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Distinct Pro-Inflammatory Species-Specific Transcriptional Changes in Human T Cells Following Pig Xenogeneic Stimulation

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ABSTRACT

Conventional T cell-directed immunosuppression is the mainstay of standard-of-care therapy to prevent graft rejection in clinical organ transplantation. However, it remains ineffective in preventing experimental and clinical organ xenograft rejection. Here, we explored the impact of allogeneic versus xenogeneic antigen stimulation on human T cell responses and gene profile. A comparable proliferative human T cell response was observed in vitro following stimulation with either human or pig cells. Yet, elevated High mobility group box-1 (HMGB1) levels were following xenogeneic but not allogeneic stimulation, suggesting a pro-inflammatory response. Next, human peripheral blood mononuclear cells (PBMC) were cultured with allogeneic human, "concordant" xenogeneic monkey, or "discordant" xenogeneic pig, intact cells, or cell lysates. Flow-sorted CD3$^+$T cells were analyzed for gene expression using NanoString. A distinct pro-inflammatory gene profile was observed in human CD3$^+$T cells following co-culture with discordant xenogeneic pig cells, but not concordant xenogeneic monkey cells or allogeneic human cells. Uniquely, stimulation with pig cells induced the expression of the transcription factor NCF4, which promotes inflammasome activation. Pig cell lysate, but not intact pig cells, induced high expression of the DNA-binding cytokine interleukin-26 gene. Collectively, these observations highlight the impact of xenogeneic stimulation of human T cells in pig xenograft recipients and concomitant inflammatory responses, which may contribute to immunosuppression-resistant xenograft rejection. Finally, the impact of genetic engineering of donor pigs on human T cell transcriptomic gene profile is yet to be determined.

1 | Introduction

Xenotransplantation remains a viable approach to bridge the gap of organ shortage by providing an alternative and unlimited source of organs [1]. Recent progress in genetic editing, immuno-suppressive therapy, organ preservation, and infection control measures have significantly enhanced long-term life-supporting pig organ xenograft survival in nonhuman primates (NHPs) [2–4]. Such remarkable progress laid the foundation for the recent clinical attempts of transplantation (Tx) of life-supporting pig organs in human patients, where expanded access authorization by the United States Food and Drug Administration (FDA),

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Abbreviations: FDA, Food and Drug Administration; HMGB1, High mobility group box-1; MLR, mixed lymphocyte reaction; NHP, nonhuman primate; PBMC, peripheral blood mononuclear cell.

that is, compassionate use was granted for patients, who were considered ineligible for an allo-Tx, to receive organ xenografts obtained from genetically engineered (GE) pigs. Two patients received heart xenografts from GE pig donors at the University of Maryland, and two patients received kidney xenografts from GE pig donors at Massachusetts General Hospital and NYU Langone, respectively. In one recipient, pig heart xenograft failure ensued less than 2 months after Tx [5]. There has been a concern that xenografts may have been lost due to rejection, despite the use of anti-CD154mAb-based immunosuppression in the second heart xenograft recipient (ATC 2024).

Currently, standard-of-care immunosuppression fails to achieve long-term xenograft protection in preclinical NHP studies [6]. Further approaches have been considered to achieve long-term xenograft survival, such as, donor pig genetic modifications, selection of recipients with low pre-Tx levels of anti-pig antibodies [7], and targeting of the CD40-CD154 co-stimulation pathway [7, 8].

Following xenotransplantation, the immune system identifies pig xenograft as foreign and initiates both innate [9, 10] and adaptive [11, 12] immune responses, which leads to a robust inflammatory reaction within the xenograft as well as systemically in the recipient, as shown in NHP studies [13]. Particularly, the magnitude of such inflammatory response is stronger in xenograft compared to allograft recipients [14]. Meanwhile, clinically available immunosuppressive therapy does not prevent systemic inflammatory responses in xenograft recipients, despite effective regulation of xeno-reactive T cell responses [15].

Here we hypothesize that xenogeneic stimulation of human T cells induces a distinct genetic profile from that induced by allogeneic stimulation. If factual, this may indicate that prevention of pig xenograft rejection requires a distinct therapeutic approach from the conventional immunosuppression standard-of-care currently used in clinical allo-Tx.

2 | Methods

2.1 | Isolation of Human, Monkey, and Pig Cells

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of blood type O healthy volunteers (Institute for Transfusion Medicine, Pittsburgh, PA, USA). Pig PBMC were isolated from whole blood of farm pigs. Monkey PBMC were isolated from whole blood of naïve juvenile rhesus monkeys. Briefly, human buffy coat, monkey or pig peripheral blood were diluted with phosphate-buffered saline (PBS) at a 1:1 volume ratio, then overlaid on Ficoll-Paque Plus (GE Healthcare Life Sciences AB), spun for 25 min at 1500 rpm (room temperature), and buffy coat collected. PBMC were treated with red blood cell lysis buffer (150 mmol/L NH_4C; 1 mmol/L $KHCO_3$; 0.1 mmol/L Na_2EDTA), and cell viability was evaluated with Trypan blue.

