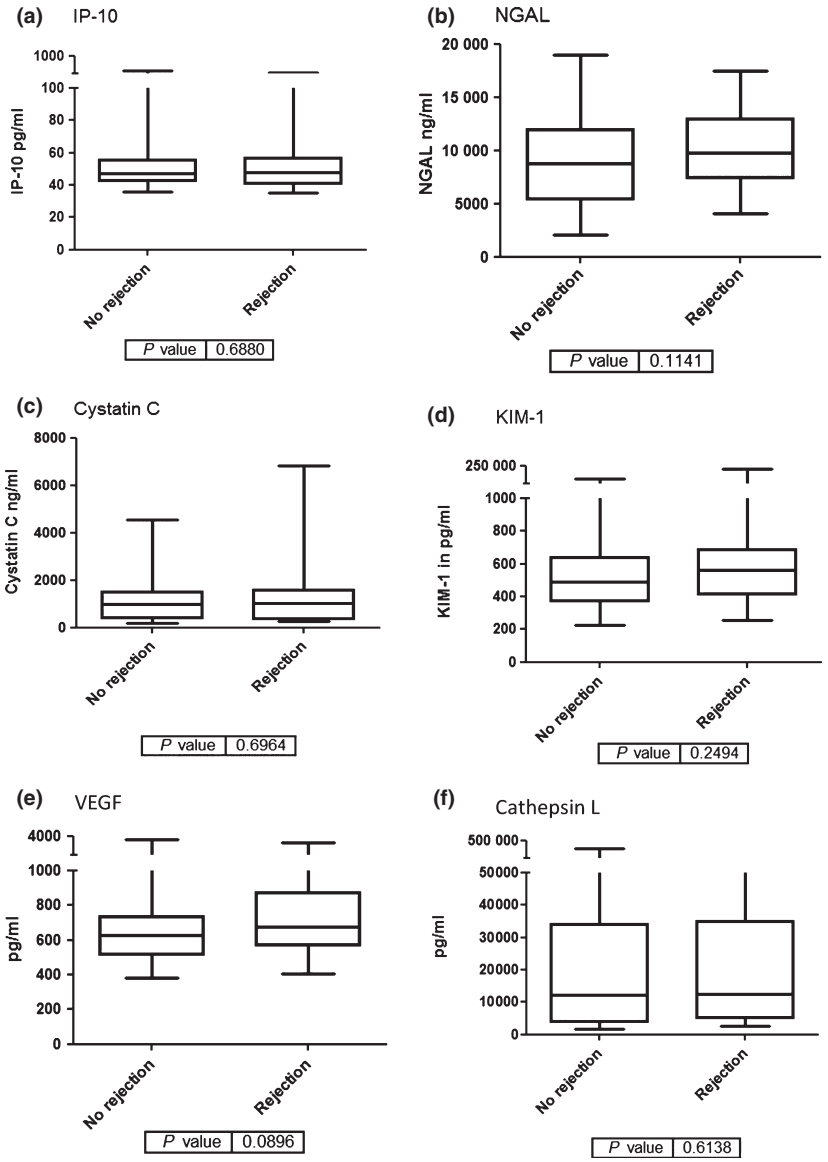

Figure 1 Graphs demonstrating no significant differences between biomarker levels pretransplant in those who did or did not develop rejection.

also undertaken using a pretransplant DSA cut-off MFI of 2000 which demonstrated significantly higher chance of rejection in those patients with a pretransplant DSA level above 2000 (28% vs. 54% $P = 0.048$) There was no significant association between IP-10 and NGAL with DSA levels on day 1 ($P = 0.77$, $P = 0.133$, respectively).

Levels of IP-10 and NGAL were also correlated with the time of onset of rejection to determine whether the magnitude of biomarker level on day 1 was predictive of the timescale of rejection. Spearman r for NGAL was −0.21 with a P value of 0.17, indicating no significant association between the level of NGAL on day 1 post-transplant and the time frame for developing rejection. For IP-10, a Spearman r value of −0.354 was generated with a P value of 0.02, demonstrating that the level of IP-10 on day 1 post-transplant is weakly associated with the time frame for developing rejection.

