Inhibition of complement component C5 protects porcine chondrocytes from xenogeneic rejection

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Objective: Tissue-based xenografts such as cartilage are rejected within weeks by humoral and cellular mechanisms that preclude its clinical application in regenerative medicine. The problem could be overcome by identifying key molecules triggering rejection and the development of genetic-engineering strategies to counteract them. Accordingly, high expression of α1,2-fucosyltransferase (HT) in xenogeneic cartilage reduces the galactose α1,3-galactose (Gal) antigen and delays rejection. Yet, the role of complement activation in this setting is unknown.

Design: To determine its contribution, we assessed the effect of inhibiting C5 complement component in α1,3-galactosyltransferase-knockout (Gal KO) mice transplanted with porcine cartilage and studied the effect of human complement on porcine articular chondrocytes (PAC).

Results: Treatment with an anti-mouse C5 blocking antibody for 5 weeks enhanced graft survival by reducing cellular rejection. Moreover, PAC were highly resistant to complement-mediated lysis and primarily responded to human complement by releasing IL-6 and IL-8. This occurred even in the absence of anti-Gal antibody and was mediated by both C5a and C5b-9. Indeed, C5a directly triggered IL-6 and IL-8 secretion and up-regulated expression of swine leukocyte antigen I (SLA-I) and adhesion molecules on chondrocytes, all processes that enhance cellular rejection. Finally, the use of anti-human C5/C5a antibodies and/or recombinant expression of human complement regulatory molecule CD59 (hCD59) conferred protection in correspondence with their specific functions.

Conclusions: Our study demonstrates that complement activation contributes to rejection of xenogeneic cartilage and provides valuable information for selecting approaches for complement inhibition.

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Introduction

Porcine cells/tissues could be obtained under controlled conditions and used for xenogeneic therapies to treat patients in need.1–3 Currently, porcine acellular matrices are used in clinical practice for reconstructive surgery,4 and together with pig cells, are used as basic raw materials in tissue-engineering research.3,5 Nevertheless, the rejection process of cell/tissue-based xenografts needs better understanding to allow the development of strategies that ensure engraftment and clinical use. In this respect, relevant advances have been made in the field of cartilage repair by identifying key molecules and pathways responsible for rejection of porcine chondrocytes.3,6,7 Porcine cartilage transplanted into primates is rejected by a slow process mediated by humoral and cellular responses in which the galactose α1,3-galactose (Gal) antigen plays a key role.8,9 These observations were reproduced in α1,3-galactosyltransferase-knockout (Gal KO) mice grafted with pig engineered cartilage, in which α1,2-fucosyltransferase (HT) expression markedly reduced Gal antigen expression, averted the elicited anti-Gal antibody response and diminished the cellular immune infiltrate within the grafts.1,3 Despite these advances, the contribution of complement to the rejection process of xenogeneic cartilage remained unclear.

Complement activation by the classical pathway is the main mechanism leading to hyperacute rejection of pig vascularized organs transplanted into primates and contributes to acute humoral
xenograft rejection (AHXR)\textsuperscript{10–12}. Accordingly, there is increasing consensus about the need to continue using transgenic approaches directed towards complement inhibition in addition to the Gal KO pigs for development of solid organ xenotransplantation\textsuperscript{11,12}. Interestingly, recent work pointed out a role of complement activation in rejection of pig corneal xenografts\textsuperscript{13}. Consistent with initial observations in mice, an increase in C3a was detected in the aqueous humor of rhesus monkeys with rejecting pig corneas. These findings support the concept that complement activation contributes to rejection of avascular xenografts, but the mechanisms are unclear.

Different complement components play different roles in the course of an inflammatory response\textsuperscript{14–17}. The classical pathway generates sequentially membrane-bound C4b, C3b and the complex C5b–9 as well as the production of anaphylatoxins C4a, C3a and C5a. C4 and C3 breakdown products are ligands for complement receptors expressed on immune cells and promote immune functions such as B cell activation and phagocytosis\textsuperscript{14,15}. The membrane attack complex (C5b–9, MAC), generated by the classical, alternative and lectin pathways, leads to cytosis, and together with the anaphylatoxins (C3a being the most potent) promotes inflammation\textsuperscript{15,17–19}. Importantly, the complement system is involved in the pathobiological process of cartilage\textsuperscript{20,21}. Chondrocytes themselves are capable of producing most complement components of the classical pathway with most found in synovial membranes and fluid\textsuperscript{16,17}. Complement activation most surely contributes to cartilage injury as both the alternative and the classical pathways are activated by released matrix fragments\textsuperscript{16,18}. In addition, further activation can be produced when trauma (or surgical intervention) involves bleeding, providing another source of complement and other potentiating proteins\textsuperscript{19}.

