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Evaluation of Transfer Stability and Vascular Concordance of Porcine Regulatory Macrophages in Xenotransplantation

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ABSTRACT

Pig regulatory macrophages (pMregs) represent the donor-derived immunoregulatory cells for xenotransplantation in the need of porcine graft resistance to the adaptive immune recognition with minimal inflammatory and coagulation-associated vascular injury. The key translational question is which parts of the pMreg profile can be considered reliable irrespective of the species identity of the donors, type of primates or humans as responders, responder burden pressure and macrophage-endothelial vascular readouts. RDCA, RTRA, JDEM, VCS, and LDFA structure the pMreg assay values into a transfer-reliability profile. $CD14^+$ pig monocytes under M-CSF and IFN-γ conditioning yielded the cells analyzed by regulatory phenotype, cytokine production, $CD4^+$ T-suppression in pigs and cynomolgus monkeys, Jurkat inhibition, FOXP3 up-regulation and human macrophage-pig endothelial transcript changes. $CD14^+$ enrichment was elevated from 12.4% to 97.5%, $DHRS9^+$ cell count was increased from 19.4% to 85.4%, IL-10 raised from 40 to 730 pg/mL, TGF-β was increased from 90 to 500 pg/mL, while IL-1β was decreased from 230 to 5 pg/mL. RDCA provided the balance-adjusted score of 0.623 suggesting the coordinated but non-equally balanced immune-vascular regulation. RTRA revealed that $CD4^+$ suppression of cynomolgus monkeys and maximal inhibition of Jurkats preserved only 42.6% of the pMreg pig $CD4^+$ reference effect, while FOXP3 induction of Jurkat kept 28.5% of the fold-change of pig FOXP3. JDEM demonstrated 74.6% loss of the 1:1 Jurkat inhibition effect due to the 1:5 pMreg:Jurkat ratio. VCS indicated the concordant transcript relief with cytokine-gene reduction of 60.2%, coagulation-associated reduction of 69.7% and concordance value of 0.926. LDFA found the major limiting readouts in the adaptive transfer and vascular quiescence rather than cell identity and regulatory secretion. Profile pMreg can thus be said to be most pronounced in terms of regulatory identity and vascular transcript agreement, but non-pig adaptive preservation, responder-burden longevity, and protein-level verification of TF/PAR-1 are the key preclinical considerations.

KEYWORDS: pig regulatory macrophages; xenotransplantation; transfer reliability; Jurkat dilution erosion; tissue factor; PAR-1; FOXP3; thromboinflammation

1 Introduction

The field of xenotransplantation is experiencing a key stage at which genome-edited porcine organs, decedent-recipient trials, and early living-recipient experience is starting to shape the biological necessities for clinical practice. The pigs continue to be a favoured source of organs due to their compatibility in size, breeding, and availability for genome engineering in terms of human needs. Most recent studies have focused on the elimination of major xenoantigens and introduction of complement and coagulation regulation genes in the donor's genome [1]. Genetic engineering techniques have developed significantly regarding these immune and vascular barriers [2]. Other recent reviews have highlighted advanced gene editing and the needs of cardiac xenotransplantation [3, 4]. Control over donor pathogenicity continues to be an element of future

donor development [5]. All these advancements decreased some barriers of xenograft acceptance, but they did not turn this process into a genetic engineering task. The xenograft will encounter such elements of recipient's physiology as macrophages, T cells, complement molecules, platelets, antibodies, cytokines, and thrombin-sensitive endothelial pathways even after its implantation. Successful long-term function thus depends not only on the absence of acute rejection, but also on the establishment of appropriate immune and vascular conditions.

Recent clinical and preclinical experience confirms this conclusion. Genome-edited pig kidneys provided a short-term function in human or decedent recipients [6, 7]. The recent experience in kidney transplantation includes the case of living-recipient function of a pig graft [8]. A pig heart transplantation also showed a short-term clinical function [9]. A six-gene-edited pig liver has recently been investigated in a living auxiliary transplant setting in a brain-dead recipient [10]. High-dimensional profiling of pig-to-human kidney xenotransplantation revealed the necessity of monitoring of coordinated tissue and immune-cell signatures [11, 12]. Further physiological and immune profiling studies have indicated that such factors as transcriptomic, proteomic, and vascular signals should be taken into consideration instead of only one rejection pathway [13, 14]. These studies highlight the importance of donor-derived immunoregulation. If there is a cellular product which can decrease recipient immune activation and inhibit coagulation-mediated inflammation, it can complement the engineered donor organs by creating an appropriate environment at the graft interface.

Macrophages are situated in the centre of this interface as they represent innate recognition, cytokine secretion, antigen presentation, tissue repair, endothelial damage, and coagulation-related signaling. Transplantation monocytes and macrophages can contribute to rejection, fibrosis, repair, or regulatory accommodation depending on their origin, stimulation, and tissue microenvironment [15]. Modern macrophage biology warns us against a simple M1/M2 classification because the states of these cells depend on the history of stimulation and specific marker pattern [16, 17]. The secretome and functional behavior of macrophages are additional parameters of their activity [18]. This idea is particularly important for xenotransplantation in which macrophage population can influence both immune rejection and graft injury related to coagulation. Donor-derived macrophage product should be characterized not only by its phenotype and regulatory function, but also by cytokine secretion, responsiveness of other cells to the product, and vascular activity reduction.

Regulatory macrophages are a good candidate for such a role. Human Mregs obtained from $CD14^+$ monocytes in the presence of M-CSF and IFN-γ have been associated with regulatory immune responses and the protein DHRS9 is a stable marker of human regulatory macrophages [19]. Human regulatory macrophages can induce $TIGIT^+$ induced regulatory T cells, establishing links between myeloid conditioning and adaptive immunity [20]. Xenotransplantation makes donor-derived version of this idea relevant. Pig Mregs can provide graft-matched regulatory cell population which is preconditioned before the encounter with recipient immune system and can regulate inflammatory signaling at the pig–human boundary [21]. This approach does not replace genetic engineering or pharmacological immunosuppression; instead it provides additional living regulatory cell population which can be characterized by its efficiency and reliability.