Overall patient survival in the cohort was 95% at 1 year, and 1-year graft survival was 93%. Of the 5 graft losses in the first year, 4 had early rejection and one had late rejection; however, this was not statistically significant ($P = 0.36$). Creatinine levels at 6 months post-transplant were significantly higher in those patients who had early rejection ($P = 0.0084$, mean 118 vs. 150). However, at 1 year post-transplant, mean creatinine was not significantly different between the two groups ($P = 0.30$, 128 vs.

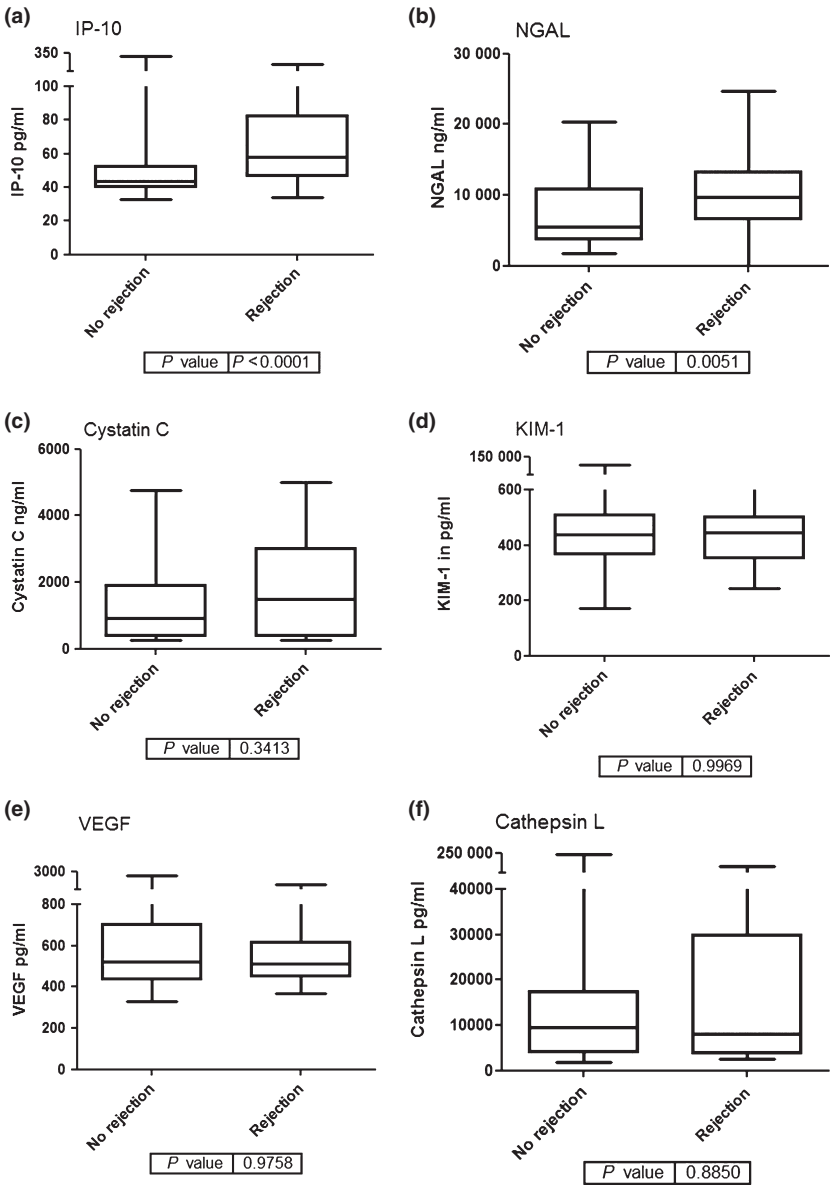

Figure 2 Graph demonstrating significantly elevated levels of IP-10 day 1 and NGAL following transplantation in those who developed acute rejection.