Lastly, a role of complement is described for arthritis\textsuperscript{16–18,20–25}. In osteoarthritis (OA), complement activity participates in the active inflammatory phase and is enhanced by pro-inflammatory cytokines which stimulate local synthesis of complement proteins\textsuperscript{16,21}. Their differential expression and function in OA takes them into consideration as biomarkers\textsuperscript{22,23}. Complement activation is also observed in rheumatoid arthritis (RA), which prompted the assessment of complement inhibitors in small animal models of the disease\textsuperscript{16,20,23}. The high efficacy of the anti-C5 blocking antibodies in this setting led to the first clinical trials\textsuperscript{24} and is the focus of further developments\textsuperscript{23}.

Here, we have assessed the contribution of complement to rejection using pig chondrocytes exposed to human serum and engineered pig cartilage grafted into Gal KO mice. This study determines the potential efficacy of targeting C5 for complement inhibition in this setting.

Materials and methods

Antibody production, purification and analysis

BB5.1 hybridoma producing the anti-mouse C5 monoclonal antibody (mAb) was obtained from Hoffmann-La Roche Ltd. (Basel, Switzerland), and antibody thereof used under kind permission of Hycult Biotech (Uden, Netherlands) who currently owns BB5.1 rights. The iso-type-control-mAb hybridoma was HPN7.1 (ATCC). Both were initially cultured in DMEM/20% FBS, gradually adapted to serum-free Hybridoma SFM medium (Life Technologies) and transferred into CellLine CL 1000 bioreactors (IBS Integra Biosciences AG, Chur, Switzerland) for antibody production. Antibody was purified using HiTrap MabSelect SuRe 1-ml columns and buffer exchanged in PBS using HiTrap Desalting columns following manufacturer’s instructions (GE Healthcare). Purity was verified by 8%-SDS-PAGE under non-reducing conditions and Coomassie-blue staining. All samples were below 1 EU/ml endotoxin.

The BB5.1 dose needed for C5 inhibition was determined by pharmacodynamic studies in Gal KO mice that we maintain highly inbred in a hybrid genetic background (B6xCAx129sv)\textsuperscript{26}. For the experiment shown, Gal KO male mice were injected i.p. with PBS, 20 mg/kg BB5.1 or control mAb at time 0 and 7 days later. Blood was collected at various indicated times prior to injection and serum obtained for complement activity determinations.

A hemolytic assay was developed to assess BB5.1 activity. Rabbit RBC were prepared from 400 μl of rabbit blood, washed twice and resuspended in 3.6 ml HBSS (Sigma). For the pharmacodynamic study, 10 μl of each serum were diluted with 40 μl HBSS in V-bottom 96-well plate and incubated with 15 μl RBC in duplicates at 37°C for 30 min. For the in vitro studies, BB5.1 was added to the mixture at the indicated concentrations. After complement activation, the plates were centrifuged (3000 rpm for 5 min) and supernatants (40 μl) were transferred to 96-well plates for absorbance determination at 415 nm in a BioTek PowerWaveXS microplate reader (BioTek Instruments, Winnoski, VT, USA).

Porcine articular chondrocytes (PAC) isolation, culture and engineering

PAC were isolated from control and HTAF\textsuperscript{27} transgenic pigs, cultured and tissue-engineered as described\textsuperscript{16}. All PAC were assayed between passages 3 and 6. PAC expressing human complement regulatory molecule CD59 (hCD59) were generated by retroviral transduction. We obtained pCR2.1/hCD152-hCD59 (Alexion Pharmaceuticals) and subcloned it into EcoRI and XhoI sites of the retroviral vector pLXSN using Takara reagents. Constructs were transfected into PG13 packing cells (ECACC) using LipofectamineTM–2000 (Life Technologies), viral supernatants were collected after 48 h, filtered and stored (–80°C). PAC were infected for 10 h with viral supernatants in the presence of 0.8 μg/mL hexadimethrine bromide (Sigma) and 48 h later, cultured in selection with 500 μg/mL G418 up to 2 weeks.