2.2 | Mixed Lymphocyte Reaction (MLR)

Co-cultures were carried out in round-bottom, 96-well plates with serum-free, AIM-V medium (Invitrogen, Carlsbad, CA, USA).
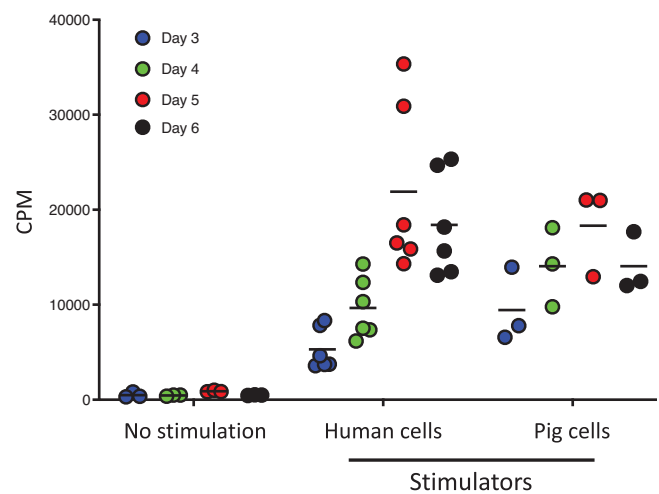

FIGURE 1 | Human PBMC response to allogeneic versus xenogeneic stimulation. In MLR, human PBMC were cultured either alone, with irradiated allogeneic human PBMC, or with irradiated xenogeneic pig PBMC. Responder cells were harvested on Days 3, 4, 5, and 6 to assess for proliferation using ^3H incorporation. CPM = counts per minute; MLR = mixed lymphocyte reaction; PBMC = peripheral blood mononuclear cell.

Human PBMC were used as responders at 0.2×10^6 cells/well. Irradiated human or pig PBMC were used as stimulators at stimulator–responder ratios of 1:1. ^3H-thymidine (1 µCi/well) was added to each well during the last 16 h of incubation. Cells were harvested on glass-fiber filter mats. Next, they were analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA, USA). The mean of triplicate results was expressed as ^3H-thymidine incorporation values (presented in units of counts per minute [CPM]).

2.3 | Luminex Multiplex Immunoassays for Cytokine Levels

Supernatants were collected from MLR cultures on Day 3. Human cytokine and chemokine levels were assessed using custom LEGENDplex Luminex kit (BioLegend, San Diego, CA, USA).

2.4 | Western Blotting for HMGB1

Western blot analysis for High mobility group box-1 (HMGB1) was performed as previously described [16]. Briefly, supernatant in MLR co-cultures was collected on Day 3 and stored at −80°C for Western blot analysis. Primary polyclonal rabbit antibody to HMGB1 (1:5000; BD Biosciences) was used. Membranes were developed with the SuperSignal West Pico Chemiluminescent Kit (Pierce) and exposed to film.

