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Characterizing the Mechanistic Pathways of the Instant Blood-Mediated Inflammatory Reaction in Xenogeneic Neonatal Islet Cell Transplantation

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Introduction. The instant blood-mediated inflammatory reaction (IBMIR) causes major loss of islets after transplantation and consequently represents the initial barrier to survival of porcine neonatal islet cell clusters (NICC) after xenotransplantation.

Methods. This study used novel assays designed to characterize the various immunologic components responsible for xenogeneic IBMIR to identify initiators and investigate processes of IBMIR-associated coagulation, complement activation and neutrophil infiltration. The IBMIR was induced in vitro by exposing NICC to platelet-poor or platelet-rich human plasma or isolated neutrophils. **Results.** We found that xenogeneic IBMIR was characterized by rapid, platelet-independent thrombin generation, with addition of platelets both accelerating and exacerbating this response. Platelet-independent complement activation was observed as early as 30 minutes after NICC exposure to plasma. However, membrane attack complex formation was not observed in NICC histopathology sections until after 60 minutes. We demonstrated for the first time that NICC-mediated complement activation was necessary for neutrophil activation in the xenogeneic IBMIR setting. Finally, using the Seahorse extracellular flux analyzer, we identified substantial loss of islet function (up to 40%) after IBMIR with surviving NICC showing evidence of mitochondrial damage. **Conclusions.** This study used novel assays to describe multiple key pathways by which xenogeneic IBMIR causes islet destruction, allowing further refinement of future interventions aimed at resolving the issue of IBMIR in xenotransplantation.

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Islet xenotransplantation is a promising treatment of type-1 diabetes.¹ Unfortunately, substantial challenges to its clinical application remain, including cell-mediated rejection and the instant blood-mediated inflammatory reaction (IBMIR).²⁻⁶ As the name implies, IBMIR occurs immediately after exposure of islet grafts to recipients blood.⁵ In clinical islet allotransplantation, IBMIR is a major cause of tissue loss, commencing on exposure of islet cells to blood after infusion into the portal vein.^{7,8} It has been estimated that up to 60% of islets are lost within a week of transplantation.⁹

At its worst, IBMIR results in portal vein thrombosis, hepatic infarction, and portal hypertension.^{10,11}

After islets are exposed to human blood, IBMIR is initiated by activation of thrombosis and complement pathways. Islets express tissue factor (TF), which leads to thrombin activation, and inhibition of TF expression has been shown to suppress thrombin production and subsequent clot formation in vitro.^{7,12} Equally, inhibition of complement activation with complement inhibitors, such as compstatin,¹³ has been shown to inhibit IBMIR in vitro.^{14,15} In islet xenotransplantation,

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there are additional factors at play, such as the presence of preformed antipig antibodies,¹⁶ which in turn leads to amplification of complement activation via the classical pathway¹⁷; and an even greater role for the alternate pathway was discovered recently.¹⁸ There have been several strategies attempting to ameliorate IBMIR in islet xenotransplantation, including genetic modification of the islet to prevent these initiating events. Recently, we demonstrated that IBMIR induced by porcine neonatal islet cell cluster (NICC) was prevented in nonhuman primates (NHP) using NICC lacking the galactose- α 1,3-galactose (α -Gal) epitope and expressing human complement regulatory factors CD55 and CD59.¹⁹

Despite these advances, IBMIR remains a major hurdle for both islet allotransplantation and xenotransplantation. A clear understanding of the early initiating events is required to develop a rational therapeutic intervention. However, it is difficult to study IBMIR *in vivo* because of the complex interaction between thrombosis, the complement system, and the innate inflammatory pathways. Furthermore, differentiating the primary from the secondary effects of IBMIR also remains a significant challenge because of the simultaneous activation of immunological and coagulation pathways.^{7,20-22}

To better understand these important initiating events, we have developed an *in vitro* model of IBMIR where we separate and add the individual blood components to study their impact on the IBMIR response. Our objective was to find which aspects were crucial for initiation of IBMIR and to identify potential targets for therapeutic strategies; the primary aim being to develop a set of assays to investigate specific components of IBMIR after exposure of NICC to human blood. The second aim was to determine how each pathway interacts and contributes to clot formation, activation of complement, and recruitment of leukocytes to determine better means of ameliorating IBMIR. In addition, post-IBMIR NICC viability was evaluated by measuring metabolic capacity using extracellular flux (XF) parameters, to establish a novel means for determining functional capacity posttransplantation. We present a novel series of experiments separating the various immunologic components responsible for IBMIR to characterize the initiation of coagulation, complement activation and neutrophil infiltration, and to evaluate their impact on NICC function.

