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Review

Indicators of impending pig kidney and heart xenograft failure: Relevance to clinical organ xenotransplantation - Review article

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ABSTRACT

In pig-to-baboon organ xenotransplantation, coagulation dysfunction and inflammation have been suggested to be associated with acute humoral xenograft rejection. We have evaluated platelet counts, plasma fibrinogen, and parameters of inflammation as indicators of xenograft failure in baboons with kidney and heart grafts from genetically-engineered pigs.

Blood chemistry, hematologic, immune, and inflammatory parameters were measured in recipient baboons (n = 16) with organs from α1,3-galactosyltransferase gene-knockout pigs expressing human complement- and coagulation-regulatory proteins.

Thrombocytopenia and reduction of plasma fibrinogen level were observed in baboons developing graft failure, and these correlated with histopathologic findings of glomerular and interstitial thrombosis, and vasculitis in the graft. Not infrequently, in baboons with pig kidney grafts, a consumptive coagulopathy developed prior to a rise in serum creatinine. In contrast, when kidney graft survival was prolonged, no changes were observed in platelet count or fibrinogen. Indicators of the inflammatory response, particularly the serum amyloid A (SAA) assay, increased when graft failure was developing. There were no changes in cellular immune parameters, e.g., T or B cell counts or phenotypes that indicated graft failure.

Therefore, in clinical xenotransplantation, noninvasive parameters (e.g., platelet count, fibrinogen level, SAA) might provide more reliable indicators of impending xenograft failure than measurements of immune parameters or even of serum creatinine.

1. Introduction

Rejection developing in a human kidney *allograft* is generally suspected by a rise in serum creatinine, and possibly by an increase in the T cell counts in cellular rejection [1–4]. Ultrasound may indicate reduced blood flow through the kidney, increased resistance index, or the development of edema [5,6]. Rejection developing in a human cardiac allograft can be suspected by the onset of early features of right heart failure, including radiographic, electrocardiographic, and echocardiographic abnormalities, and by increases in troponin T and I [7–9]. Because the rate of rejection is slower in an allograft than a xenograft, these warning signs usually allow time for the diagnosis to be confirmed by percutaneous needle biopsy (of the kidney) or percutaneous transvenous endomyocardial biopsy (of the heart).

In our experience, failure of a pig renal or cardiac *xenograft* develops more rapidly than in an allograft, in part because it remains mainly a humoral mechanism rather than a cellular one [10,11]. The mechanism may change when “triple-knockout” pigs (in which all three known carbohydrate xenoantigens, against which humans have natural ‘preformed’ antibodies, have been genetically deleted) expressing multiple protective human transgenes become available, but to date graft failure has been an antibody-mediated event, whether the antibodies are natural (preformed) or T cell-dependent elicited antibodies [12].

How will impending graft failure be diagnosed clinically? Based on our experience to date in pig-to-nonhuman primate models, we suggest it will be based largely on monitoring for (i) features of coagulation

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Abbreviations

C-RP	C-reactive protein
fT3	free triiodothyronine
GTKO	α1,3-galactosyltransferase gene-knockout
LDH	lactic dehydrogenase
SAA	serum amyloid A
WT	wild-type (genetically-unmodified)

dysregulation, and (ii) increases in the inflammatory response.

In the 1990s, Platt and his colleagues predicted that thrombotic coagulopathy would be problematic in xenotransplantation [13–15]. Robson et al. demonstrated that pig endothelial cells induce human platelet aggregation through thrombin generation and complement activation [16]. Later, Lin et al. demonstrated that the initiation of consumptive coagulopathy was associated with the activation of platelets to express tissue factor [17].

To our knowledge, apart from early studies in the pig-to-dog and dog-to-pig transplantation models [18–21], the earliest definitive reports of a coagulopathy developing after pig organ transplantation in a nonhuman primate were by Ierino and Kozlowski and their respective colleagues in the late 1990s [22,23].

Ierino et al. reported that a bleeding diathesis was clinically evident 5–12 days after wild-type (WT, i.e., genetically-unmodified) pig kidney transplantation [22]. Profound thrombocytopenia, fibrinogen depletion, and production of high levels of the fibrin degradation product, D-dimer, were observed. Importantly, the authors observed that these disturbances rapidly resolved after excision of the rejecting xenograft (kidney). Similar features of thrombocytopenia and coagulopathy were also reported by Kozlowski in the WT pig kidney- and heart-to-baboon transplant models [23].

Most of these recipient baboons had undergone intensive pre-transplant immunological 'conditioning' with nonmyeloablative or myeloablative irradiation that may have been a factor in the development of thrombocytopenia, but Buhler et al. demonstrated that thrombocytopenia occurred even in the absence of intensive 'conditioning' therapy [24,25].