2.5 | Flow Sorting of T Cells for RNA Analysis

Human PBMCs were cultured with either human PBMC, monkey PBMC, or pig PBMC stimulators. In tandem, cell lysates from human, monkey, and pig stimulator cells were also prepared using the freeze–thaw technique. Same simulator cell numbers (human, monkey, or pig) were used for cultures using intact cells
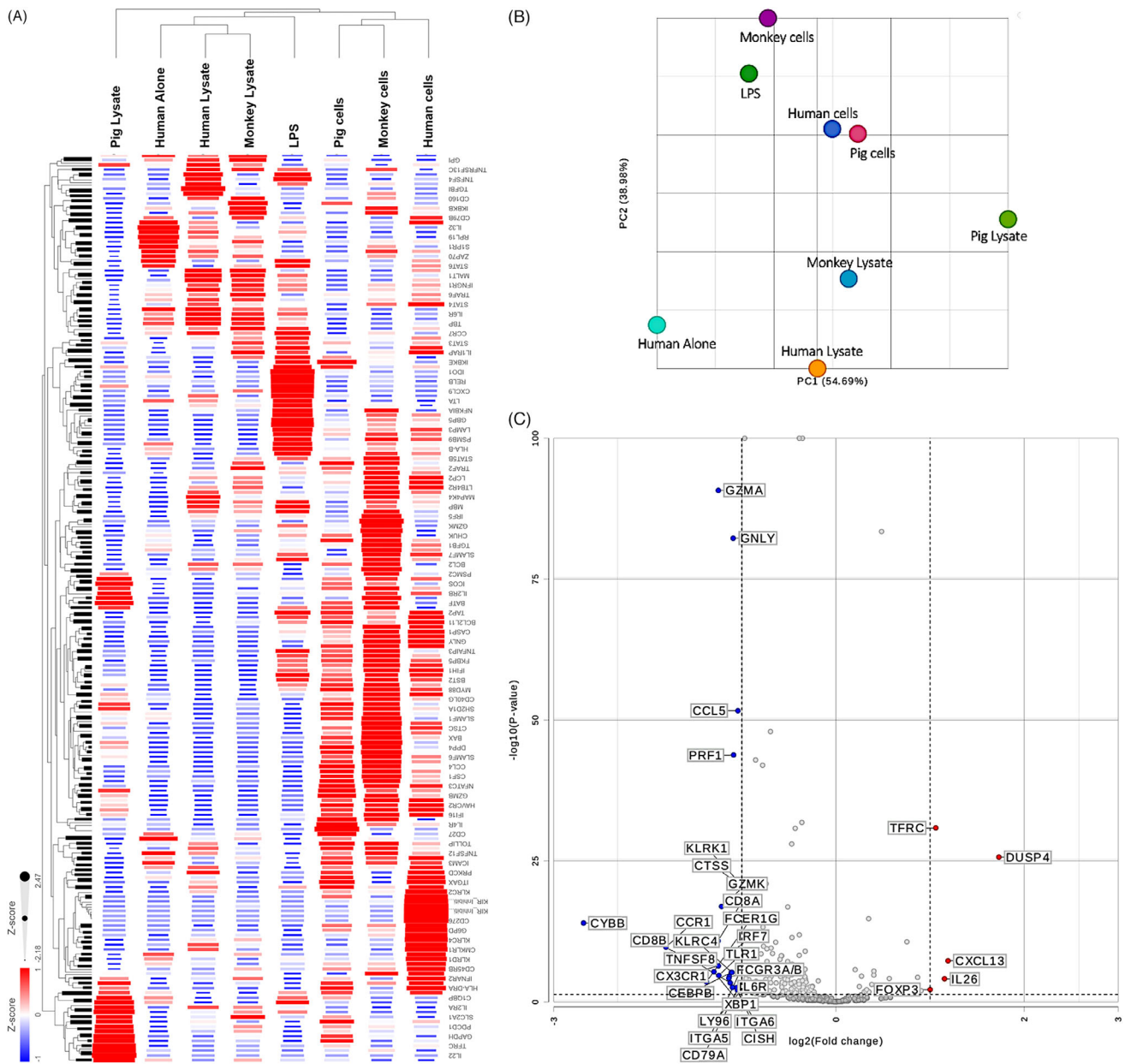
FIGURE 2 | Gene expression in xeno- versus allo-stimulated human T cells. (A) Hierarchical clustering of samples and genes based on detected gene expression using the NanoString nCounter GX Human Immunology V2 assay. Dendrograms represent sample (columns) clusters. (B) Principal component analysis of samples was performed to validate hierarchical clustering results. (C) Volcano plot of differential gene expression in human T cells co-cultured with pig lysates versus human T cells co-cultured with pig cells. Thresholds for $p < 0.05$ and |fold change| >1.5 are represented with dotted lines.

or lysate stimulation and used at 1 (responder):1 (stimulator) ratio to co-cultures. All stimulator cells (including cells used for lysate preparation) were labeled with PKH (Sigma) and irradiated (as for the MLR experiments). For lysate preparation, cell suspensions were exposed to multiple cycles (5–10 cycles) of freezing (dry ice) followed by thawing at room temperature. The absence of intact cells was confirmed using Trypan blue staining. If intact cells were detected, freeze–thaw cycles were repeated. As a control, human PBMC were either cultured alone or stimulated with lipopolysaccharide (LPS) (10 ng/mL) 24 h before harvest. Responder cells were harvested on Day 5 and washed twice with sodium azide-free medium; CD3$^+$T cells were flow-sorted for RNA analysis (Figure S1).