METHODS

Source of Neonatal Porcine Islet Cell Clusters

Outbred piglets were used, because the focus of this study was to induce the maximum IBMIR response *in vitro* to determine the contribution of each of its component in the unmodified response prior to testing individual genetic modifications. Neonatal islet cell clusters were isolated from 1- to 5-day-old piglets, and production of NICC was performed as described previously.²³ Procedures were approved by the local animal ethics committee and conducted in compliance with state and federal legislations.

Human Blood

Whole blood was collected from healthy volunteers into citrated or ethylenediaminetetraacetic acid tubes. Citrated blood was centrifuged for 10 minutes at 150g to obtain platelet-rich plasma (PRP), or 10 minutes at $2 \times 2000g$ to obtain platelet-poor plasma (PPP). The average concentration

of PRP ranged from ~ 300 to 500×10^9 cells/L. Neutrophils were purified from the ethylenediaminetetraacetic acid tubes using the EasySep Human Neutrophil Enrichment Kit (Stemcell Technologies, USA).

Thrombin Generation Assay

Thrombin generation assay of PRP or PPP was performed using an automated thrombogram with fluorometer (FLx800; BioTek Instruments, USA) and BIOTEK Gen-5 software. The PRP concentration used for the thrombin generation assay was determined by serial titration to be 6×10^9 cells/L. Because this was an *in vitro* assay, concentrations above this level resulted in spontaneous thrombin generation. The assay was performed as follows: 40 μ L PRP or PPP was pipetted into 96-well plates with 100, 250, 500, or 1000 islet equivalents (IEQ) of NICC in 40 μ L Hanks buffered salt solution (HBSS) without CaCl_2 with 5% human serum albumin (HSA) (Albumex 20), or 40 μ L HBSS/HSA control. Adding 50 μ L of a mixture of 1 mM fluorogenic substrate (Z-GGR-AMC) and 15 mM CaCl_2 (Technoclon, Austria)^{24,25} started the reaction, fluorescence was measured at 1-minute intervals at 37°C (ex. 390 nm, em. 460 nm).

Complement Activation and Soluble Membrane Attack Complex Detection

One thousand IEQ NICC in 500 μ L HBSS/HSA was added to 500 μ L PPP in a nontissue culture-treated 24-well plate (BD Biosciences, USA). Cells were incubated with 120 rotations per minute agitation for 30 or 60 minutes at 37°C. Complement activation and soluble membrane attack complex (sMAC) levels was measured in PPP samples using a cytometric bead array for human anaphylatoxin kit (BD Biosciences) or using enzyme-linked immunosorbent assay (BD OptEIA Human C5b-9) (BD Biosciences). Complement activation assays using freshly isolated PRP (~ 300 - 500×10^9 cells/L) did not induce significantly higher C3a, C4a or C5a after exposure to NICC compared with PPP; therefore, all subsequent assays of NICC-induced complement activation used PPP to simplify the interpretation of the findings.

MAC Deposition

After exposure to PPP, NICC pellets were washed 3 times with HBSS/HSA, then embedded in optimal cutting temperature (Tissue-Tek, USA). Tissue sections were stained with fluorescent antibodies for C5b-9 MAC (Dako, Denmark) (1:100) and glucagon (Dako) (1:200).