Pig $CD14^+$ monocytes were stimulated in vitro with M-CSF and IFN-γ to produce pMregs which were evaluated for their phenotype, cytokine secretion, adaptive suppression, FOXP3 expression, and vascular transcript modulation using human macrophages and pig endothelial cells [22]. The obtained phenotype was $CD14^+CD16^+CD163^+PD\text{-}L1^+DHRS9^+CD32^-CD169^-$, with high levels of IL-10 and TGF-β and low level of IL-1β. The functional tests included suppression of pig $CD4^+$ T cells, cynomolgus monkey $CD4^+$ T cells, and Jurkat cells, FOXP3 induction, and reduction of IL-1β, IL-6, IL-12, TNF-α, tissue factor, and PAR-1 in the macrophage–endothelial cell assay [22]. These tests covered aspects of donor cell identity, recipient-relevant adaptive response, cell-ratio resistance, and vascular inflammation-coagulation interactions.

The research question thus becomes more specific and stricter than the one of general regulatory property of pMregs. It is about those aspects of pMreg profile which are most robust in the terms of transferability,

ratio resistance, and vascular concordance, as well as about the major criteria of preclinical evaluation. RDCA evaluates the overall regulatory potential, RTRA shows how much suppression of donor cells remains in non-pig responder systems, JDEM characterizes the Jurkat ratio response under increasing responder burden, VCS distinguishes cytokine-gene inhibition from coagulation-related gene inhibition, and LDFA reveals the regions of score support and inhibition. Together these evaluations connect cell identity, soluble regulation, adaptive transfer, ratio resistance, and vascular quiescence in one interpretation.

The graft-interface picture shown in Figure 1 presents pMreg identity, adaptive suppression, Jurkat ratio response, and vascular transcript inhibition in one biological space. This presentation highlights the fact that the cell product is being evaluated at the interface of donor-derived regulation and recipient's immune and endothelial stress.

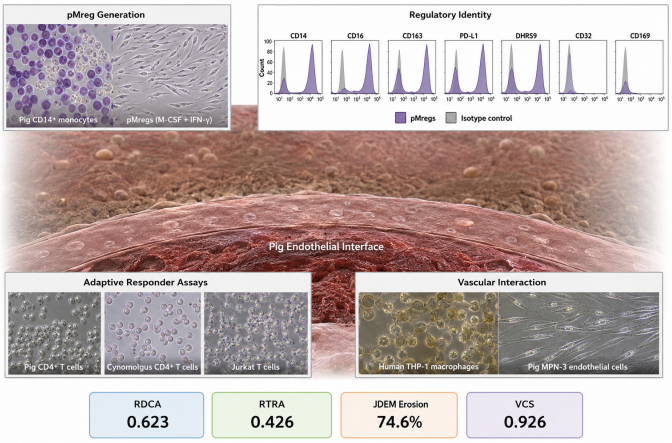

Figure 1. pMreg evidence landscape.

2 Materials and Methods

2.1 Cell systems and analytic scope

The regulatory phenotype of pig peripheral blood $CD14^+$ monocytes stimulated by M-CSF followed by IFN-γ treatment was induced as described previously [22]. Pig classically activated macrophages were generated through M-CSF, IFN-γ, and LPS treatments separately, allowing the inflammatory comparative group for IL-10, TGF-β, and IL-1β secretion. Human THP-1 cells were first differentiated into macrophages and next polarised towards the M1-like phenotype, before exposing them to the pig endothelial cells, while MPN-3 cell line served as the xenogeneic endothelial stimulus. The use of THP-1-derived macrophage systems in studies on regulatory macrophages supports their utility as standardised human myeloid systems until the need for primary cell validation is addressed [23]. Pig $CD4^+$ T cells, cynomolgus monkey $CD4^+$ T cells, and Jurkat cells represented adaptive-response systems employed for the analysis of suppression and FOXP3 expression [22, 24].

Four biological domains were selected as the focus of the analysis. They include phenotypic domain that encompasses CD14 enrichment, $DHRS9^+$ cell prevalence, and DHRS9 mRNA expression. Secretory domain comprises IL-10, TGF-β, and IL-1β secretion where IL-10 and TGF-β are deemed positive increases and IL-1β as positive decrease. Adaptive domain includes pig $CD4^+$ proliferation, cynomolgus monkey $CD4^+$ proliferation, the amount of Jurkat cells at three different pMreg:Jurkat ratios and FOXP3 expression. Inflammatory cytokine transcripts and coagulation-related transcripts measured in human macrophages exposed to pig endothelial cells under the conditions of pMreg are the components of the vascular domain. These four domains were chosen since they cover the minimal requirements of a donor-derived macrophage product in terms of biological claims: cellular identity, regulatory secretion, responder-cell inhibition, and vascular quiescence.

The calculations performed were done conservatively. Percentages were considered as percentage points and only interpreted within the respective assay context. The values were rounded up to the same precision used in the assay tables without adding extra digits. The inversions were made only when the biological interpretation of the result was unambiguous, i.e. proliferation, Jurkat-cell counts, inflammatory cytokine transcripts, TF and PAR-1 as negative, while DHRS9, IL-10, TGF-β, and FOXP3 as positive. No replicate-level variances, p-values, and confidence intervals were estimated, which means that the coordinates are effect descriptors but not substitutions for the replicate-level statistical inference.

This organization in Table 1 maintains a link to biological function. While phenotype and secretion provide evidence for a plausible starting position in terms of regulation, the adaptability and vascular blocks decide whether this position is still meaningful outside the donor organism. This way, the integration score is thus not a replacement of the individual

scores but serves to balance out potential dominance by an especially robust domain.

Table 1. Evidence blocks and analytic roles.