2.6 | RNA Isolation and Gene Expression Analysis

RNA was isolated and quantified using NanoString nCounter GX Human Immunology V2 assay, which accounts for around 600 genes (NanoString Technologies, Seattle, WA, USA) and normalized using nSolver by following the manufacturer's instructions. Normalized gene expression data were imported to Partek Flow v11.0.23.1105 for filtering and downstream analysis. Genes whose expression did not vary between samples were excluded from downstream analysis. Unsupervised hierarchical clustering was performed on both the gene expression and samples. Principal component analysis (PCA) was performed for dimensionality reduction. Differential gene expression
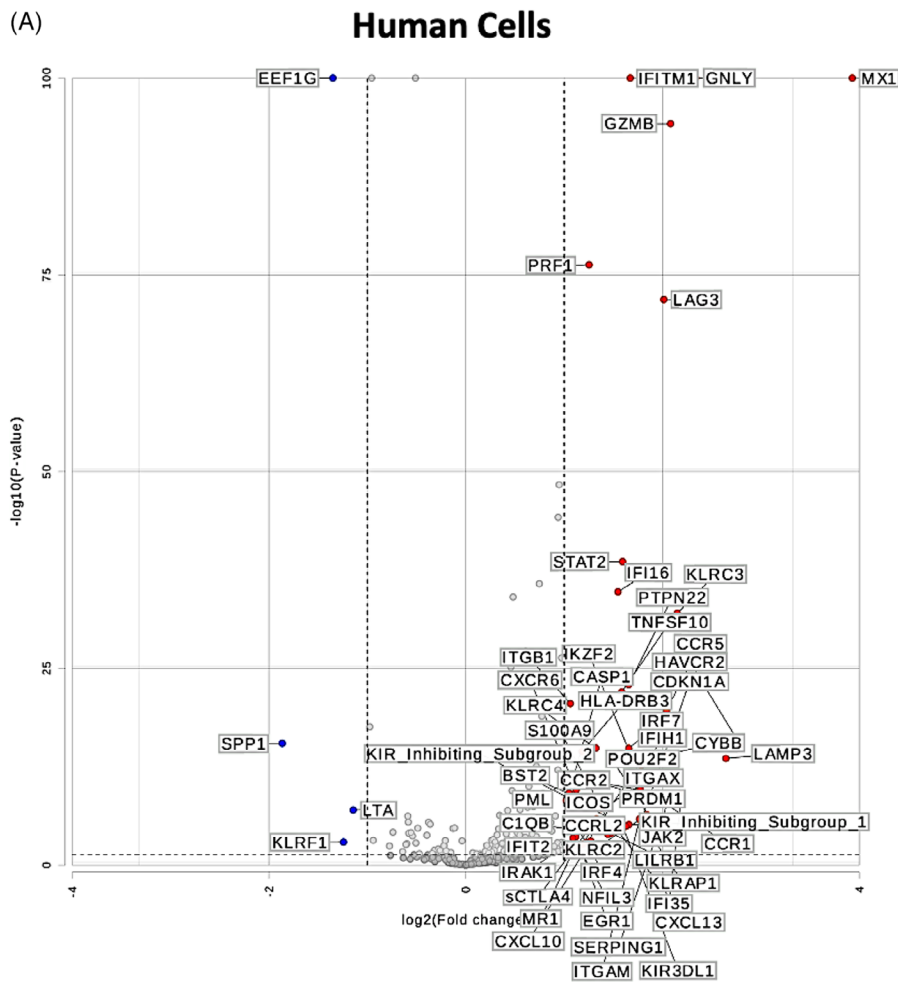
FIGURE 3 | Differential gene expression analysis uncovers uniquely expressed genes by pig-stimulated human T cells. Volcano plots of differential gene expression in human T cells co-cultured with (A) human allogeneic cells, (B) LPS, (C) pig cells, and (D) pig lysates as compared to human T cells cultured alone. Thresholds for $p < 0.05$ and |fold change| >1.5 are represented with dotted lines. Genes that are differentially expressed in (C) or (D) and similarly expressed (|fold change| <1.5) in (A) and (B) are outlined in red.

was performed using gene specific analysis (GSA) in Partek Flow. Volcano plots were drawn to visualize differential gene expression between different samples.

2.7 | Statistical Analyses

Significance was determined by paired, two-tailed Student's *t*-tests. Analyses were carried out using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant.

3 | Results

3.1 | Human PBMC Response to Xenogeneic Pig Cells Versus Allogeneic Human Cells

Three individual human responder PBMC were cultured with either irradiated human allogeneic PBMC ($n = 6$) or irradiated pig xenogeneic PBMC ($n = 3$). After culture, cells were harvested on Days 3, 4, 5, and 6 to assess the proliferative response of human

PBMC to human versus pig stimulators (Figure 1). Proliferation of human responder cells gradually increased to reach a maximum level on Day 5, followed by decreased proliferation on Day 6. Notably, human PBMC proliferation in response to human stimulators was not significantly greater than that to pig stimulators.

It has been shown that the pro-inflammatory HMGB1 protein is released following co-culture of human T cells with pig endothelial cells [17]. On Day 3, we assessed HMGB1 production in the supernatant of human and pig co-cultures (Figure S2). High levels of HMGB1 were detected in supernatants of human PBMC cultured with pig cells, but not with human cells. These observations suggest a stronger pro-inflammatory response following xenogeneic pig stimulation of human PBMC.