Neutrophil Activation

Neutrophil activation was determined by mixing freshly purified neutrophils (5×10^5 cells) with either NICC (1000 IEQ) alone, NICC and PPP (200 μ L), or NICC, PPP and 200 μ M Compstatin (Tocris Bioscience, UK). Complement-specific neutrophil activation was determined by mixing neutrophils with NICC alone or in combination with 0.8, 1.2, 2.0, 3.0, or 4.0 mg of rabbit complement sera (Sigma, USA). Neutrophil populations were gated by size and positive selection on CD45 and CD16 (BD Biosciences). Neutrophil activation was measured by median fluorescence intensity (MFI) of CD66b and CD11b (BD Biosciences). Change in the MFI of neutrophil-bound TF (Affinity Biologicals, Canada) indicated neutrophil expression of TF. Cumulative analysis on all neutrophils (CD45+ and CD16+) was performed, as well as independent analyses on single cells and doublets

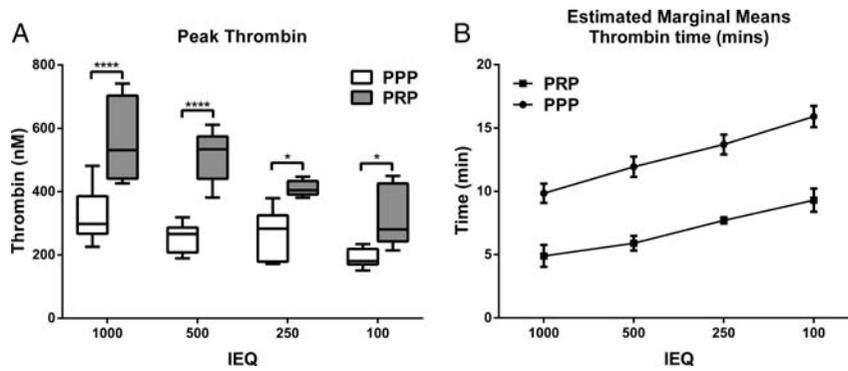


FIGURE 1. Platelets exacerbate NICC-induced thrombin generation. A, Peak thrombin concentration after incubation of NICC with PPP (white) or PRP (grey). Whiskers represent 5% to 95% spread of the data. B, Time (minute) required to reach maximum thrombin level after exposure of NICC to either PPP or PRP. Data represent mean \pm SEM of at 6 to 8 independent experiments. **** $P < 0.0001$, * $P < 0.01$.

(Figure 5). Neutrophil activation assay using freshly isolated PRP ($\sim 300\text{--}500 \times 10^9$ cells/L) did not induce higher levels of CD66b, CD11b, or TF, therefore all subsequent assays used only PPP to simplify the interpretation of the findings.

Extracellular Flux Assay

Extracellular flux analyses were performed using the XF24 Extracellular Flux Analyzer in 24-well islet plates (Seahorse Bioscience, USA). One thousand IEQ NICC in 500 μ L HBSS/HSA were mixed with 500 μ L PPP in a 24-well plate and agitated at 120 rotations per minute at 37°C for 1 hour. Control group consists of 1000 IEQ NICC in growth media. After incubation, NICC were washed twice with HBSS/HSA and resuspended in Modified XF media (Seahorse Bioscience, USA) (XF media, 0.1% fetal calf serum, 3 mM glucose). Neonatal islet cell clusters (200 IEQ per well) were rested in modified XF media in the islet plate for 1 hour at 37°C in a CO₂-free incubator. Extracellular flux analysis was performed as per Wikstrom et al⁴³ (Supplementary methods and Table S1, SDC, <http://links.lww.com/TXD/A25>). After XF analysis, NICC collected for DNA content analysis using Quant-iT PicoGreen dsDNA Assay (Life Technologies, USA).

Statistics

All analyses were performed using SPSS-22 (IBM Corp., USA). Two-sided tests with a 5% significance level ($P < 0.05$) were used throughout. General linear models were used to assess the joint effects of the factors on the outcome (Figure 1,

factors were NICC IEQ and PPP or NICC IEQ and PRP; outcomes were peak thrombin and thrombin time; Figure 2, factors were NICC and time; outcomes were concentrations of C3a, C4a and C5a; Figure 7, factors were NICC and complement concentrations; outcomes were MFI of CD66b, CD11b and TF; Table 1, factors were PPP or PRP; outcome was lag time). The following outcomes: peak thrombin; concentrations of C3a, C4a, C5a; MFI of CD66b, CD11b and TF, were log-transformed to approximate normality and to stabilize the variance before analysis. Parameter estimates from the fitted models were back-transformed to present the results, as fold-changes, using the original scale of measurements. Shapiro-Wilk tests were used to check that the residuals from the fitted-models satisfied the underlying assumption of normality in all cases. Paired Student *t* test was performed to determine statistical significance in Figure 4, Figure 8, and Table 2. Data in square brackets represent the 95% confidence interval, lower and upper limit, respectively.