Evidence block	Primary readouts	Coordinate role	Translational interpretation
Phenotypic identity	CD14 enrichment; DHRS9$^+$ cells; DHRS9 mRNA	Phenotypic acquisition	Starting-cell product readiness and lineage-consistent regulatory identity
Secretory state	IL-10; TGF-β; IL-1β	Secretory inversion	Soluble regulatory maturity and inflammatory de-escalation
Adaptive response	Pig and monkey CD4$^+$ proliferation; Jurkat cell number; FOXP3$^+$ cells	Adaptive restraint	Cross-species transfer retention and responder-burden durability
Vascular interaction	IL-1β, IL-6, IL-12, TNF-α, TF, PAR-1	Thrombo-inflammatory quiescence	Cytokine–coagulation concordance at the macrophage–endothelial interface
Integrated profile	Four domain scores	Balanced regulatory dominance	Identification of limiting domains by omission sensitivity

2.2 Regulatory dominance coordinate analysis

RDCA was applied for converting the heterogenous data into corrected bounded coordinates. For those parameters where a high pMreg value indicates a good regulatory change, like IL-10, TGF-β or DHRS9, the response ratio was defined as

$$R_i^{(+)} = \frac{x_{\mathrm{pMreg},i} + \varepsilon}{x_{\mathrm{ref},i} + \varepsilon}, \tag{1}$$

where $x_{\mathrm{pMreg},i}$ is the pMreg value, $x_{\mathrm{ref},i}$ is the comparator value, and ε is a small stabilizing constant. For variables in which a lower pMreg value represented a favourable regulatory displacement, such as IL-1β, proliferation, Jurkat-cell number, TF, or PAR-1, the ratio was inverted:

$$R_i^{(-)} = \frac{x_{\mathrm{ref},i} + \varepsilon}{x_{\mathrm{pMreg},i} + \varepsilon}. \tag{2}$$

Each response ratio was mapped to a bounded coordinate:

$$q_i = \max\left(0, \frac{\log_2(R_i)}{1 + \log_2(R_i)}\right). \tag{3}$$

The transformation preserves directionality and constrains the effect of extreme fold-changes. The coordinate of a readout equals zero when the pMreg treatment does not shift towards the desired direction and converges to one when there is a large desired shift. The scores of the domains were calculated as follows:

$$S_d = \frac{1}{n_d} \sum_{i=1}^{n_d} q_{di}. \tag{4}$$

The final balanced RDCA score penalized dispersion across domains:

$$\mathrm{RDCA} = \bar{S}\left(1 - \frac{\sigma_S}{\bar{S}}\right), \tag{5}$$

where $\bar{S}$ is the average score of the four domains and σ_S is the population standard deviation of the scores. It is essential to use this penalty term since the presence of one dominant domain and some less prominent domains cannot imply that the product is always regulatory.

2.3 Regulatory transfer retention analysis

RTRA is a test used to determine how much adaptive suppression is left when shifting away from pig responder cells. Fractional inhibition is computed for each suppressive test as

$$E_s = 1 - \frac{P_{\mathrm{pMreg},s}}{P_{\mathrm{stim},s}}, \tag{6}$$

where $P_{\mathrm{stim},s}$ denotes the stimulated comparator and $P_{\mathrm{pMreg},s}$ is the pMreg condition comparator. The inhibition of CD4$^+$ of the pig was used as the reference point of the donor species. Retention for the responder setting s was computed as

$$T_s = \frac{E_s}{E_{\mathrm{pig}}}. \tag{7}$$

1 is perfect retention of pig inhibition; less than 1 indicates inhibition decay. For the non-pig inhibition summary, the product square root of cynomolgus monkey CD4+ retention and 1:1 Jurkat retention is:

$$\mathrm{RTRA}_{\mathrm{gm}} = \sqrt{T_{\mathrm{monkey}} T_{\mathrm{Jurkat1:1}}}. \tag{8}$$

FOXP3 transfer was analyzed separately because it is an enrichment readout rather than a proliferation-inhibition readout:

$$\mathrm{FTR} = \frac{F_{\mathrm{Jurkat}}/F_{\mathrm{Jurkat,ref}}}{F_{\mathrm{pig}}/F_{\mathrm{pig,ref}}}. \tag{9}$$

This is an assessment of whether the FOXP3 enrichment in human T cell line can approach that in the porcine T cell scenario.

2.4 Jurkat dilution-erosion modelling

JDEM applied the three different scenarios of Jurkat pMreg:responders to assess the durability of the suppression under varying responder load. The dilution-erosion constant was defined as

$$\mathrm{JDEM}_{\mathrm{erosion}} = \frac{E_{1:1} - E_{1:5}}{E_{1:1}}, \tag{10}$$

where E1:1 is the inhibition at the most optimal ratio of pMreg:Jurkat and E1:5 is the inhibition at the least available pMreg. A number close to zero means stability in the effect despite dilution, whereas a number close to one shows almost full loss of the original effect. Simple log burden slope was also determined as:

$$E(r) = \alpha + \beta \log_2(r), \tag{11}$$

where r is the relative load of Jurkat compared to pMreg. A negative β value implies a continuous decline in inhibition with increasing responder load.

2.5 Vascular concordance separation

VCS was applied for separating cytokine-gene relief from coagulation-gene relief in the macrophage-endothelial system study. The mean cytokine-gene relief was determined by

$$C = \frac{1}{4} \sum_{g \in \{\mathrm{IL1}\beta,\mathrm{IL6},\mathrm{IL12},\mathrm{TNF}\alpha\}} \left(1 - \frac{x_{\mathrm{pMreg},g}}{x_{\mathrm{PEC},g}}\right), \tag{12}$$

and mean coagulation-associated gene reduction was calculated as

$$G = \frac{1}{2} \sum_{g \in \{\mathrm{TF},\mathrm{PAR1}\}} \left(1 - \frac{x_{\mathrm{pMreg},g}}{x_{\mathrm{PEC},g}}\right). \tag{13}$$

The vascular relief ratio measured the relative strength of coagulation-associated relief compared with cytokine-gene relief:

$$\mathrm{VRR} = \frac{G}{C}. \tag{14}$$

The vascular concordance value measured whether both components moved together:

$$\mathrm{VCS} = 1 - \frac{|G - C|}{G + C}. \tag{15}$$

The closer the value is to 1, the more concordant it is; otherwise, it shows that the vascular benefit is restricted to one domain only.

2.6 Leave-domain fragility auditing

The LDFA test was carried out in order to evaluate the influence of each individual domain on the combined RDCA score. The balanced score without the k^{th} domain was calculated for each domain:

$$D_{(-k)} = \bar{S}_{(-k)} \left(1 - \frac{\sigma_{S(-k)}}{\bar{S}_{(-k)}}\right). \tag{16}$$

In the case where omission decreased the score, the omitted domain was viewed as supportive of the integrated profile. In cases where omission increased the score, the omitted domain was considered a limiting domain since inclusion of that domain limited the adjusted balance.