3.2 | Unique Pro-Inflammatory Transcriptional Changes in Human T Cells Following Stimulation With Xenogeneic Pig Cells

Next, we aimed to evaluate the impact of allogeneic (human) versus xenogeneic (monkey or pig) stimulation on gene expression
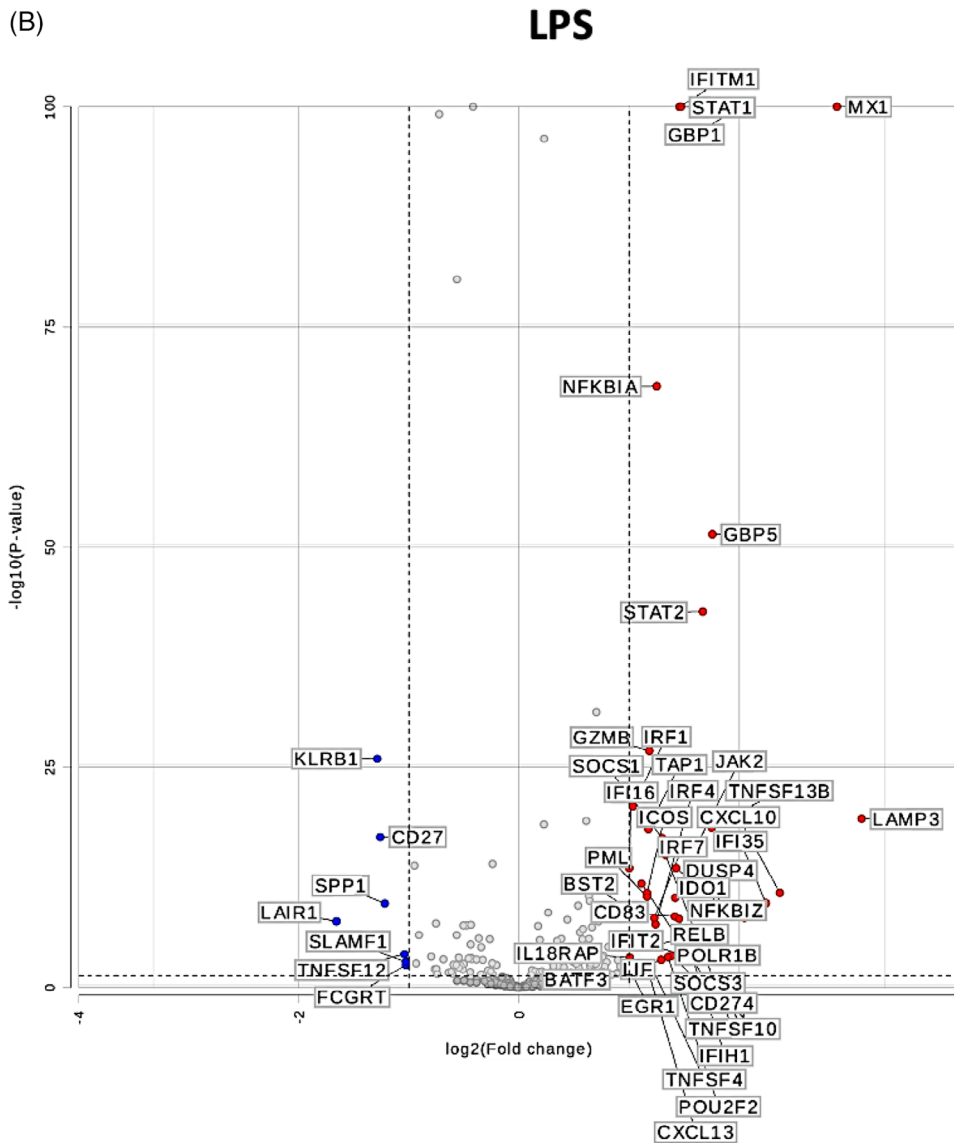
FIGURE 3 | (Continued)

in human CD3$^+$T cells. Hierarchical clustering was performed on both genes and samples to assess in an unsupervised fashion whether exposure to allogeneic or xenogeneic, intact cells or cell lysates, leads to disparate gene expression changes in human CD3$^+$T cells (Figure 2A). As controls, gene expression in human CD3$^+$T cells cultured either alone or with LPS were measured.

Gene expression profiles of human CD3$^+$T cells cultured with cell lysates were different from those from intact cells, regardless of whether it was allogeneic human, xenogeneic (concordant) monkey, or xenogeneic (discordant) pig cells (Figure 2A). The dendrograms on the rows clearly cluster human CD3$^+$T cells co-cultured with pig, monkey, or human cells. Another cluster contained samples from human CD3$^+$T cells cultured with monkey lysates or human lysates. Most surprising was the sample of human CD3$^+$T cells cultured with pig lysates as it did not cluster with other samples and most of the genes were expressed less than in the other samples, but few were uniquely upregulated. We performed PCA to project our samples in a 2D space based on gene expression profiles in different samples (Figure 2B).

Distinctly, gene expression in human CD3$^+$T cells cultured with pig lysate was quite different from all other samples as the pig lysate sample was solitarily located at the right edge of the PCA space.

Furthermore, differential gene expression analysis visualized by volcano plot in Figure 2C indicates that, as compared to stimulation with pig cells, pig lysate stimulation led to upregulation of interleukin (IL)-26, CXCL13, DUSP4, and TFRC and lacked the upregulation of genes such as GZMA, PRF1, CD8A, XBP1, and CX3CR1 seen in CD3$^+$T cells stimulated by pig cells.