Knosalla investigated these phenomena in more detail (Table 1) [26]. In baboons that received thymic irradiation (but not whole body irradiation), the three parameters that most closely correlated with WT or CD55-transgenic (human complement-regulatory protein) pig *kidney* graft failure were decreases in platelet count and plasma fibrinogen, and rises in lactic dehydrogenase (LDH). After WT or CD55-transgenic pig *heart* transplantation, the key parameters were falls in platelet count and plasma fibrinogen, and rises in LDH and aspartate aminotransferase (AST) and/or creatine phosphokinase (CPK). Although useful, monitoring of troponin T was less valuable than the other parameters as its rise occurred later in the course of heart graft failure [27,28]. Although other clinical and laboratory features of graft failure developed at a later stage - and then fairly rapidly - the hematological and biochemical abnormalities identified by Knosalla began to develop before any significant graft dysfunction was occurring.

In the first series of heterotopic heart transplants carried out in baboons using α1,3-galactosyltransferase gene-knockout (GTKO) pigs, Kuwaki and Tseng and their colleagues documented falls in platelet count and plasma fibrinogen as the graft was failing, even if this was several weeks or months after the transplant [29,30]. This group confirmed that rapid recovery of both platelet count and fibrinogen occurred after graft excision. In subsequent studies, thrombocytopenia and a fall in fibrinogen have commonly been associated with a failing graft [11,31,32].

We have now reviewed our published and unpublished data to investigate whether the parameters determined by Knosalla et al. remain relevant today in baboons receiving kidney or heart grafts from pigs that have ***multiple*** genetic modifications, e.g., GTKO ± expression of one or more human complement-regulatory proteins (e.g., CD46, CD55) +/− one or more human coagulation-regulatory proteins (e.g., thrombomodulin, endothelial cell protein C receptor, tissue factor pathway inhibitor). Although there was some variation, the indicators of impending graft failure were generally consistent, irrespective of the genetic manipulations made to the organ-source pig. We have particularly investigated features indicating coagulation dysregulation or systemic inflammation.

2. Coagulation dysregulation

2.1. Genetically-engineered pig kidney xenotransplantation

After the transplantation of kidneys from GTKO pigs (+/− expression of protective human transgenes) into baboons receiving conventional (i.e., approved by the US Food and Drug Administration [FDA]) immunosuppressive therapy (e.g., based on tacrolimus), we have observed early dramatic falls in platelet count (Fig. 1A) and plasma fibrinogen (Fig. 1B). There was a rise in LDH in some baboons (Fig. 1C), but no remarkable increase in AST (Fig. 1D).

Not infrequently, the fall in platelet count *preceded* a rise in serum creatinine (Fig. 2A), and on other occasions the fall coincided with the rise in creatinine, *even though the creatinine level remained within the normal range* (Fig. 2B). The reduction in platelet count and fibrinogen generally began approximately 2–3 days before any increase in serum creatinine was documented. Furthermore, the fall in platelet count sometimes occurred when the histology of the kidney showed features of a thrombotic microangiopathy, but few other abnormalities, e.g., no cellular infiltrate (Fig. 2C). Lin et al. reported that the fibrinogen level began to fall after platelets were activated, prior to any rise in serum creatinine [17].

In contrast, when the baboons received costimulation blockade therapy (based on either an anti-CD154mAb or an anti-CD40mAb), kidney graft survival was prolonged to months, with no histopathological features of rejection, and no reduction in platelet count (Fig. 2D) or plasma fibrinogen (Fig. 2E). On occasions, fibrinogen level increased when a life-threatening infection developed (Fig. 2E).

We have demonstrated that there is a negative correlation between platelet count or fibrinogen level and histopathological findings in the pig kidney graft, features of thrombotic microangiopathy (i.e., glomerular thrombi, interstitial thrombi, and vasculitis [Fig. 3A–H]) [33] indicating that reductions in platelet count and fibrinogen reflect kidney histopathology. Thrombocytopenia and a fall in fibrinogen indicate the development of thrombi in the graft, a precursor of graft

Table 1
Hematological and biochemical parameters predicting graft failure after wild-type pig organ transplantation in baboons[a].

Parameter	Platelet count (decrease of > 150,000/μl over 3 days or to < 50,000/μl)	Fibrinogen (decrease of > 80 mg/dL over 3 days or to < 80 mg/dL)	AST (> 300U/L)	LDH (> 600U/L)	CPK (> 500 U/L)
Kidney (n = 6)	+	+	-	+	-
Heart (n = 7)	+	+	+	+	+

AST = aspartate aminotransaminase; CPK = creatine phosphokinase; LDH = lactic dehydrogenase.
[a] Data from Knosalla C et al. Am J Transplant 2003;3:1510–1519.