3 Results and Discussion

3.1 Primary values indicate a mature regulatory macrophage initial condition

The pMreg sample had a clear cellular identity signal prior to consideration of any function transfer. $CD14^+$ cell population expanded from 12.4% before enrichment to 97.5% post-selection, showing an overall 7.86 fold increase of the target monocytes. $DHRS9^+$ cells expanded from 19.4% to 85.4%, and mRNA of DHRS9 increased by 3.40 folds. Such values matter since DHRS9 is a stable marker for human regulatory macrophages, and its expression determines how similar pig cells are to the larger category of Mreg [19]. The pMreg phenotype is $CD14^+CD16^+CD163^+PD\text{-}L1^+DHRS9^+CD32^-CD169^-$. This is indicative of regulatory macrophages, and not conventional inflammatory macrophages [22]. The reason being that the morphology and the culture conditions for macrophages can affect the macrophage phenotype, therefore the interpretation should not be one parameter only but multiparameter [16, 25].

Table 2. Primary pMreg values.

Readout	Comparator	pMreg value	Unit	Direction-corrected movement
$CD14^+$ monocyte enrichment	12.4	97.5	% cells	7.86-fold increase
$DHRS9^+$ cells	19.4	85.4	% cells	4.40-fold increase
DHRS9 mRNA	1.0	3.4	relative level	3.40-fold increase
IL-10 secretion	40	730	pg/mL	18.25-fold increase
TGF-β secretion	90	500	pg/mL	5.56-fold increase
IL-1β secretion	230	5	pg/mL	46.0-fold decrease

The values in Table 2 demonstrate an asymmetric yet internally consistent starting pMreg profile. The most pronounced directional change in values is a 46.0-fold

reduction in IL-1β, while a 18.25-fold increase in IL-10, along with CD14 enrichment and DHRS9 acquisition, confirm that the secretion pattern belongs to the intended state of a monocyte-derived cell. This combination is significant since a cytokine shift in the absence of stable cell identity would be less compelling evidence for preclinical qualification.

The identity and secretion pair plot in Figure 2 explains why the starting product is considered a mature condition of regulatory macrophages. Moreover, the figure emphasizes that the most valuable data is not from one measurement type, as phenotypic enrichment, DHRS9 acquisition, secretion of anti-inflammatory cytokines, and suppression of IL-1β go in one regulatory direction.

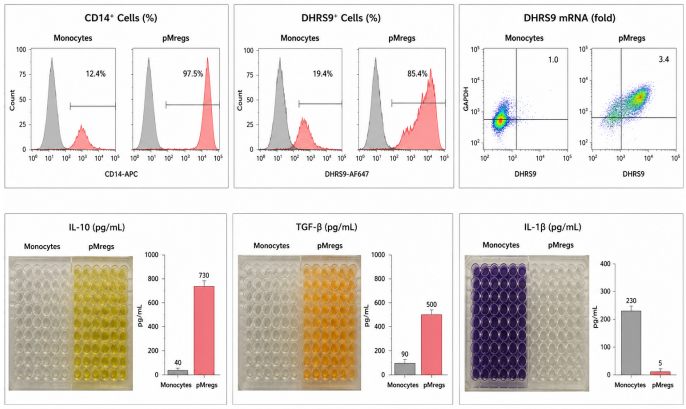

Figure 2. pMreg identity and secretion.

Secretion profile provided additional support for the interpretation of the starting product as a mature regulatory state. Secretion of IL-10 was increased from approximately 40 to 730 pg/mL compared to the pM1 comparator, secretion of TGF-β from approximately 90 to 500 pg/mL, and IL-1β was suppressed from approximately 230 to 5 pg/mL. Such a combination is more informative than individual cytokines, as IL-10 and TGF-β are known to have regulatory immune functions and regulate T-cell activity, while IL-1β is an important proinflammatory mediator, which can enhance macrophage and endothelial cell activity [26]. Thus, a pMreg profile with elevated IL-10, TGF-β, and minimal IL-1β further confirms the hypothesis about their regulatory function. However, a cytokine secretion profile does not indicate xenotransplantation suitability of the donor-derived macrophage product [21]. A suitable macrophage product should additionally affect recipient-relevant responder cells [28, 32].

3.2 RDCA reveals coordination in regulation but not full balance

RDCA provided a summary for the four domains as a balanced coordinated profile, with the phenotypic acquisition profile at 0.689, secretory inversion at 0.789, adaptive restraint at 0.633, and thrombo-inflammatory quiescence at 0.633. The unweighted mean for the four domains was 0.686, with a balance factor of 0.907 and an RDCA score of 0.623. From this score, there was a clear indication of multiple axis regulation since all the domains changed positively without any domain falling to zero. However, this score does not support exaggeration. A score of 0.623 cannot be taken as maximal evidence of preclinical sufficiency since it is a coordinated yet unbalanced profile.

The RDCA values in Table 3 provide the key result of the paper. Secretory inversion is the highest scoring coordinate, yet both the adaptive and the vascular domains score low and thereby drag the balance-adjusted score below the mean. This is the reason why the pMreg profile should be classified as being coordinated but not complete: all domains are favourable, but not all domains are equally strong.

Table 3. RDCA scores.

Component	Value	Interpretation
Phenotypic acquisition	0.689	Lineage-identity layer before recipient-facing functional testing
Secretory inversion	0.789	Strongest regulatory maturity signal
Adaptive restraint	0.633	Suppression present but weaker than internal regulatory identity
Thrombo-inflammatory quiescence	0.633	Vascular signal favourable but still a validation target
Unpenalized mean	0.686	Average multi-domain displacement before dispersion correction
Balance factor	0.907	Moderate dispersion across the four domains
Final RDCA score	0.623	Coordinated regulation with identifiable limiting translational readouts

The balance profile in Figure 3 reinforces the numerical RDCA classification. Secretory inversion scores highest on the x-axis, whereas adaptive restraint and thrombo-inflammatory quiescence score lower on the y-axis, thus making the integrated score favourable but not maximal.