In an effort to corroborate gene expression changes in human CD3$^+$T cells with functional analysis, we measured cytokine levels on Day 3 in human PBMC co-cultures with either pig or human cells (shown in Figure S3). By gene expression, IFNγ was upregulated in both human CD3$^+$T cells cultured with allogeneic or pig cells, while TNFα was downregulated in allo-stimulated CD3$^+$T cells but upregulated in pig-stimulated CD3$^+$T cells (Figure S3A). Congruently, while IFNγ cytokine levels were not
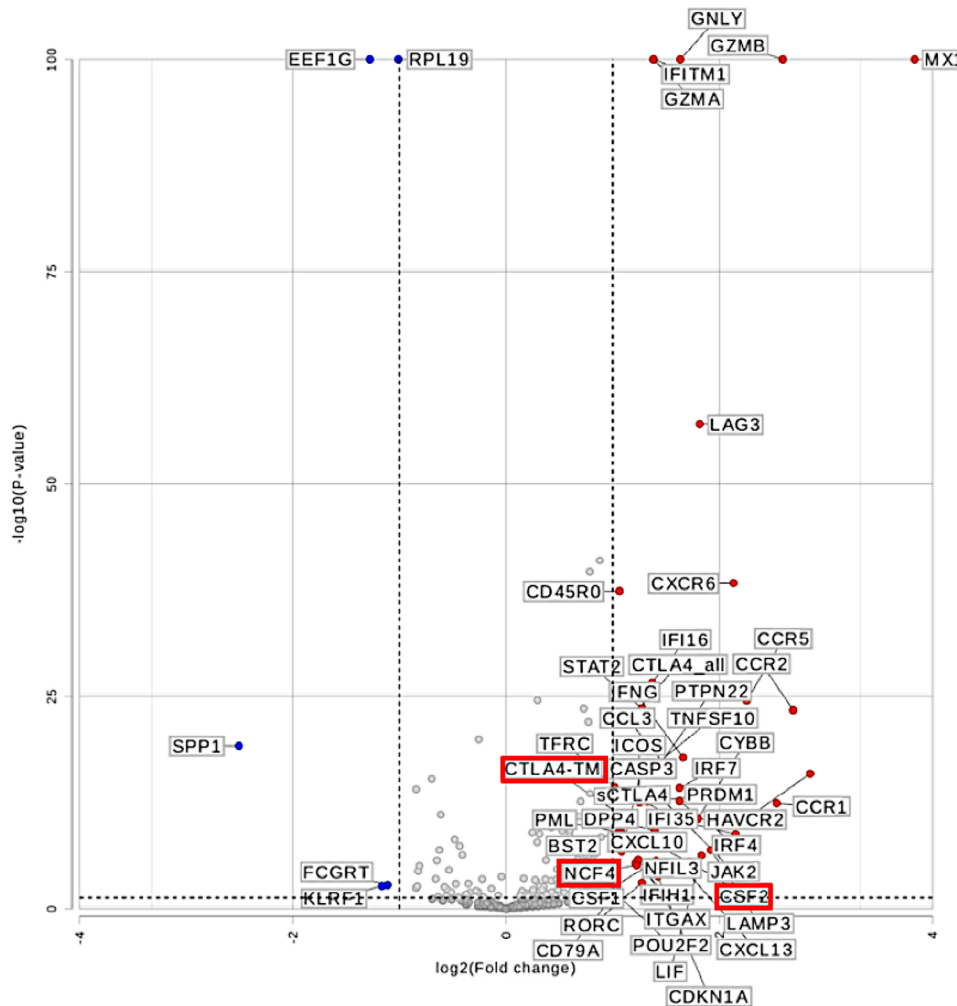
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significantly different, TNFα cytokine levels were significantly higher in the pig cell-stimulated PBMC sample (Figure S3B).

that are uniquely downregulated due to pig lysate stimulation include KLRK1, TCF7, CD48, HLA-C, CD45RA, CCR7, ITGA5, FCGR2A/B, CD8B, CEBPB, PECAM1, SLAMF7, and IL6R.