RESULTS

Thrombin Generation in PPP or PRP After Exposure to NICC

To determine the role of platelets in the initiation of IBMIR, thrombin generation kinetics were measured after exposure of NICC to PPP or PRP (6×10^9 cells/mL). Addition of NICC was highly thrombogenic, as evidenced by a

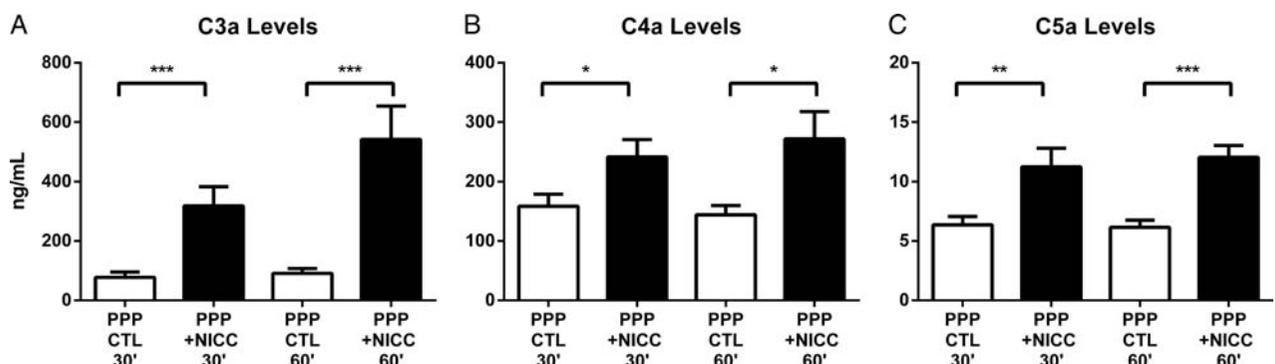


FIGURE 2. NICC induced complement activation even in absence of platelets. Complement breakdown products were measured in PPP in the presence (white bars) and absence (black bars) of 1000 IEQ of NICC, for 30 or 60 minutes at 37°C. A, Generation of complement C3a, (B) C4a generation, and (C) C5a generation. Data are cumulative means for 7 to 9 independent experiments expressed as average means of all experiments \pm SEM; results were expressed as concentration of each complement per mL of human plasma. *** $P < 0.0005$, ** $P < 0.01$, * $P < 0.05$.

TABLE 1.
Thrombin generation parameters after exposure of NICC to either PPP or PRP

Lag phase, min	1000 IEQ	500 IEQ	250 IEQ	100 IEQ
PPP	4.39 ± 1.12	5.27 ± 1.61	5.93 ± 1.66	7.56 ± 2.02
PRP	3.30 ± 0.45	3.43 ± 0.52	3.60 ± 0.55	4.30 ± 0.84
<i>P</i>	0.0539	0.0080	0.0019	0.0018

Data represent mean ± SEM of at 6-8 independent experiments.

very short thrombin lag phase (Table 1). Addition of platelets significantly accelerated thrombin lag phase. Thrombin peak time analyses demonstrated that maximum thrombin concentration was reached at 5.9 minutes (3.8, 8.0) faster in PRP compared with PPP ($P < 0.0005$), consistent over all NICC concentrations (Figure 1B). Thrombin peak level was increased significantly by a mean of 73% (52, 98) when platelets were added ($P < 0.0005$) (Figure 1A), indicating that platelets strongly contributed to the xenogeneic IBMIR response against NICC.