This RDCA result is significant, since it avoids drawing an incorrect conclusion. Considering secretory inversion alone would make the pMregs

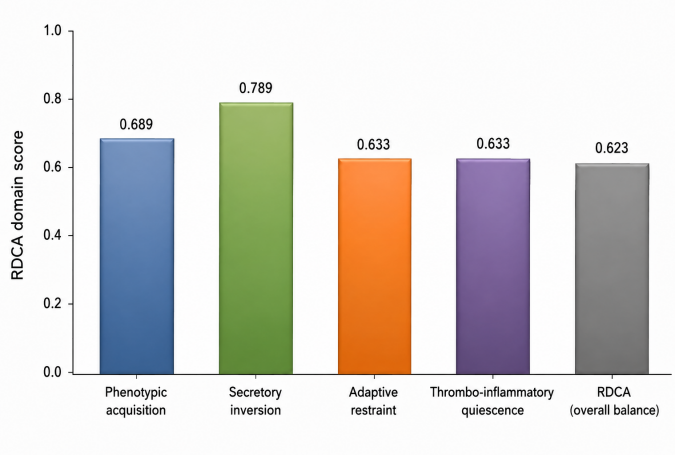

Figure 3. RDCA balance profile.

appear extremely strong. Considering the vascular domain alone would make the pMregs look promising but incompletely validated. Considering the adaptive restraint alone would make the pMregs appear functional, yet attenuated. Only considering a balanced coordinate score makes all three conclusions possible at once. This is critical in the context of xenotransplantation, since the most important source of failure is unlikely to be the weakest single assay, but rather the combination of modest adaptive restraint, endothelial activation and coagulation dysregulation. While genetic engineering eliminates many barriers, complement and coagulation pathways interact post-transplantation [29, 30]. Recipient macrophages and T cells interact with these pathways [31]. This means that pMreg product needs to be evaluated according to its ability to create a multi-domain regulatory environment rather than to produce one particular marker or one particular cytokine.

Balanced score also makes it easier to tell apart two types of promise. One is the biological plausibility: the pMreg condition alters phenotype, cytokine secretion, adaptive function and vascular transcriptome in regulatory directions. Another one is the translational sufficiency: the weaker domains need to score high enough to matter against recipient environment. The former type of promise is more clearly supported by RDCA analysis, while the latter is less clear. This helps to prevent the paper from over-interpreting the results of in vitro study and at the same time to recognize the regulatory signature of the pMreg product.

3.3 Adaptive suppression transfers beyond pig cells but loses substantial strength

The highest adaptive effect was found to occur in pig $CD4^+$ T cells. There was a decrease in proliferation from 74.3% to 7.8%, which corresponds to 89.5% inhibition. Monkey $CD4^+$ T cell proliferation decreased from 69.7% to 43.1%, which corresponds to 38.2% inhibition. Inhibition of 1:1 Jurkat cells was 38.1%, which is almost identical to the effect found in monkey $CD4^+$. Relative to the pig effect used as the reference point, inhibition in both the monkey $CD4^+$ and 1:1 Jurkat conditions maintained about 42.6% of the pig inhibition. Thus, the non-pig geometric retention estimate was 0.426. Transfer of FOXP3 was more compromised: Pig $FOXP3^+$ enrichment was 11.70 times compared to 3.34 times for Jurkat, yielding a transfer ratio of 0.285. Transfer retention values are given in Table 4.

Table 4. Transfer retention values.

Responder setting	Comparator	pMreg condition	Effect	Retention relative to pig inhibition
Pig $CD4^+$ proliferation	74.3%	7.8%	89.5% inhibition	1.000
Monkey $CD4^+$ proliferation	69.7%	43.1%	38.2% inhibition	0.426
Jurkat cell number, 1:1	1.05	0.65	38.1% inhibition	0.426
Jurkat cell number, 1:2	1.45	0.95	34.5% inhibition	0.385
Jurkat cell number, 1:5	1.55	1.40	9.7% inhibition	0.108
Pig $FOXP3^+$ enrichment	2.06%	24.1%	11.70-fold	reference
Jurkat $FOXP3^+$ enrichment	2.67%	8.91%	3.34-fold	0.285 versus pig FOXP3 fold-change

These numbers represent cross-species positivity versus cross-species retention. Both the monkey effect and the Jurkat effect are biological, but each retains only 0.426 of the pig $CD4^+$ inhibitory reference. FOXP3 transfer is lower at 0.285, suggesting that regulatory instruction in the human T cell-line setting is less effective than proliferation suppression.

The visual representation of the transfer-retention relationship in Figure 4 brings out the pattern of attenuation. The pMreg effect can still be detected in non-pig responder systems, but the non-pig inhibitory readouts are clustered significantly lower than the pig $CD4^+$ reference effect, and FOXP3 induction follows the same low-transfer pattern.

This is an important biological distinction between cross-species positivity and cross-species retention because, as discussed above, the pMreg effect inhibited all of the responder systems tested, allowing

for the conclusion of cross-species function. However, cross-species retention provides information about the degree of efficiency of the non-pig inhibition relative to the pig reference inhibition under the same assay conditions, which is essential for the xenotransplantation context because the recipient immune system is not pig-derived. In recent studies of the spatially organized nature of innate and adaptive signals in xenotransplantation, human and decedent-recipient profiling showed [11, 12]. Moreover, the studies suggested that the monocyte/macrophage immune activity is possible even in the absence of adaptive immunity in pharmacological suppression [13, 14]. Therefore, a donor-derived pMreg solution must work in the immune environment of primates or humans, in which signaling mechanisms such as direct antigen recognition, indirect antigen presentation, costimulation, and inflammatory cytokines differ from the donor-species case. This attenuation does not rule out the usefulness of pMregs; it defines a qualification target for further development. Subsequent assays should determine whether pMreg dose, activation timing, co-culture time, endothelial localization, or costimulation blockade combinations could increase non-pig retention.