3.3 | Pig Cells and Pig Lysate Induce a Distinct Gene Expression in Human CD3$^+$T Cells

To test whether the gene expression changes in human CD3$^+$T cells are unique to stimulation with pig cells or lysates and not shared with allogeneic or LPS stimulation, we performed differential gene expression analysis as represented in the volcano plots in Figure 3. While using gene expression in human CD3$^+$T cells cultured alone as a universal control, genes that are differentially expressed in CD3$^+$T cells cultured with pig cells (Figure 3C) or lysate (Figure 3D) and stable (|fold change| <1.5) in allogeneic stimulation (Figure 3A) and LPS stimulation (Figure 3B) were pinpointed (outlined in red in Figures 3C and 3D). Both types of stimulation led to upregulation of CTLA4-TM and CSF2. However, pig cell stimulation uniquely led to upregulation of NCF4, while pig lysate stimulation led to unique upregulation of IL-26 and TUBB. As seen in Figure 3D, downregulation of many genes is witnessed in human T cells co-cultured with pig lysate. Those

4 | Discussion

Conventional immunosuppression remains the mainstay of standard-of-care therapy in clinical Tx, where T cell-directed immunosuppression has been successful in the prevention of allograft rejection. However, comparable immunosuppression fails to prevent xenograft rejection in experimental and clinical models. This observation suggests that xenogeneic immune responses may either be stronger or inherently distinct from allogeneic responses. Early studies have demonstrated that in vivo xenogeneic T cell responses are stronger than allogeneic T cell responses [18]. This discrepancy between in vitro and in vivo cellular responses to xenogeneic antigens remains unexplained.

In this study, human PBMC were cultured with human cells or pig cells. The proliferative response of human PBMC to pig cells was not stronger than that to human cells. However, HMGB1 release
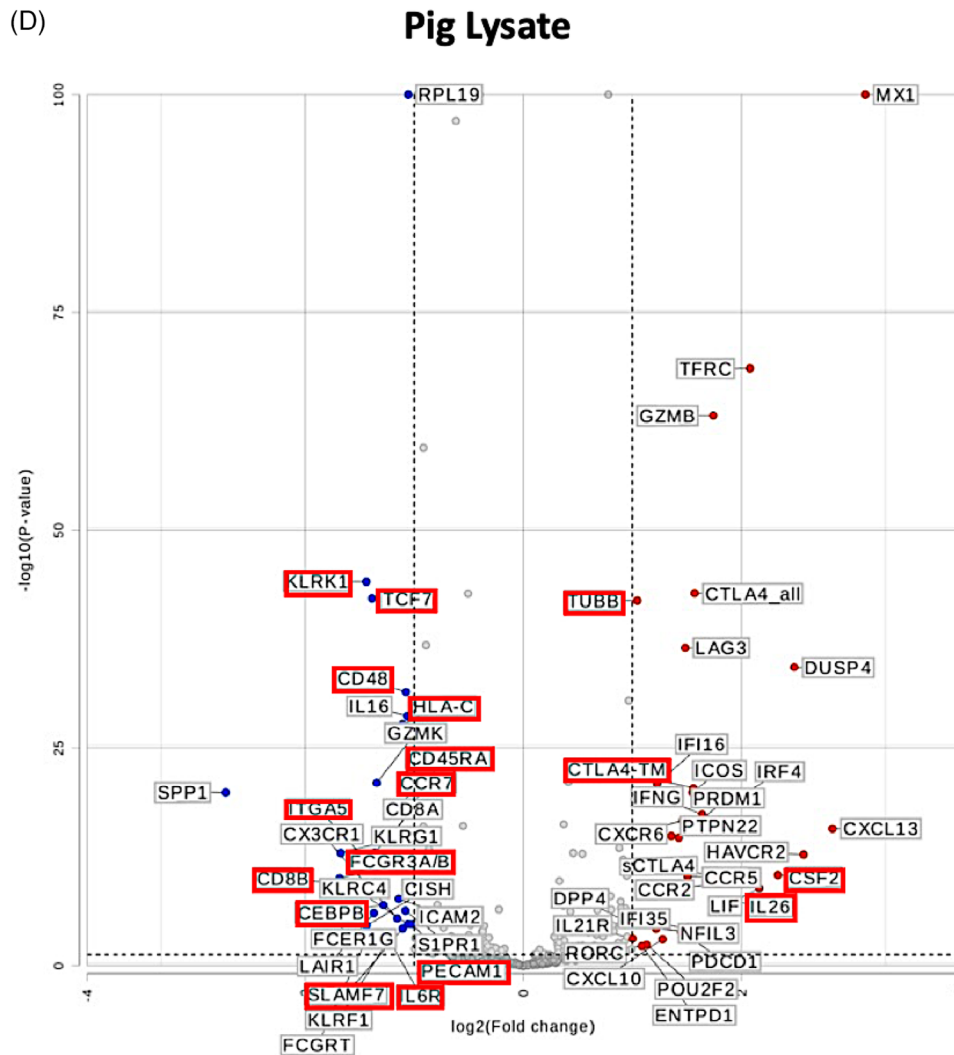
(D)

Pig Lysate

FIGURE 3 | (Continued)

was observed following human PBMC culture with pig cells, but not human cells, suggesting a pro-inflammatory response. HMGB1 is a critical mediator of inflammatory, immune and metabolic responses [19]. In vitro, human T cells induce significant HMGB1 release after co-culture with pig endothelial cells [17]. In vivo, neutralization of extracellular HMGB1 suppresses B cell activation and delays xenograft rejection [20].