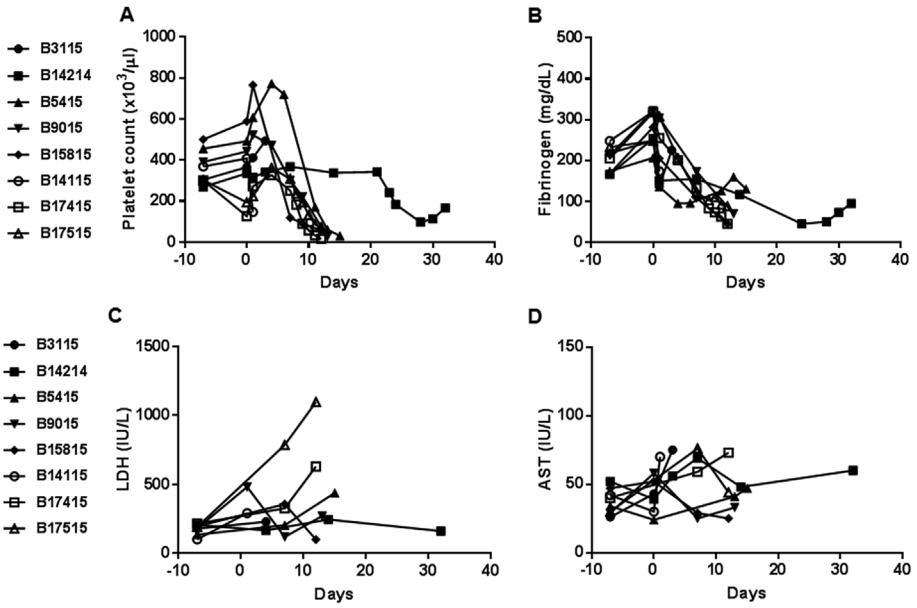

Fig. 1. Changes in key parameters in baboons developing genetically-engineered pig kidney graft failure. Falls in platelet count (normal range in baboons = 358–480 x10^3/μl [mean: 419 x10^3/μl]) (A) and plasma fibrinogen (normal range in baboons = 157–223 mg/dl [mean: 190 mg/dl]) (B), a slight, but inconsistent, rise in serum lactic dehydrogenase (LDH) (normal range in baboons = 278–290 IU/l [mean: 284 IU/l]) (C), but no remarkable change in serum aspartate aminotransaminase (AST) (normal range in baboons = 31–43 IU/l [mean: 37 IU/l]) (D) were observed.

failure.

2.2. Genetically-engineered pig heart xenotransplantation

After genetically-engineered pig heart xenotransplantation, where there is no specific marker of graft failure comparable to serum