139). Graft survival curves can be seen (Fig. 5), showing so significant difference between those who did or did not have early rejection ($P = 0.32$).

Discussion

Following the first description of HLA-incompatible transplantation nearly 30 years ago, protocols for desensitization have been refined and outcomes continue to improve [14].Transplantation across HLA-specific antibody barriers is now increasingly routine but limited by poorer long-term outcomes that may, in part, be due to the effects of rejection. The potential of biomarkers to aid identification of patients at higher risk of rejection, in advance of a rejection episode, could further enhance transplantation in this situation.

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a normal biologic process, a pathogenic process or a pharmacologic response [15]. As such, they provide the potential to detect immunological changes occurring following transplantation and highlight cases in which this process is enhanced and may lead to rejection.

The analysis of biomarkers of rejection usually involves the comparison of biomarker levels at the time of diagnosis of rejection (e.g. upon biopsy for rising creatinine) with biomarker levels in a cohort with stable renal function.

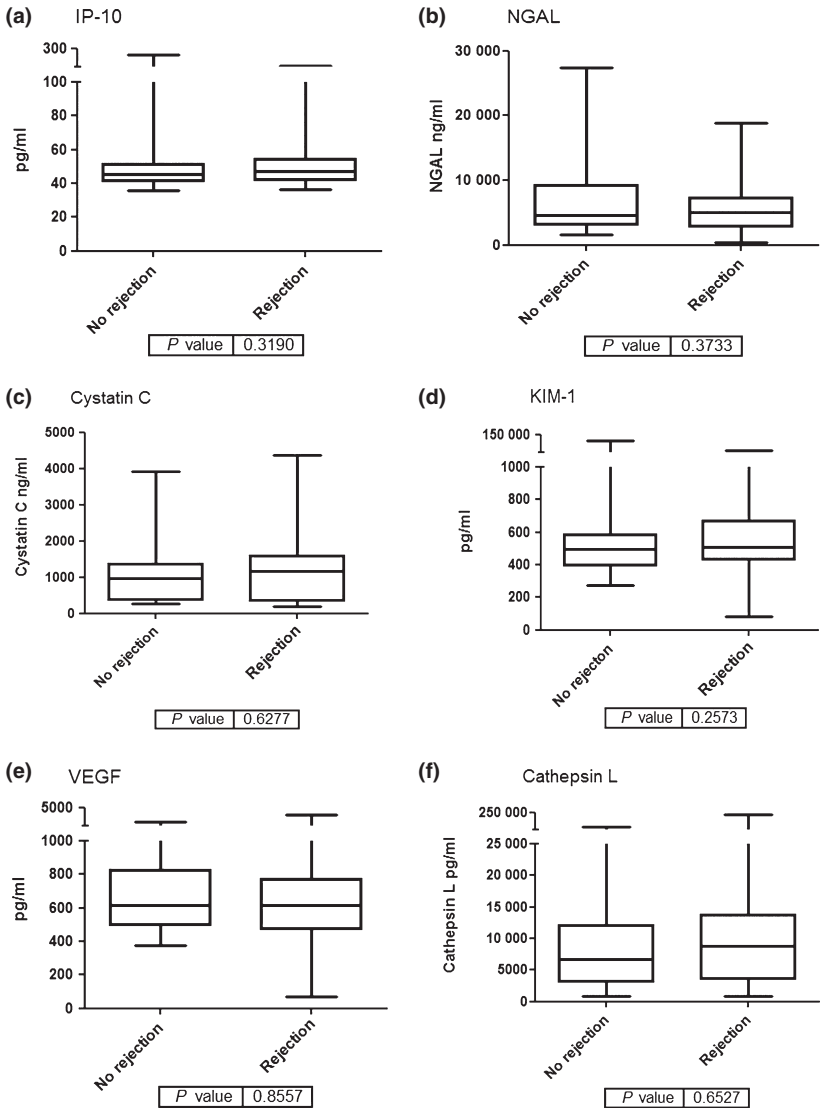

Figure 3 Comparison of biomarker levels on day 30 demonstrating no significant differences between biomarker levels of those who developed acute rejection and those who did not.