Next, human PBMC were cultured with allogeneic human, "concordant" xenogeneic monkey, or "discordant" xenogeneic pig, intact cells or cell lysates. Flow-sorted CD3+T cells were analyzed for gene expression using NanoString, where utilizing direct detection avoids reverse transcription and amplification biases and is less prone for gene drop-out as compared to single cell methods, which ensured that our observations are unlikely to be due to technical artifacts.

Following culture with intact pig cells or pig cell lysate, distinct expression of certain genes was observed in human CD3+T cells. Uniquely, intact pig cell stimulation led to upregulation of *NCF4* (Neutrophil Cytosolic Factor 4), which plays a key role in inflammasome assembly and activation and further promotes effector T cell function [21]. Notably, inflammasome activation drives

macrophage expression of the procoagulant protein tissue factor and systemic activation of coagulation [22]. In NHP xenograft recipients, macrophages infiltrating pig xenografts express tissue factor [9, 23]. Also, the expression of *CSF2* (granulocyte-macrophage colony stimulating factor) which stimulates survival and differentiation of myeloid cells, and *CTLA4-TM* (membrane-bound cytotoxic T-lymphocyte protein 4) a key regulator of T cell activation, was upregulated in CD3+T cells, compared to stable expression (|fold change| <1.5) following allogeneic stimulation (Figure 3A). In parallel, TNFα cytokine levels were significantly higher following culture with the pig cells, than following human cells (Figure S3B). On the other hand, IL-26 gene expression was upregulated in response to pig cell lysate stimulation. Of note, IL-26 is a recently identified member of the IL-10 cytokine family [24] with pro-inflammatory [25, 26] and DNA-binding properties [27, 28]. This was associated with downregulation of *SLAMF7* (lymphocyte activation molecular family 7) a key suppressor of inflammation during sepsis [29]. Additionally, *TCF7* (T cell factor 1; TCF-1) expression was downregulated, where inflammation-induced TCF-1 downregulation is known to facilitate effector CD8+T cell differentiation [30]. Furthermore, concomitant downregulation of CD45RA and CCR7 expression suggests T cell differentiation towards terminally-differentiated

effector memory T cell phenotype. Interestingly, gene expression of *CEBPB*, *PECAM1*, and *IL6R*, which play key roles in the inflammatory responses, was also downregulated after stimulation with pig cell lysate.

In vitro "proliferative" human T cell anti-pig responses are no stronger than anti-human responses [31]. Lack of GAL (Galactose α1,3 Galactose) expression is associated with reduced human T cell proliferation in vitro [32]. However, these in vitro observations have not been mirrored with improved survival of GE GAL-knockout organs using standard-of-care immunosuppression [15]. While GAL-knockout pigs provide the backbone for donor pigs used in preclinical NHP studies and in the recent clinical xeno-Tx cases, combined with various genetic manipulations to prevent complement activation and dysregulation of coagulation [33], additional factors may promote immunosuppression-resistant xenograft rejection, such as, innate inflammatory responses [13].

Concomitant innate immune responses may play a role in augmenting xenogeneic T cell responses beyond their allogeneic counterparts [34, 35], which may preclude T cell regulation, as shown in allo-Tx models [36]. In the recent decedent human xenograft recipients, kidney xenografts demonstrated strong evidence of innate immune cell activation [37]. These observations were further supported in vitro [38] and in vivo [13]. In response to inflammation, innate immune cells upregulate tissue factor expression and promote activation of coagulation [39, 40]. Mutually, coagulation factors augment inflammatory responses [41–43]. In response to xenografts, in vivo reciprocal amplification of inflammation and activation of coagulation may further impede immune cell regulation, requiring higher levels of immunosuppression [44]. Hence, application of conventional immunosuppressive regimen may not be ideal for human xenograft recipients.

Certainly, the current study has some limitations. First, endothelial cells, the likely cell target after Tx were not used as stimulators in these experiments. We aimed to avoid any variability related to antigen expression due to different passages of endothelial cell obtained from wild type pigs versus commercially available human or monkey endothelial cells. Second, cells derived from GE pigs could provide further insight into the impact of various genetic modifications, particularly, transgenic expression of anti-inflammatory human genes. Third, these observations may not reflect the in vivo dynamics of T cell activation in human allograft versus xenograft recipients. Nonetheless, they demonstrate the nonconformity of T cell responses following allogeneic versus xenogeneic stimulation. Finally, xenogeneic cellular responses observed in this system may not be exclusively T cell-dependent, as other autologous immune cells, such as, monocytes, may have indirectly influenced human CD3$^+$T cell genetic profile in response to xenogeneic stimulation.

Collectively, data presented herein underscores the inflammatory responses by human immune cells following exposure to discordant xenogeneic pig antigens, but not allogeneic human antigens. These observations justify the monitoring of inflammatory markers and further therapeutic targeting of innate inflammatory responses following clinical xenotransplantation.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.