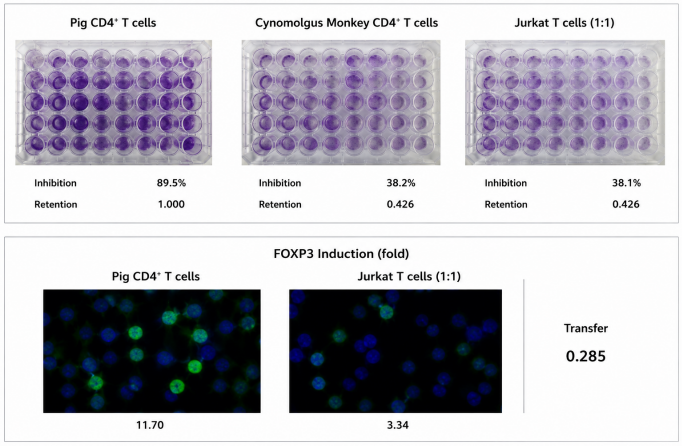

Figure 4. Cross-species transfer retention.

One possible explanation for transfer attenuation is that pig T cells and pig macrophages have species-specific receptor, ligand, and cytokine interaction environments, which makes suppression more effective in the donor-species assay. As the responder system switches to cynomolgus monkey $CD4^+$ cells or Jurkat cells, the pMregs may continue to produce suppressive signals, but their receptor compatibility, antigen-presentation geometry, costimulatory balance, cytokine responsiveness, and contact-dependent interactions may be less compatible than in the donor-species case. This interpretation is also in line with the general problem of xenotransplantation: molecular compatibility can be improved through genetic engineering, but cross-species immune communication remains imperfect. The important thing in product development is not whether this is surprising; it is whether this attenuation can be reduced through product conditioning or combination therapy.

The discovery of attenuation in FOXP3 induction requires careful analysis. FOXP3 induction is relevant to xenogeneic response modulation by regulatory T-cell pathways because human pig-specific regulatory T cells can be generated in controlled culture systems [26]. These numbers suggest that pMregs can induce $FOXP3^+$ cells in both pig and Jurkat systems, but the ratio of the fold-changes in the latter case retains only 28.5%. However, since the Jurkat cells are a transformed T cell line, this result should not be interpreted as an equivalent of human primary regulatory T cell induction. This should rather be seen as an indication of the standardized human line signal that demonstrates the possibility of cross-species regulation and the need for primary human $CD4^+$ T cell assays.

3.4 Suppression of Jurkats is susceptible to reduction from responder burden

The JDEM model demonstrated that as Jurkat burden increased compared to pMreg burden, the effect of suppression declined. Suppression was achieved at 38.1% under the 1:1 scenario, 34.5% in the 1:2 scenario, and 9.7% in the 1:5 scenario. The erosion rate was 0.746, implying that 74.6% of the 1:1 scenario effect was reduced in the 1:5 scenario. The log-burden rate was -0.126.

From the ratio response values in Table 5, it is clear that Jurkat responder burden effect is not equally stable across cell ratios. Both the 1:1 and 1:2 conditions are very close, but the 1:5 condition falls to 9.7% inhibition. In this assay, responder burden stress dominates. The erosion value has an implication: it describes how much of the favourable 1:1 effect is lost due to pMreg availability becoming limiting.

Figure 5 represents the erosion profile for the three conditions of Jurkat cells on a single ratio response curve. The erosion profile clearly shows that it is not only important to have a suppression at 1:1 but it is

Table 5. Jurkat erosion values.

pMreg:Jurkat condition	Jurkat comparator	Jurkat value with pMregs	Derived interpretation
1:1	1.05	0.65	38.1% inhibition; strongest human-line effect
1:2	1.45	0.95	34.5% inhibition; moderate retention
1:5	1.55	1.40	9.7% inhibition; marked erosion
Dilution-erosion value	–	–	74.6% loss of the 1:1 inhibitory effect by 1:5
Log-burden slope	–	–	$\beta = -0.126$ fractional inhibition units per $\log_2$ burden unit

also important that there is a fast drop-off of the effect when responder availability goes up.

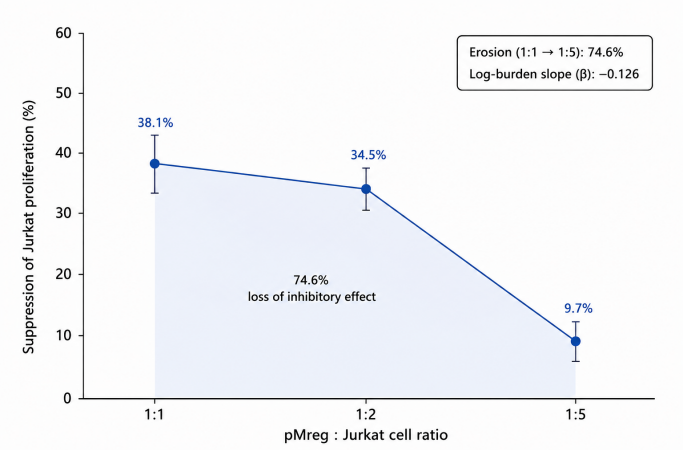

Figure 5. Jurkat responder-burden erosion.

Responder-burden erosion is not a mere statistical parameter, it is a product parameter. Under transplantation conditions, regulatory cells will most likely not come across responders at a fixed ratio. The quantity and activation of recipient T cells, macrophages, and endothelial targets may vary in case of ischemia-reperfusion injury, infection, antibody-mediated injury, and systemic inflammation. Products that suppress only when regulatory cell availability is high may need local administration, repeated treatments, or even additional pharmacologic immunosuppression. Therefore, the erosion result obtained for Jurkat is a way to convert laboratory findings to a translational problem, namely what is the required level of pMreg availability to provide prespecified recipient suppression.

This should not be taken to extremes because only three ratio points are used, and Jurkat cells are not the same as primary human T cells. Still, the slope of the erosion curve is a useful screening statistic. It shows that further investigations of the product need to focus not only on the existence of the suppressive effect but on how much of it is preserved when responder availability changes. If the optimized product improves 1:5 effect without degrading 1:1 effect, the erosion coefficient will decrease and reliability profile will become better. If on the contrary, the additional priming results in increased maximum suppression but the erosion coefficient increases, the product will remain unreliable under high responder availability conditions.