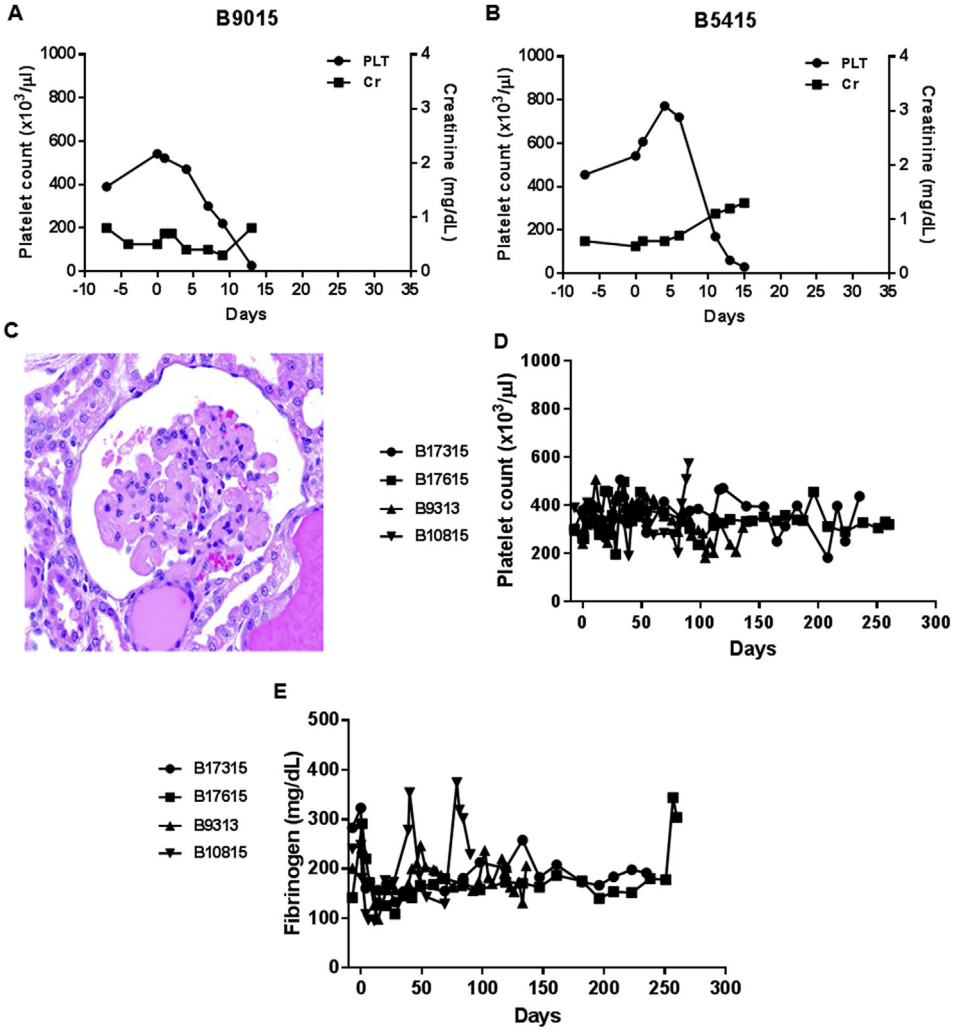

Fig. 2. Correlation between a fall in platelet count and a rise in serum creatinine in individual baboons with genetically-engineered pig kidney grafts. (A) The reduction in platelet count began before any rise in serum creatinine, and thrombocytopenia was profound even when the serum creatinine remained well within the normal range (The normal range of serum creatinine in baboons, humans, and pigs is 0.6–0.8 mg/dL, 0.5–1.4 mg/dL, and 0.6–1.6 mg/dL, respectively). (B) The fall in platelet count and rise in serum creatinine occurred simultaneously, though thrombocytopenia developed much more rapidly than the rise in serum creatinine, which again remained within the normal range. (C) Microscopic appearance of a kidney in a baboon (B17415) in which consumptive coagulopathy was developing (day 12 post-transplantation) showed features of thrombotic microangiopathy (occluding thrombi within glomerular capillary loops), but no cellular infiltration. (The platelet count had fallen to 15,000/μl, but the serum creatinine remained at 0.8 mg/dL). No reductions in platelet count (D) or plasma fibrinogen (E) were observed in baboons that survived long-term after genetically-engineered pig kidney transplantation (and when immunosuppressed with an anti-CD40mAb-based regimen). (The rise in fibrinogen in one case after day 257 was associated with a systemic infection.)