Complement Plays a Central Role in Xenogeneic IBMIR Against NICC

To determine the conditions necessary for complement activation after exposure of NICC to human blood, the formation of the complement fragments C3a, C4a, and C5a were measured after exposure of 1000 IEQ of NICC to PPP in vitro. Complement levels were increased significantly ($P < 0.0005$) in PPP when exposed to NICC (Figure 2) compared with control PPP not exposed to NICC at 30 and 60 minutes. Complement C3a (Figure 2A) levels were increased 4.41-fold (2.35, 7.44) at 30 minutes and 5.70-fold (3.16, 10.29) at 60 minutes after exposure of NICC to PPP. To a lesser degree, complement C4a (Figure 2B) concentration was also significantly increased ($P < 0.05$) at 30 minutes (1.52-fold [1.01, 2.28]) and at 60 minutes (1.75-fold [1.15, 2.66]) after exposure to NICC. Similarly, complement C5a (Figure 2C) levels were significantly increased both at the 30 minutes (2.08-fold [1.34, 3.22], $P < 0.01$) and 60 minutes (2.00-fold [1.5, 2.64], $P < 0.0005$) time points. The levels of C3a, C4a, and C5a were higher at 60 minutes than at 30 minutes, but did not increase significantly over the period.

Complement MAC Forms Around Islets Exposed to PPP

To determine whether NICC exposure to PPP leads to development of the C5b-9 complex, we measured C5b-9 production after 30- or 60-minute exposure to PPP, and by examining C5b-9 attachment to NICC by immunohistochemistry. At 60 minutes, C5b-9 formation (Figure 3A, red) was colocalized around glucagon staining cells (Figure 3A, green) within the NICC, but at earlier time points (ie, 30 minutes) C5b-9 was not identified on NICC (Figure 3C). In NICC, where C5b-9 was identified, islet morphology was degraded substantially with cellular surface destruction and fragmentation of NICC observed in all experiments. Inhibition of C3a formation, using 200 μM compstatin reduced C5b-9 formation (Figure 3B). Neonatal islet cell clusters not exposed to PPP showed no sign of nonspecific binding to the C5b-9 antibody (Figure 3D). Confirming these findings, soluble C5b-9 complex concentration in the PPP was increased significantly ($P < 0.001$) after 60-minute exposure to NICC when compared with controls (Figure 4).

NICC Induces Complement-Dependent Neutrophil Activation

Neutrophil activation measured by fluorescence-activated cell sorting determined surface expression of adhesion molecules CD66b and CD11b. Neither NICC alone nor PPP alone were able to induce neutrophil activation (Figure 5, black dotted line vs blue). Neutrophil activation was observed only when both PPP and NICC were added to the reaction (Figure 5, red). The average MFI was quantified and normalized against nonstimulated neutrophils. Both CD66b (1.64-fold [1.56, 1.84]) and CD11b (1.51-fold [1.56, 1.84]) were increased significantly ($P < 0.0005$) when neutrophils were exposed to NICC with PPP compared with neutrophils exposed to NICC alone (Figure 6A).

The single cell neutrophil population demonstrated a significant increase ($P < 0.0005$) in CD66b expression (1.55-fold [1.45, 1.66]) and CD11b expression (1.43-fold, [1.28, 1.59]) when exposed to NICC with PPP, compared to neutrophils exposed to either NICC alone or PPP alone (Figure 6C). Similarly, the doublet cell population (Figure 6B) also showed a significant increase ($P < 0.0005$) in CD66b (1.48-fold, [1.36, 1.61]) and CD11b (1.18-fold, [1.04, 1.34]) when exposed to NICC with PPP, compared with neutrophils exposed to either NICC alone or PPP alone.

Complement has been shown to play an important role in neutrophil activation and chemotaxis.²⁷ In our model, we tested if complement was responsible for neutrophil activation by using compstatin to inhibit complement activation. Compstatin significantly reduced ($P < 0.0005$) CD66b and CD11b expression on the surface of neutrophils in the presence of NICC and PPP (Figure 6A), indicating that complement was necessary for neutrophil activation. This was consistent both in the single cell (Figure 6C) and doublet (Figure 6B) populations. Interestingly, in some cases, compstatin reduced the level of neutrophil activation markers below basal levels. This may indicate a role for nonspecific complement activity in regulating neutrophil activation.