3.5 Vascular effects are concordant across cytokine and coagulation-associated transcripts

VCS analysis showed that pMregs reduce inflammatory cytokine transcripts and coagulation-associated transcripts in the macrophage–endothelial system. Reductions in inflammatory cytokine genes were heterogeneous: IL-12 decreased by 86.3%, IL-6 by 60.7%, IL-1β by 51.7%, and TNF-α by 41.9%. The mean reduction was 60.2%. Reductions in coagulation-associated genes were less heterogeneous: TF decreased by 70.4% and PAR-1 by 69.0%, resulting in mean reduction of 69.7%. The vascular relief ratio was 1.16 indicating that there was slightly more reduction in coagulation associated transcripts than in cytokine genes. The vascular concordance value was 0.926 indicating that there was concordance between the changes in cytokine and coagulation-related transcripts.

Table 6. Vascular concordance values.

Gene group/readout	Pig endothelial condition	pMreg condition	Decrease	Group role
IL-1β	5.8	2.8	51.7%	Cytokine
IL-6	2.8	1.1	60.7%	Cytokine
IL-12	8.0	1.1	86.3%	Cytokine
TNF-α	1.55	0.90	41.9%	Cytokine
Tissue factor	2.7	0.80	70.4%	Coagulation-associated
PAR-1	3.55	1.10	69.0%	Coagulation-associated
Mean cytokine reduction	–	–	60.2%	C
Mean coagulation reduction	–	–	69.7%	G
Vascular relief ratio	–	–	1.16	G/C
Vascular concordance value	–	–	0.926	$1-\lvert G-C\rvert/(G+C)$

The vascular values from Table 6 represent concordance rather than cytokine inhibition in isolation. While IL-12 represents the largest reduction in cytokine-gene expression, both TF and PAR-1 experience reductions of almost equal size. The average reduction of coagulation genes is greater than the average reduction of cytokine-genes resulting in a

vascular relief ratio of above one.

The vascular plot from Figure 6 demonstrates that pMreg associated relief is not restricted to a single inflammatory transcript. Both cytokine-associated and coagulation-associated readouts experience reductions, and this explains the large vascular concordance value.

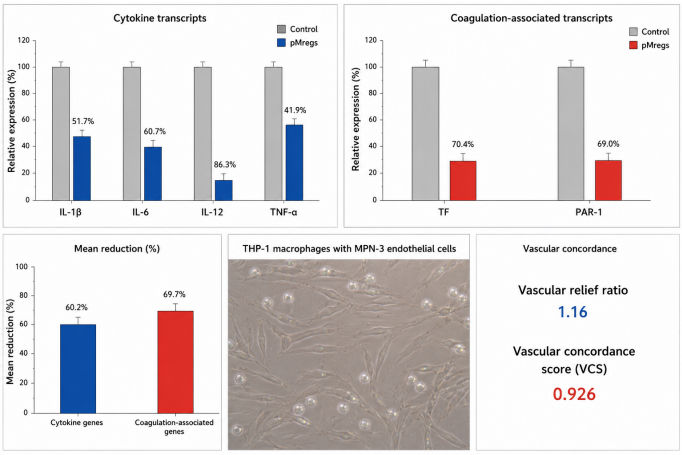

Figure 6. Vascular transcript concordance.

The vascular data is one of the most robust translational signals for several reasons. First, xenograft damage involves the interplay of both inflammation and coagulation, not the presence of cytokines. Activation of the complement system and coagulation system is deeply intertwined in xenotransplantation [28, 29]. Systemic inflammation may occur before and increase coagulation activation in xenotransplantation patients [32]. Modern solid organ xenotransplantation reviews focus on the need to pay attention simultaneously to both immune activation and vascular compatibility [4, 33]. Pathogen safety and clinical monitoring should also be considered rather than one specific barrier [5]. TF plays an important role in the initiation of extrinsic coagulation pathway, and it also connects coagulation and inflammation signaling [34]. PAR-1 belongs to a class of protease-activated receptors which affect the vascular and immune response by thrombin and other proteases [35]. The concomitant reduction of TF and PAR-1 therefore indicates that pMregs might target thromboinflammatory pathways involved in xenograft damage.

The vascular concordance result additionally indicates that pMregs may target the macrophage activation status rather than the single transcript. If the pMregs had been reducing the level of expression of only one cytokine, the result might be perceived as narrow anti-inflammatory effect. The simultaneous reduction of the levels of IL-1β, IL-6, IL-12, TNF-α, TF, and PAR-1 indicates broad macrophage de-escalation upon exposure to pig endothelial cells. It is critical since the pig endothelial cells might serve as the starting point for xenogeneic reaction, while human macrophages may enhance damage through cytokines and procoagulant mediators. Therefore, the preparation with a regulatory effect on both parts of the reaction may turn out to be very helpful in combination with pig genetic manipulation in relation to coagulation and complement pathways.

The robustness of this conclusion is limited by the type of readout, however. The vascular values represent transcript-level readouts in an in vitro model involving human THP-1 derived macrophages and pig endothelial cells. Transcript reductions are encouraging but do not prove by themselves the decreased protein level of tissue factor, decreased factor Xa generation, decreased thrombin generation, preserved anticoagulant properties of the endothelium, or improved graft perfusion. The proper interpretation is that pMregs generate a coherent vascular transcript signal which allows for functional coagulation testing. A more comprehensive validation strategy might include measurements of TF protein level, PAR-1 protein or signaling, thrombin generation kinetics, platelet interaction, endothelial activation markers, complement deposition, and macrophage cytokine release in the primary human cell model.

3.6 Fragility of the domains highlights adaptive and vascular domains as constraints

LDFA was able to define which domains are useful for the combined score and which domains are limiting it. The removal of phenotypic acquisition lowered the balanced score to 0.611 from 0.623, showing that phenotype contributes positively to the score. Removal of secretory inversion resulted in no change in the score with the score remaining at 0.625. Adaptive restraint removal raised the score to 0.639, and thrombo-inflammatory quiescence removal also raised the score to 0.639. This is not an indication that adaptive or vascular domains have no importance. It is the exact opposite – that these domains are directionally positive but weaker than the other domains.