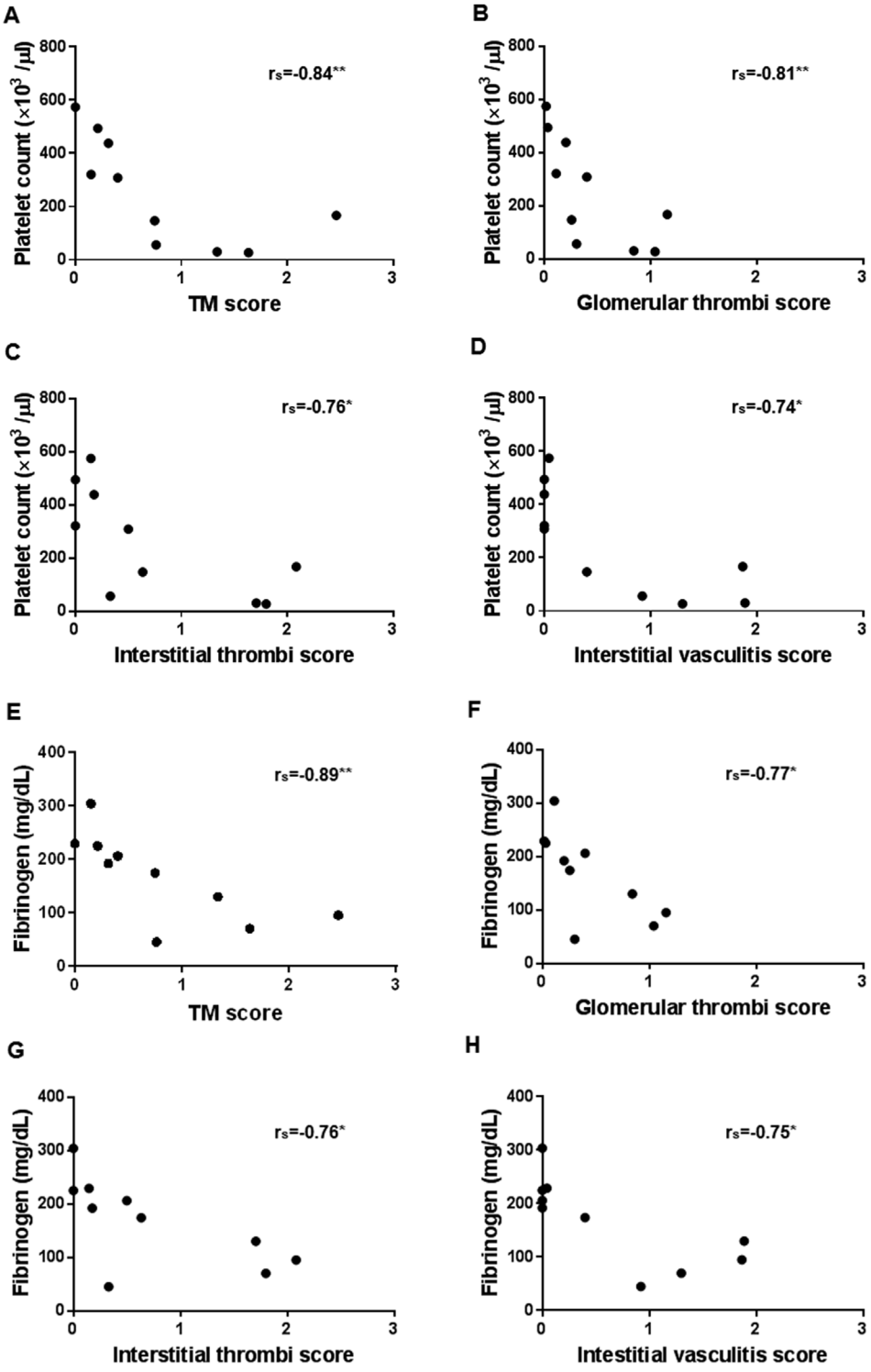

Fig. 3. Correlation between platelet counts or plasma fibrinogen levels and the extent of thrombotic microangiopathy (glomerular thrombi, interstitial thrombi, and interstitial vasculitis). There was a negative correlation between platelet count and (A) thrombotic microangiopathy ($p < 0.01$), (B) glomerular thrombi ($p < 0.01$), (C) interstitial thrombi ($p < 0.05$) and (D) interstitial vasculitis ($p < 0.05$). Similarly, negative correlations were documented between fibrinogen level and (E) thrombotic microangiopathy ($p < 0.01$), (F) glomerular thrombi ($p < 0.05$), (G) interstitial thrombi ($p < 0.05$) and (H) interstitial vasculitis ($p < 0.05$).

creatinine, with the possible exception of troponin T and I [26–28,30,34,35], a falling platelet count (Fig. 4A) and plasma fibrinogen (Fig. 4B) may prove even more valuable as indicators of impending graft failure and, as with pig kidney xenotransplantation, were generally associated with histopathological features of a developing thrombotic microangiopathy (Fig. 4C). A rise in LDH was also usually observed (Fig. 4D).

There is some evidence that features of a consumptive coagulopathy are less obvious when hearts with multiple genetic manipulations (including human coagulation-regulatory proteins) have been transplanted [36]. Baboons receiving grafts from pigs with six genetic modifications maintained significantly higher post-operative fibrinogen levels than those with grafts from pigs with fewer modifications [36].

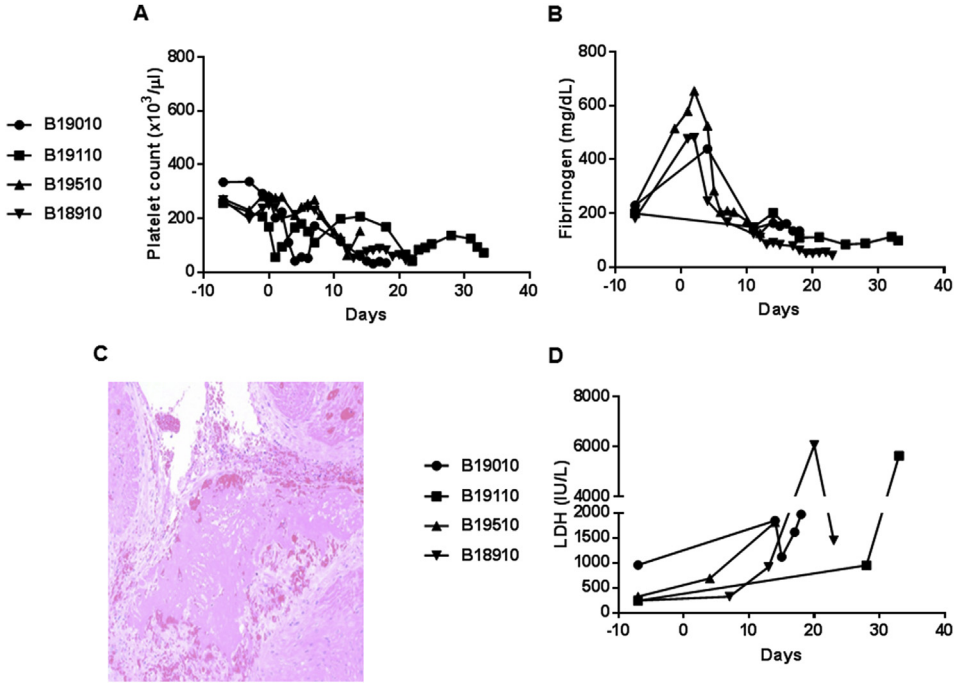

Fig. 4. Falls in (A) platelet count and (B) plasma fibrinogen level in baboons with heterotopic pig heart grafts in which (C) thrombotic microangiopathy was developing. (D) Increases in serum LDH were also observed.

3. Systemic inflammation

3.1. Genetically-engineered pig kidney xenotransplantation

After genetically-engineered pig kidney or artery patch xenotransplantation, Ezzelarab provided evidence of a persistent systemic inflammatory response in immunosuppressed baboons, which he termed the "systemic inflammatory xenograft response" (SIXR) [37]. We have therefore monitored markers of a systemic inflammatory response, e.g., C-reactive protein (C-RP) [11,38–40], extracellular serum histones [39], serum amyloid A (SAA) [39,40].

Although C-RP initially appeared to be a good indicator of the inflammatory response, it was found to be less reliable when the baboon recipient was treated with the IL-6R blockade agent, tocilizumab. After an initial rise (associated with the surgical procedure), the C-RP could fall to within the normal range even when other parameters indicated that graft failure was developing (Fig. 5A). This was again generally associated with conventional (FDA-approved) immunosuppressive therapy.