Transplant studies

Following procedures accepted by local ethical committee, 6–8-months-old male Gal KO mice were equally distributed between experimental groups to receive either control or HT-transgenic cartilage with or without complement inhibition. Ten-mg pieces of engineered cartilage were implanted subcutaneously as described\textsuperscript{1}. Seven mice received control or HT grafts without further treatment. The other transplant cohorts were subjected to systemic complement inhibition with BB5.1 (six mice each) for 5 weeks without additional immunosuppression. The treatment was administered i.p. once a week, starting at day –1, with a dose of 20 mg/kg mAb in PBS. No loses or complications were observed in the study. Grafts were collected 5 weeks post-transplantation, formalin-fixed and embedded in paraffin for histology. Sections were H&E stained and evaluated independently by two investigators for signs of rejection.

Cell death determinations

Confluent PAC were incubated in 12-well plates with 20% and 40% normal human serum (NHS) or the corresponding heat-inactivated serum (hiHS) in HBSS at 37°C. All cells were harvested after 1, 8 and 24 h by 30-min treatment with collagenase II followed by trypsin at 37°C, washed and incubated with 2 μg/mL propidium iodide (PI) in PBS for FACS analysis in a FACScalibur (BD Biosciences). For apoptosis determination (sub-G1 analysis as
described\(^6\), confluent PAC were incubated with 20% and 40% NHS or hiHS in HBSS for 24 h at 37°C.

**Complement deposition**

Confluent PAC were trypsinized, washed and incubated with 20% and 40% NHS or hiHS in HBSS for 30 min at 37°C. GAS914 (Novartis, Basel; Switzerland) was added into selected wells in saturating concentration (100 \(\mu\)g/mL) to block anti-Gal antibodies. For complement deposition determination, cells were stained with FITC-conjugated anti-human C3 and anti-human C4 antibodies (Cappel ICN, Costa Mesa, CA, USA), and anti-human SC5b-9 antibody (Quidel Corp., San Diego, CA, USA) followed by FITC-conjugated goat anti-mouse IgG antiserum (Life technologies). To assess human antibody deposition, we used FITC-conjugated anti-human IgG and anti-human IgM antibodies (Life Technologies). MFI was determined in a Gallios flow cytometer with Kaluza software (Beckman Coulter).

**Expression analyses**

PAC characterization (including activation markers) was conducted by FACs as described\(^3,6\). To activate PAC, confluent PAC were incubated in T25 flasks with 20% and 40% NHS in HBSS, as well as hiHS and 10 ng/ml human TNFα (R&D Systems) controls for 24 h, or with human C5a (100 and 1000 ng/ml, R&D Systems) for 12 h. For transduced PAC, a large population with high expression levels was obtained from G418-selected cells by cell sorting in MoFlo-XDP high-speed cell sorter (Beckman Coulter). Immunofluorescence of hCD59 was performed with anti-hCD59 mAb (BRA10G, Biodesign International, Kennebunk, ME, USA) and goat anti-mouse IgG FITC-conjugated antiserum as secondary antibody.

**Cytokine determinations**

Confluent PAC, either control or genetically modified, were incubated in 12-well plates at 37°C with 20% and/or 40% NHS in
HBSS. PAC untreated, treated with 10 ng/ml human TNFα (R&D Systems) or exposed to hiHS were included as controls. After 8- and/or 24-h incubations, culture supernatants were collected and used for pig IL-6 (pIL-6) and IL-8 (pIL-8) determinations (in duplicates) by ELISA with Quantikine immunoassays (R&D Systems). These ELISA do not produce significant cross-reactivity with human IL-6 and IL-8. Similar procedures were conducted to study the effect of human C5a, as well as that of anti-human C5 mAb 557 and 561 (Hycult Biotech) at 10 µg/ml and GAS914 at 100 µg/ml.

Statistical analysis

Values are presented as the mean of single values corresponding to each animal or independent experiment (analysis unit) and the 95% confidence interval (95% CI) calculated for each data set. For the ELISA results, the mean of duplicates of each condition was used. Statistical analysis was conducted using two-tailed Satterthwaite’s t test when comparing two groups, with exception of one specific experiment for which a paired t-test was applied. ANOVA (applying Tukey) was used for multiple comparisons. Differences were considered statistically significant at \( P < 0.05 \).