This strategy does not identify the rejection process at an earlier time point than standard current practice. The ability of a single biomarker or a panel of biomarkers to predict rejection earlier than current methods would expedite diagnosis and treatment of the episode. It may also identify those patients at higher risk of rejection and guide surveillance. As increased glomerular margination can be detected as early as 30 min following perfusion in biopsy specimens of those who later go on the develop rejection, it is not unreasonable to suggest that a more peripheral investigation such as a blood test could detect changes that similarly predict rejection soon after transplantation [12].

This study demonstrates that the biomarker IP-10 has a good ability to predict rejection in the first 30 days in this group of transplants, as evidenced by the AUC of 0.73. Whilst levels of NGAL were significantly different between the two groups, its ability to differentiate was inferior, with an AUC of 0.67.

The correlation statistics between IP-10, NGAL and the time frame for developing rejection suggests that higher levels of IP-10 on day 1 may be associated with more rapidly developing rejection.

Patients who subsequently developed acute rejection demonstrated a trend towards higher rates of DGF than those without rejection, but this was not significant. NGAL and IP-10 levels have been suggested to be elevated in DGF in other cohorts, and the differences between levels in this cohort were examined to ensure this was not causative in

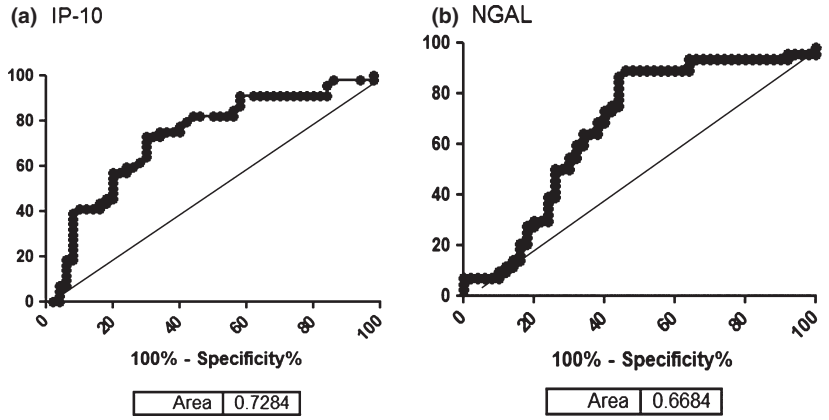

Figure 4 Receiver operator characteristic (ROC) for NGAL and IP-10 to predict rejection in first 30 days from levels on day 1.

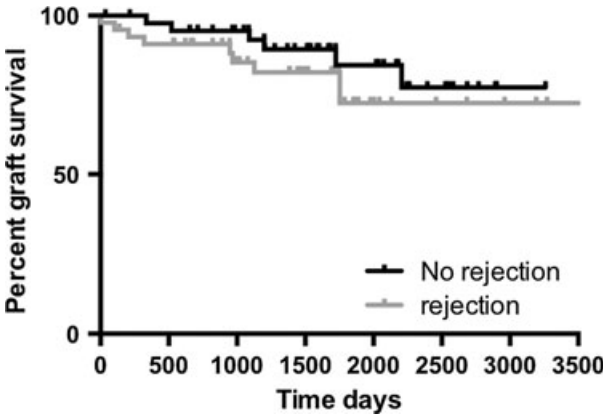

Figure 5 Graft survival between those with early rejection and those who did not have early rejection.

the association with acute rejection. Analysis revealed that NGAL and IP-10 levels were not significantly different on day 1 between those who had DGF or primary function overall, and similarly, no significant differences were seen between NGAL and IP-10 levels in those patients with rejection whether they developed DGF or not. This implies that the elevated levels of IP-10 and NGAL on day 1 in those who later develop rejection are not a function of their higher rates of DGF. Whilst there is evidence in the literature suggesting that NGAL is associated with DGF, the lack of association between IP-10/NGAL and DGF in this study could be explained because of the high proportion of live donors and the lower percentage of DGF in this population.