The SAA, though providing only a rough estimate of the extent of inflammation, increased whenever graft failure was developing (even when IL-6R blockade was induced) (Fig. 5B), and appeared a more useful marker than C-RP, where the result was more variable [41]. Inflammation indicated by the SAA assay correlated with expression of

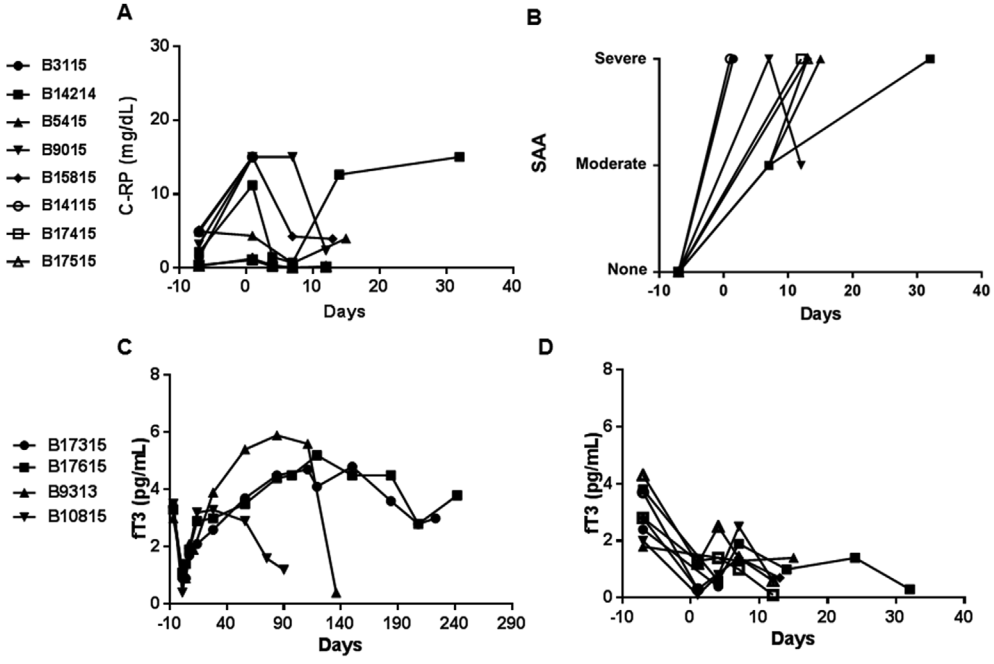

Fig. 5. Markers of the inflammatory response in baboons developing early pig kidney graft failure. (A) Serum C-reactive protein (C-RP) (normal level in humans = < 0.5 mg/dL) immediately increased after the transplant, but this was followed by a variable and inconsistent recovery despite graft failure. (B) The serum amyloid A (SAA) increased whenever early graft failure was developing. (C and D) Serum levels of free triiodothyronine (fT3) (normal range in baboons = 2.2–4.0 pg/mL [mean: 3.1 pg/mL]) decreased immediately after kidney transplantation (which occurred in every case) and recovered when kidney graft survival was prolonged (C), but did not recover when early graft failure occurred (D). (Note the difference in the days of survival on the x axis.)

amyloid A in the native liver and with deposition of amyloid A in the pig kidney graft [41].

However, we have observed that the SAA rises in other pathologic conditions, e.g., systemic infection, and intussusception, and so it is a non-specific indicator of any inflammatory state. Nevertheless, it is a simple and rapid test that, when used prospectively, alerts us to the fact that an inflammatory response is occurring, enabling further investigation to delineate its cause.

The level of D-dimer (that is considered to be a marker of fibrin degradation [42], but we suggest may also be a marker of inflammation [38,43–45]), may rise when a kidney xenograft is failing, but follows a variable and inconsistent pattern [11,38,40,46].

In contrast, serum levels of free triiodothyronine (fT3), which we suggest is another marker of an inflammatory response [11,39,47,48], immediately fell after kidney xenotransplantation, but then recovered until the xenograft was failing (Fig. 5C and D) [48]. When some extent of kidney xenograft survival was achieved, the serum fT3 level on post-transplant day 1 was higher in baboons with longer survival compared to baboons in which survival proved to be short (0.9 vs 0.6 pg/mL). Whether this interesting observation can be used as a reliable predictor of outcome remains uncertain.

3.2. Genetically-engineered pig heart xenotransplantation

Monitoring of baboons with pig heart xenografts has been less comprehensive at our center (where we have concentrated attention in recent years on kidney xenotransplantation). C-RP increased and remained elevated throughout the post-transplant course (Fig. 6A). SAA rose whenever graft failure was developing (Fig. 6B). An immediate rise in D-dimer was demonstrated, which was sustained for the duration of the experiment, but which was not helpful in indicating graft failure (Fig. 6C) [38]. Serum fT3 levels immediately fell post-transplantation, and did not recover when early graft failure occurred (Fig. 6D). Serum histones are known to increase in inflammatory states, and rose in most cases when early consumptive coagulopathy or systemic infection developed (Fig. 6E).