To test whether complement alone was sufficient to induce neutrophil activation, rabbit complement was added to the reaction, with or without NICC. After the addition of complement, the MFI of neutrophil activation markers (except CD66b) was not significantly different to those obtained when neutrophils were incubated in the absence of complement (Figure 7). However, when neutrophils were exposed to rabbit complement and 1000 IEQ NICC, there was a significant increase in neutrophil CD66b expression when compared with neutrophils exposed to complement in the absence of NICC ($P < 0.001$) (Figure 7A). CD11b

TABLE 2.
OCR analyses of NICC after 1-hour exposure to PPP

	Control	PPP	<i>P</i>
	pmol/min per μg DNA	pmol/min per μg DNA	
Glucose response	36.50 ± 6.86	26.25 ± 3.902	0.131
Coupling efficiency	91.50 ± 6.357	58.00 ± 3.189	0.004
Proton leak	37.50 ± 1.443	26.75 ± 3.224	0.049
Max capacity	173.8 ± 20.38	107.50 ± 8.109	0.023
Spare capacity	25.50 ± 10.33	12.25 ± 7.993	0.159

Data represent mean ± SEM of 4 independent experiments, each performed in 10 replicates.

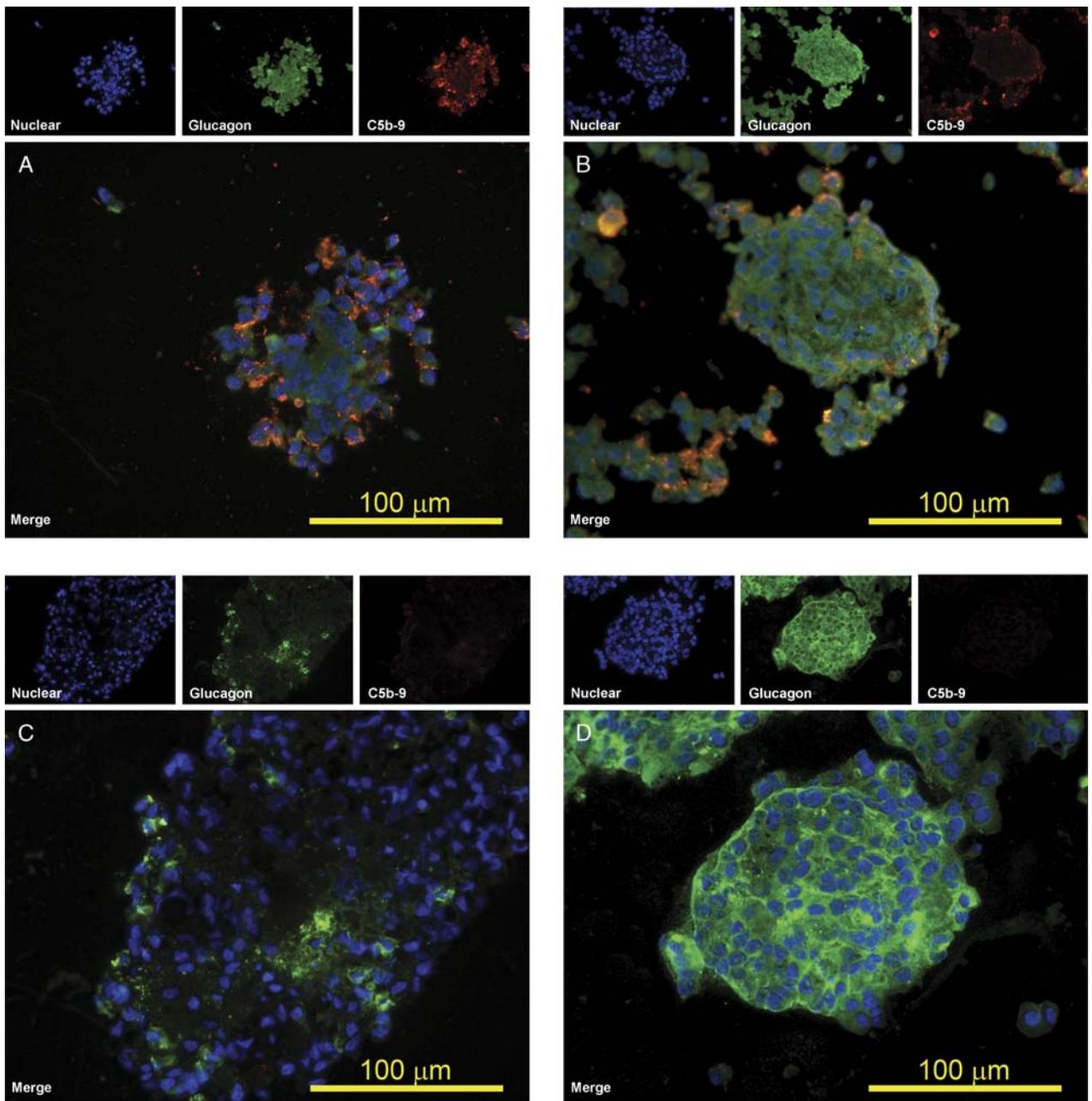


FIGURE 3. IBMIR induced C5b-9 (MAC) formation on NICC. Representative micrographs of NICC after treatment with PPP for 30 or 60 minutes: Immunofluorescence staining for C5b-9 (red), glucagon (green) and DAPI (blue). A, C5b-9 deposition on NICC after 60-minute exposure to PPP, showing diminished cellular structure; C5b-9 deposition was reduced when 200 μ M compstatin was included in the reaction (B). C5b-9 deposition was not detected after 30-minute exposure to PPP (C). D, Control NICC not exposed to PPP. Data represent of 6 to 9 different experiments.