Results

Anti-C5 therapy reduces the severity of rejection of xenogeneic cartilage in Gal KO mice

To assess the effects of C5 inhibition in a pig-to-mouse model of chondrocyte xenotransplantation, we used the anti-mouse C5 mAb BB5.1. BB5.1 efficacy was assessed in a hemolytic assay incubating Gal KO mouse serum containing natural anti-Gal antibodies with rabbit erythrocytes, which express Gal antigen. In vitro, BB5.1 strongly inhibited the serum hemolytic activity at 20, 10 and 5 µg/
Human complement causes moderate PAC death

To study whether human complement kills porcine chondrocytes, control PAC were treated with 20% and 40% NHS or hiHS internal controls for various times and analyzed for PI incorporation [Fig. 2(A)]. All experiments with NHS included the corresponding hiHS devoid of complement activity by heat inactivation. As described, rejection of the HT-transgenic grafts from untreated and BB5.1–treated cohorts were more difficult to appreciate, the anti-C5 treatment seemed to provide some improvement by preventing tissue damage [Fig. 1(E)].

Porcine chondrocytes activate human complement

We next determined deposition of human IgM, IgG and various complement components on PAC incubated with 20% NHS or hiHS (Fig. 3). Cell death was simultaneously assessed to exclude related effects. Thus, PAC incubated with hiHS showed mainly IgM and IgG reactivity. NHS pre-treatment with saturating concentrations of GAS914 (to completely block anti-Gal antibodies) markedly reduced immunoglobulin deposition on alive PAC [Fig. 3(A)]. Substantial C4, C3 and C5b-9 reactivity was also detected on alive cells after NHS exposure [Fig. 3(B, C)]. Furthermore, deposition of these complement components increased at 40% serum and was up to three-fold higher in dead (PI-positive) cells (data not shown). Lastly, GAS914 decreased complement deposition in similar proportion to antibody on NHS-treated PAC (60–70% for C4/C3, 40–50% for C5) [Fig. 3(B, C)]. Remarkably, complement deposition remained well above background on both alive and dead cells incubated with NHS plus GAS914.

Expression analyses of PAC exposed to human complement. Mean with 95% CI of mean fluorescence intensity associated to SLA-I, pICAM-1 and pVCAM-1 cell-surface expression. (A) PAC untreated or exposed to 20% NHS and hiHS for 24 h (n = 4 independent experiments). Statistical differences were calculated between the treated and untreated PAC and significance is indicated (P values shown in figure). Expression of pVCAM-1 was also significantly higher in NHS-treated than hiHS control (P = 0.03). (B) PAC untreated, exposed to TNFα for 24 h or to human C5a at 100 and 1000 ng/ml for 12 h (n = 4 independent experiments). Statistical differences were calculated between the treated and untreated PAC and significance is indicated (P values shown in figure). Statistically significant differences were also attained for SLA-I and pVCAM-1 between the two C5a doses (P = 0.04).

Fig. 4.
Human complement activates porcine chondrocytes

To assess whether complement activation had an effect on expression of relevant activation markers, we incubated control PAC with 20% and 40% NHS or hiHS for 24 h and measured swine leukocyte antigen 1 (SLA-1), porcine ICAM-1 (pICAM-1) and VCAM-1 (pVCAM-1) on the cell surface. Both adhesion molecules were significantly up-regulated by 20% [Fig. 4(A)] and 40% NHS (data not shown) relative to untreated PAC, whereas SLA-1 followed a similar trend. However, increases were also observed after exposure to hiHS. Thus, pVCAM-1 was the only marker tested which attained statistically significant differences over the corresponding hiHS control. These results led us to assess the direct effect of human C5a on expression of PAC activation markers [Fig. 4(B)]. PAC responded to 12-h human C5a treatment by increasing SLA-1, pICAM-1 and pVCAM-1 in a dose-dependent manner, up to three-fold for SLA-1 and pICAM-1 and 13-fold for pVCAM-1 at high C5a concentration. None of these markers reached expression levels as high as after 24-h TNFα treatment.

Porcine chondrocytes release inflammatory mediators after complement activation

We measured the release of pIL-6 and pIL-8 after incubation of PAC with 20% and 40% NHS for 8 and 24 h [Fig. 5]. NHS at 20% sufficed to trigger pIL-6/pIL-8 release by PAC, although 40% serum produced a stronger response. The effect was complement-mediated as low cytokine secretion was detected after incubation with hiHS. Interestingly, PAC responded early on with significant pIL-6/pIL-8 increases 8 h after serum exposure, whereas TNFα needed a longer treatment time to attain the full stimulatory effect (2–3-fold higher than with NHS).

To assess the contribution of Gal antigen to this response, we determined PAC cytokine release after 24-h exposure to 40% NHS with and without GAS914. GAS914 led to a significant reduction in pIL-6/pIL-8 secretion relative to NHS alone (about 55%).