4. The adaptive immune response

Monitoring of readily-available parameters of the cellular immune response, e.g., counts of white blood cells, lymphocytes, T and B cells and their subsets, have largely proven to be unhelpful in determining whether xenograft failure is developing, as no significant changes are seen during early graft failure. $CD3^+$T cell and $CD22^+$B cell counts on day 7 were not significantly different between baboons in which kidney xenograft survival was short (days) or prolonged (months) and

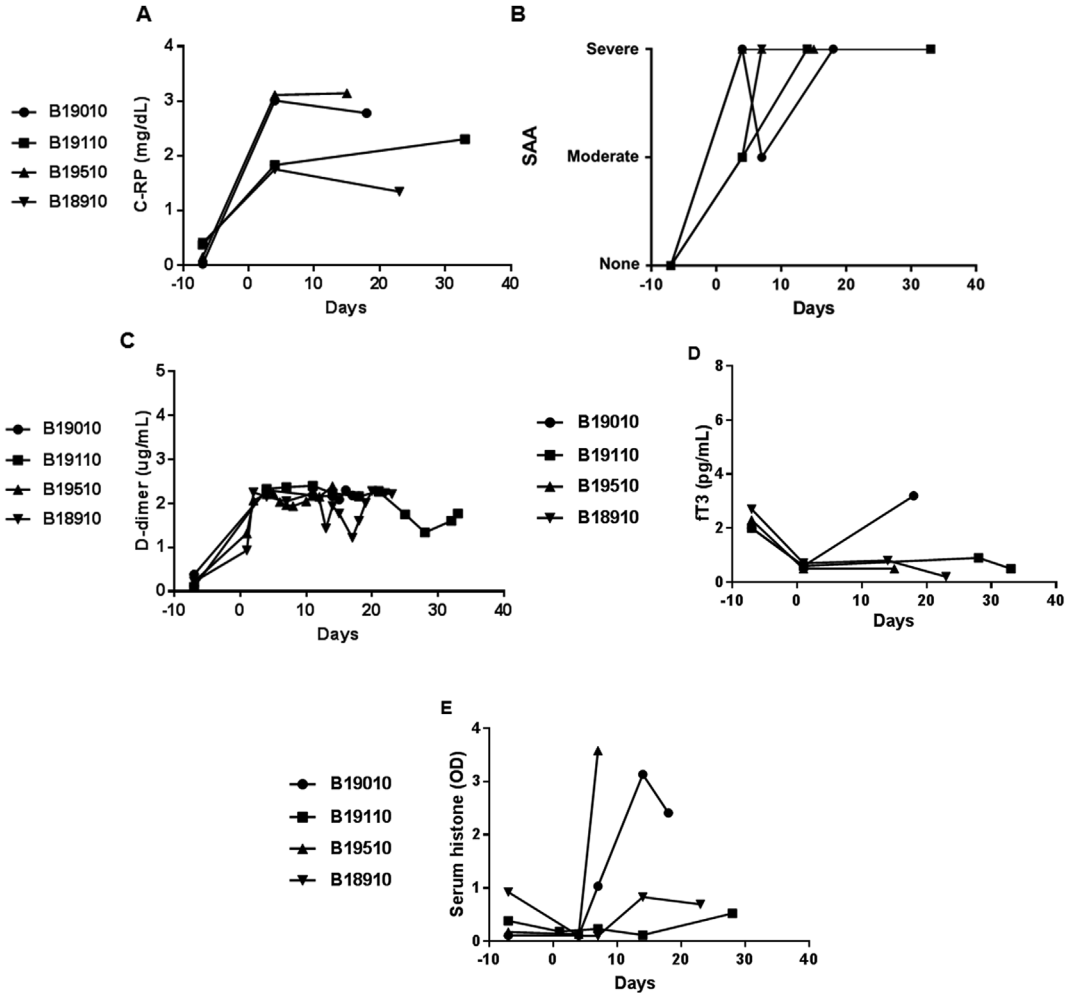

Fig. 6. Markers of the inflammatory response in baboons developing pig heart graft failure. (A) An immediate sustained rise in serum C-RP levels was observed. (B) The SAA increased when graft failure was developing. (C) A rise in D-dimer (normal level in humans = < 0.45 μg/mL) was maintained throughout the post-transplant course whether graft failure occurred or not. (D) There was an immediate post-transplant decrease in fT3 in all baboons, and the level did not recover when early graft failure occurred except in one case. (E) Serum histone levels increased to > 3 when consumptive coagulopathy or systemic infection developed, but otherwise remained within or just above the normal range (normal level in baboons = < 1).

therefore not predictive of outcome (Fig. 7A and B).

Serum levels of circulating anti-pig antibodies, e.g., anti-nonGal antibodies, are almost never informative, as any increase in antibody production usually results in increased antibody binding to the organ xenograft (and therefore an increase in serum IgM or IgG only becomes obvious when a non-life-supporting graft is excised). Biopsies of the graft may demonstrate IgM, IgG, and/or complement deposition (not shown), but this may not necessarily indicate impending graft failure, and, when it does, it often is too late for the xenograft to be rescued.