The omission values from Table 7 show that the limiting domains are not biologically negative. Adaptive restraint and thrombo-inflammatory

quiescence are better than nothing, but their scores are lower than the best identity and secretory domains. Their omission thus improves the balance-adjusted score somewhat, indicating that these are the limiting domains that will determine the utility of the product in clinic.

Table 7. LDFA omission values.

Calculation	Balanced score	Change from full RDCA	Interpretation
Full RDCA	0.623	Reference	Coordinated but incomplete regulation
Omit phenotypic acquisition	0.611	−0.012	Phenotype supports the integrated result
Omit secretory inversion	0.625	+0.002	Secretory strength is high but not uniquely score-defining
Omit adaptive restraint	0.639	+0.016	Adaptive transfer is a limiting component
Omit thrombo-inflammatory quiescence	0.639	+0.016	Vascular quiescence is favourable but still limiting

The omission profile in Figure 7 confirms that the limiting domains are not absence domains. Adaptive restraint and thrombo-inflammatory quiescence are positive features, but they are weaker than the best identity and secretory domains, so their omission slightly improves the balance-adjusted score.

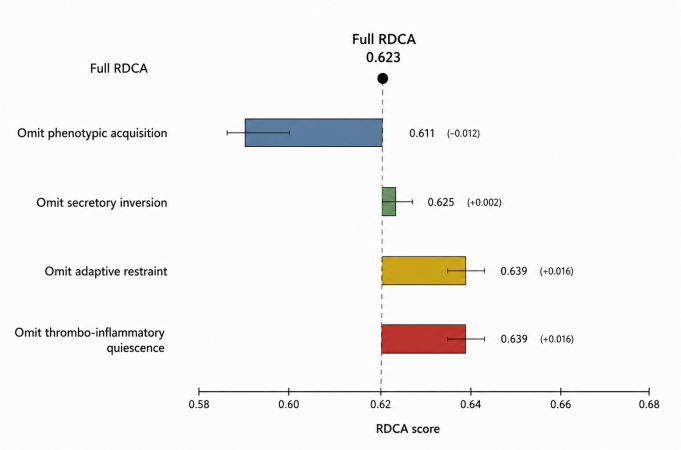

Figure 7. LDFA domain displacement.

The fragility analysis gives a clear indication of the direction to go in practical terms. The identity and secretion domains are already good, so the best tests will be those which check whether the adaptive restraint ability of the product can be improved and whether the vascular transcript signals translate into function inhibition of thromboinflammation. This is consistent with the current status of xenotransplantation research, where genome editing and early clinical trials have overcome some initiating barriers [2, 8]. Cellular and vascular incompatibilities, however, remain unresolved [30]. A pMreg product that improves the weak domains could complement genetically edited organs by adding an immunoregulatory live layer in contact with the graft.

The integrated profile can thus be described as good identity, good soluble regulatory maturity, moderate cross-species adaptive transfer, high responder-burden erosion in the Jurkat model and good but transcript-level vascular function. This is not a negative outcome. This is a more precise development profile. The concept of the pMregs is viable because the cells fulfil several requirements set for the regulatory macrophages and because they regulate immune and vascular functions. The translational challenge is that the strongest domains are those related to the regulatory phenotype of the pig, while the most important functions must act in the recipient primate/human system. Translational readiness will increase with improving the non-pig retention to the reference pig value, with reducing the slope of the Jurkat or primary human $CD4^+$ T cell erosion and with proving that reductions in the TF and PAR-1 transcripts translate to reductions in the coagulation function.

The same values give a preclinical testing priority list. The minimum potency panel should consist of CD14 positivity, DHRS9 positivity, IL-10, TGF-β, and low IL-1β. Recipient relevant adaptive potency should be measured using primary human $CD4^+$ T cells in addition to monkey cells and Jurkat cells, with retention calculated against the pig reference assay. Ratio-response experiments should substitute the use of a single co-culture condition, since the 1:5 Jurkat result indicates that responder burden exposure may reveal fragility not seen at 1:1 ratio. The vascular function should be measured also at other levels than mRNA, such as TF protein, thrombin generation, PAR-1 activity, endothelial activation markers and platelets interaction. The profile should be then recalculated after changes in cell conditioning.

4 Conclusion

The research question posed was which elements of the pMreg profile are the most robust, considering the criteria of transfer reliability, responder-burden durability, and vascular concordance for a donor-derived regulatory macrophage product. The answer is clear-cut. The best evidence comes from the regulatory starting point, which showed an increase

in $CD14^+$ enrichment from 12.4% to 97.5%, $DHRS9^+$ cells from 19.4% to 85.4%, IL-10 and TGF-β production greatly increased, and IL-1β became very low. The results make it legitimate to refer to the product as a regulatory macrophage condition rather than an incompletely specified monocyte condition.

When the durability facing the recipient is considered, the answer needs to be somewhat qualified. RDCA produced 0.623, indicating coordinated immune and vascular regulation, yet RTRA indicated that the cynomolgus monkey $CD4^+$ cell inhibition and the strongest Jurkat inhibition were capable of maintaining no more than 42.6% of the pig $CD4^+$ reference effect. FOXP3 transfer was lower at 28.5%, and JDEM revealed that 74.6% of the 1:1 Jurkat inhibitory effect was lost in the 1:5 condition. This answers the adaptive part of the research question: pMreg suppression is transferable beyond the pig responders, yet it lacks donor-species strength and is sensitive to responder cell burden.

The vascular answer is less restrictive but still needs functional validation. VCS revealed that the reduction of cytokine gene transcripts and the coagulation gene transcripts happened coordinately, showing mean reductions of 60.2% and 69.7%, and a concordance value of 0.926. This is supportive of a consistent macrophage-endothelial de-escalation of IL-1β, IL-6, IL-12, TNF-α, TF, and PAR-1. Then, the LDFA found adaptive restraint and thrombo-inflammatory quiescence to be the critical indicators of the comprehensive profile. The ultimate conclusion will thus not be a blanket statement about the regulatory nature of pMregs. It will be the one stating that pMregs have a clearly established regulatory identity and a credible vascular transcript signal, although the decisive preclinical tasks are to increase non-pig adaptive retention, maintain suppression under greater responder burden, and validate TF/PAR-1 transcript relief into protein-level and activity-level control of thromboinflammatory injury.

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