Although in this cohort there was no statistically significant difference in graft survival between those who had early rejection and those who did not, it is interesting to note that of the 5 early graft losses (within the first year), 4 had early episodes of rejection.

IP-10, also known as CXCL10, is a small chemokine secreted by several cell types including monocytes, endothelial cells and fibroblasts in response to interferon-gamma. It has been postulated to have roles including promotion of T-cell adhesion to endothelial cells, antitumour activity and inhibition of angiogenesis [16]. It elicits a response by binding to CXCR3 and a role for CXCR3 in mediating alloresponses, and graft destruction has been suggested [17]. IP-10 expression may correlate with intragraft immune activation, and measuring IP-10 levels might allow stratification of patients' immune risk and allow tailoring of immunosuppression [18–20]. IP-10 is an initiator of the response to antigen, driving T-cell proliferation [21]. Urinary levels of IP-10 have been demonstrated to be higher in those kidney transplant recipients with acute rejection [22].Urine samples from patients presenting for biopsy because of deterioration in renal function and compared levels of IP-10 (amongst other potential biomarkers) to levels amongst a group of stable function patients demonstrated a sensitivity of 86% and specificity of 91.3% for IP-10 in detecting rejection [22].

High pretransplant levels of serum IP-10 in immunologically uncomplicated transplants have been correlated with worse graft outcomes and rejection in the first 30 days [23]. In our study, pretransplant levels were not different although day 1 post-transplant levels of IP-10 did show predict rejection within the first month. IP-10 levels at day 30, following treatment of the rejection episode, normalized to the same as the levels of those without rejection implying that IP-10 may be a marker for appropriate levels of immunosuppression.

The difference in IP-10 levels demonstrated in this and other cohorts also raises the possibility of IP-10 as a potential target or pathway, as well as a marker of immunological activity.

NGAL levels were also significantly higher on day 1 post-transplant in the group who subsequently went on to reject. NGAL is a member of the lipocalin protein family which has a role in transport, enzyme synthesis, immunomodulation and cell regulation and signalling. It has emerged as a potential biomarker for renal injury [24] and has been examined in the setting of acute kidney injury particularly in patients undergoing cardiopulmonary bypass [25–27]. Urinary levels of NGAL have also been examined in the setting of renal transplantation as mechanisms to help predict DGF [28]. Urinary NGAL levels have been linked to acute allograft rejection with higher NGAL levels in those patients presenting with rejection than stable allograft function [29].

Pretransplant DSA levels have been previously demonstrated to correlate with the development of rejection, with higher rates of rejection amongst patients with pretransplantation DSA's of above 2000u [30]. In this study, rejection rates were significantly higher in the group whose pretransplant antibody levels were above 2000. However, levels at other time points provided no additional information. This may be due to the duel effects of antibody removal prior to transplantation combined with the absorption of antibody by the kidney immediately following transplantation.

It is important to consider that these biomarkers can be predictive of rejection when detected as early as 1 day post-transplant. In the majority of our cohort at this time point, there is very little, if any, detectable DSA, due to reasons outlined earlier such as antibody absorption via epitopes expressed on the kidney and the dilution of blood volume following surgery. As such, the level of DSA in the very early post-transplant period is of little predictive value, thus augmenting the importance of the discovery of new informative biomarkers.

This large, single-centre study of HLA-incompatible transplant patients demonstrated that serum IP-10 and NGAL have good predictive ability for the development of acute rejection in the first 30 days following transplant and may allow the targeted surveillance of IP-10 or NGAL identified 'higher risk' patients to provide early diagnosis and expeditious treatment of rejection episodes.

Whilst this study focuses on the potential role of IP-10 and NGAL in HLAi transplantation, they may also have a role in immunologically uncomplicated transplantation and would benefit from further study in a broader recipient group.

Authorship

MF, DL, MC, DB, NI and ARA: designed research. MF, DL and RH: performed research. MF, DL and RH: collected data. MF, DL, DB and NI: analysed data. MF, DL, MC, RH, DB, NI and ARA: wrote paper.

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