expression on neutrophils also demonstrated a significant increase ($P < 0.001$) when exposed to 1000 IEQ NICC with rabbit complement sera at all complement concentrations compared with controls (Figure 7B).

TF Expression on Neutrophils

Surface TF expression on neutrophils was measured using fluorescence-activated cell sorting, after exposure to NICC with or without PPP. Tissue factor was increased ($P < 0.0005$) when neutrophils were exposed to NICC with PPP (1.22-fold [1.12, 1.33]) (Figure 6C). Treatment with compstatin reduced TF expression on NICC/PPP-activated neutrophils

down to basal levels. Both single cells (1.13-fold [1.07, 1.19]) and neutrophil doublets (1.56-fold [1.36, 1.65]) were found to have significantly increased ($P < 0.0005$) TF expression, after exposure to NICC and PPP, when compared with controls. Compstatin treatment significantly reduced TF expression ($P < 0.0005$) on both single cell and doublet neutrophils (Figures 6B and C). However, although TF expression on single neutrophils returned to baseline/untreated values after compstatin treatment, TF expression on doublets was only partially reduced and remained significantly higher compared with untreated neutrophils (1.25-fold [1.13, 1.37], $P < 0.0005$). Additionally, when neutrophils were exposed to purified rabbit complement sera and NICC; significant

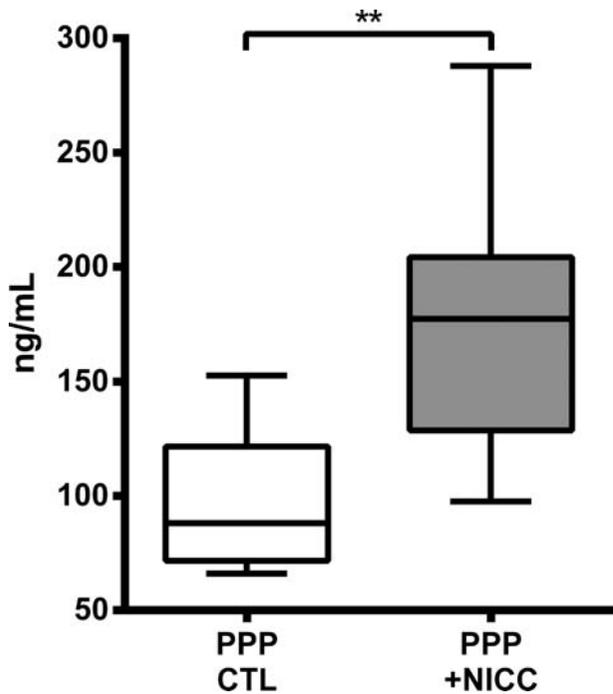


FIGURE 4. Soluble MAC was detected in plasma samples after IBMIR. The concentration of soluble C5b-9 in plasma supernatant was measured after 60-minute incubation of 1000 IEQ of NICC with PPP. Data represent 9 independent experiments. Whiskers represent 5% to 95% spread of the data. Labels of significance: ** $P < 0.01$.