5. Comment

Once clinical organ xenotransplantation trials are initiated (which will most likely first be of pig kidneys or hearts), we suggest that meticulous and frequent monitoring of the platelet count and plasma fibrinogen will provide a reliable indicator of whether graft failure is developing. Changes in these parameters develop before any rise in serum creatinine or troponin T. Immune parameters are not informative. The concept that the thrombocytopenia and fall in plasma fibrinogen are associated with the accumulation of platelets and fibrin within the graft is supported by our histopathological observations and the fact that urgent excision of a heterotopic heart graft (i.e., a non-life-supporting graft) is followed by rapid recovery (< 48 h) of the platelet count and plasma fibrinogen [22,26,29,30,49].

We suggest that little has changed since Knosalla's original observations. A decrease in platelet count of > 150,000/μl over 3 days or to < 50,000/μL should be considered a sign that graft failure is impending, particularly if associated with a decrease in fibrinogen of > 80 mg/dL over 3 days or to < 80 mg/dL. An increase in LDH to > 600U/L supports this conclusion.

These observations, supported by indicators that a systemic inflammatory response is increasing or sustained, particularly indicated by a rise in SAA, should prove valuable when xenotransplantation is introduced clinically. These parameters would at least suggest that further investigation of graft status is indicated, e.g., ultrasound to determine the state of blood flow in a pig kidney xenograft, or a decrease in right ventricular contractility (with possible right ventricular dilatation) in a pig heart xenograft. Early detection of graft dysfunction may allow therapeutic efforts to be made to reverse it.

Ethical approval

All animal care was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council (8th edition, revised 2011) and was conducted in an AAALAC accredited facility. Protocols were approved by the University of Pittsburgh and University of Alabama at Birmingham Institutional Animal Care and Use Committee.

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Conflicts of interest

David Ayares is an employee of Revivicor, Inc.

Trial RegistryNumber

N/A.

Guarantor

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Provenance and peer review

Not commissioned, externally peer-reviewed.

Data statement

The data will be made available on request.

CRediT authorship contribution statement

Hayato Iwase: Data curation, Writing - original draft, Writing - review & editing. **Abhijit Jagdale:** Data curation, Writing - original draft, Writing - review & editing. **Takayuki Yamamoto:** Data curation, Writing - original draft, Writing - review & editing. **Guoqiang Zhang:** Data curation, Writing - review & editing. **Qi Li:** Data curation, Writing - review & editing. **Jeremy Foote:** Data curation, Writing - review & editing. **David Ayares:** Data curation, Writing - review & editing. **Burcin Ekser:** Data curation, Writing - original draft, Writing - review & editing. **Hidetaka Hara:** Data curation, Writing - original draft, Writing - review & editing. **David K.C. Cooper:** Data curation, Writing - original draft, Writing - review & editing.

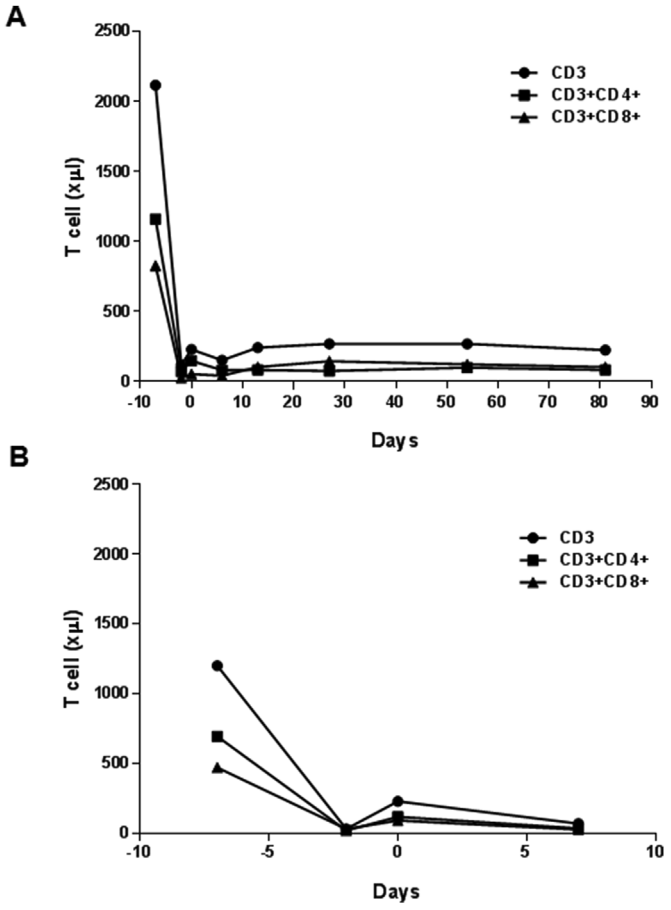

Fig. 7. There was no significant increase in T cell count whether kidney graft survival was prolonged (A) or short (B).

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