Both C5a and C5b-9 trigger pIL-6/pIL-8 secretion by chondrocytes

The role of C5 was investigated in this setting. First, we confirmed that human C5a directly triggered pIL-6/pIL-8 release in PAC [Fig. 6(A)]. The anti-human C5 mAb 561 specifically inhibited this effect. We also found that anti-C5 mAb 557 and 561 were both capable of partially inhibiting NHS-mediated pIL-6/pIL-8 release by PAC, although 557 displayed a significantly higher inhibitory activity [Fig. 6(B)]. As the effect of these antibodies had not been fully clarified, we also assessed their capacity to inhibit C5b-9 generation by determining the protection conferred to porcine aortic endothelial cells exposed to NHS. The 557 mAb showed a partial inhibitory activity of complement-mediated lysis, whereas 561 had no effect at this level (data not shown). As these results indicated the involvement of C5b-9, we next studied these effects in genetically-engineered PAC expressing hCD59. High expression of hCD59 was attained by cell sorting (mean fluorescence intensity of 95 relative to 7 of mock control), which resulted in lower cytokine secretion when compared to mock-transduced PAC [Fig. 6(C)]. The combination of C5b-9 blockade by hCD59 expression with that of C5a by anti-C5 mAb consistently led to the highest reduction in pIL-6/pIL-8 release [Fig. 6(C)]. Counteracting anti-Gal antibodies with GAS914 similarly protected hCD59-expressing PAC.

These results lead us to propose a model of how C5 activation affects cartilage which could also apply to OA and RA pathogenesis [Fig. 7].

Discussion

In this work we found evidence for a relevant contribution of complement activation in rejection of xenogeneic chondrocytes. Targeting C5 reduced cellular rejection of pig cartilage implanted subcutaneously into Gal KO mice. To elucidate the mechanisms, cellular assays were conducted with PAC exposed to NHS that showed that porcine chondrocytes are highly resistant to complement-mediated lysis. Instead, PAC responded to complement activation by up-regulating activation markers and secreting pro-inflammatory cytokines/chemokines that surely exacerbate rejection. These findings are relevant for developing cell therapies, particularly with chondrocytes. Xenogeneic chondrocytes/cartilage could be exposed to host antibody and complement in multiple clinical scenarios such as during the repair of facial and laryngeal cartilage defects (modeled in our subcutaneous approach), and when treating articular lesions (due to surgical intervention and/or disease). Our demonstration that genetic engineering of the donor cell controls complement effects indicates this is a surmountable hurdle and encourages further development. Moreover, the simple in vivo model and cellular assays utilized here provide valuable information for better understanding the hurdles of articular cartilage repair and inflammatory disease.

The anti-C5 therapy resulted in improved graft survival that was accompanied by a reduced cellular immune infiltrate. The high BB5.1 efficacy could be explained by its potent anti-inflammatory
activity resulting from inhibiting complement-mediated lysis and C5a production\textsuperscript{20}. Notably, BB5.1 does not target C5a receptors. C5a is a very potent chemotaxin for neutrophils and monocytes that through C5aR (CD88) promotes their extravasation and activation\textsuperscript{15,27}. Their release of multiple inflammatory mediators such as TNF\textsubscript{a} and IL-1\textsubscript{b} results in cartilage extracellular matrix degradation and chondrocyte activation\textsuperscript{6,17,28}, further amplifying inflammation. Furthermore, local complement activation augments antigen presentation and T cell responses\textsuperscript{15}, which may affect survival of xenogeneic cartilage. These mechanisms are all probably involved to some degree in rejection of xenogeneic cartilage grafted in the joint. In a similar way to our model in which the avascular cartilage is surrounded by a vascularized bed of connective tissue, the cellular response could reach the articular cartilage through the synovial membrane, as well as from the bone marrow for full-thickness defects. Nevertheless, the direct effect of complement activation on xenogeneic chondrocytes was unknown despite it may be especially relevant in the joint. Therefore, we also addressed this question.

Our experiments demonstrated that PAC are particularly resistant to complement-mediated lysis. This finding is consistent with the slow rejection process described for pig cartilage in discordant xenotransplantation models\textsuperscript{3,8}. The effect cannot be explained by the low levels of Gal antigen on PAC, as porcine corneal endothelial cells show low Gal expression and are highly susceptible to NHS-mediated lysis\textsuperscript{29}. On the contrary, it appears as a particularity of chondrocytes and is consistent with recent observations that certain cartilage matrix components (NC4, PRELP) inhibit C9-polymerization and MAC formation\textsuperscript{30,31}. Our PAC were studied at low passage and produce many extracellular matrix components, possibly explaining the sublytic C5b-9 deposition after NHS exposure. The low proportion of cell death observed was most probably
from residual MAC-mediated lysis as little apoptosis was detected. It would be interesting to clarify the C5b-9 signals affecting chondrocyte apoptosis and the role of extracellular matrix components in future studies.

PAC responded to complement activation by up-regulating cell-surface activation markers and secreting pIL-6 and pIL-8. Both C5a and C5b-9 contributed to PAC inflammatory response and support the concept that exposure to low amounts of antibody and complement may suffice to activate chondrocytes and exacerbate rejection. This could have implications beyond xenotransplantation. In fact, we found no previous reports about C5a direct activity on chondrocytes. In HUVEC, C5a increases expression of various adhesion molecules, chemokines and cytokines. In the xenogeneic setting, porcine C5aR has been reported to respond poorly to human C5a, but this is unclear from our results. As in humans, we confirmed C5aR expression in PAC by RT-PCR (data not shown). We did not assess expression of C5a-decay-R C5L2, but our findings show a predominantly pro-inflammatory effect of C5a on chondrocytes suggesting a minor or no participation of C5L2. Our data also demonstrated a major contribution of sublytic C5b-8/ C5b-9 in this setting. Thus, the pro-inflammatory response of chondrocytes to C5a and C5b-9 reveal another mechanism by which C5 enhances cellular rejection of xenogeneic cartilage. The participation of donor C5 (generated by PAC) was not assessed, but we cannot discard it may play a minor role. In this regard, we confirmed the inability of BB5.1 to inhibit pig complement activity in a hemolytic assay (data not shown). Although C5a and C5b-9 are the major effectors of complement-mediated injury, it would be interesting to assess the effect of C3 in future studies in this setting. C3b deposition and/or local C5a generation may provide additional signals that favor tissue damage.

We propose a model of C5 acting at various levels to promote inflammation and cartilage rejection. In general, our results agree with the critical role described for C5 in OA and RA animal models. However, this work encourages to broaden the applicability of C5 inhibition to the chondrocyte transplant setting. Furthermore, we believe our findings may also be helpful for better understanding the molecular bases of inflammatory joint diseases. In particular, a direct effect of complement components on chondrocytes may explain some of the protective effects previously observed in mice deficient for C5aR and C6. Our results with CD59-expressing chondrocytes also agree with a CD59 requirement in the joint to prevent disease severity. Thus, the processes that trigger the initial complement activation may differ in xenogeneic cartilage rejection and inflammatory joint diseases, but subsequent effects may be shared. Interestingly, our finding that chondrocytes release IL-6 in response to C5a and C5b-9 may help to understand the enhanced degradation of cartilage extracellular matrix after complement activation in OA, which in turn could further amplify complement activation through specific matrix components. This could add up to other inflammatory and catabolic mechanisms reported to be enhanced by sublytic MAC in chondrocytes, whereas the role of C5a on these additional processes remains to be elucidated. Furthermore, the release of the potent chemokine IL-8 and the up-regulation of adhesion molecules by C5a would promote cellular immunity.

The Gal-antigen blockade was insufficient to prevent the PAC response, further emphasizing the need to control complement activation. Although Gal KO PAC were not studied, the results with GAS914 were clear. Our current findings are highly relevant for the design of genetic modifications that avert rejection of xenogeneic chondrocytes. We endorse the use of strategies that prevent local complement regulatory molecules together with Gal-antigen blocking strategies. This is a matter of debate, but we reach the same conclusion as others working in solid organ xenotransplantation that confront the problem of AHXR.
particular, it demonstrates the efficacy of anti-C5 therapy in a xenogeneic model in the absence of hyperacute rejection and encourages its systemic use if local complement control cannot prevent humoral rejection. The high safety and efficacy of anti-C5 therapy in clinical allotransplantation further supports its use.\textsuperscript{15,36} Targeting C3 for long systemic complement inhibition poses major hurdles by compromising defense mechanisms against pathogens, but the additional control of local C3 byproducts may be of interest. The development of similar approaches for articular disease may also provide additional tools to promote survival of porcine chondrocytes used for cartilage repair.

Author contributions

RS and MP-C contributed equally to this work in research design, performance and analysis, JLB participated in research design and performance and reviewed the writing, RM contributed to research design and CC contributed at all levels.

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Conflict of interest statement

The authors declare no conflict of interest.

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