increases in TF expression were achieved only at a high concentration of complement sera (3 mg, $P < 0.01$ and 4 mg, $P < 0.001$ (Figure 7C).

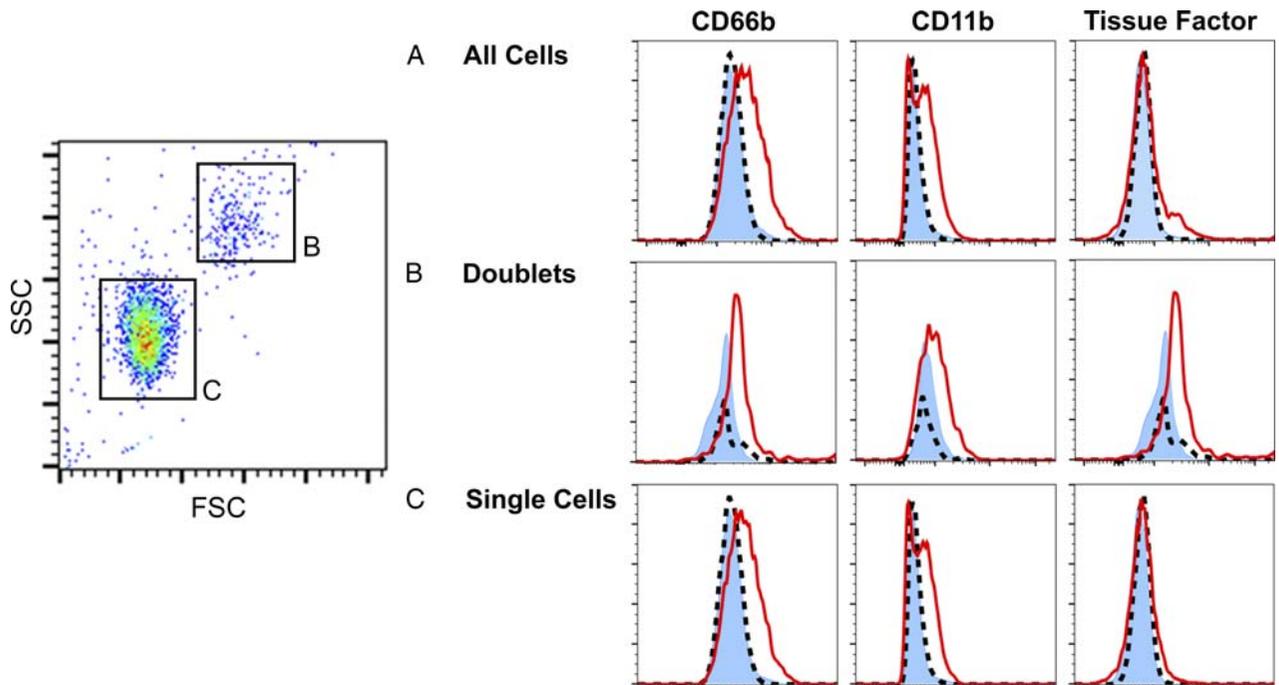


FIGURE 5. PPP is required for neutrophil activation during IBMIR in response to NICC. Representative scatterplot showing both single cells and doublet neutrophil populations. A, B and C, Representative histograms of surface CD66b, CD11b and tissue factor on neutrophils after 60 minutes incubation with or without PPP and NICC. Results are from: all cells (A), doublets (B) or single cells (C); conditions were: black dotted line, no fill—neutrophils alone, blue fill—neutrophils + NICC, red line, no fill—neutrophils + PPP + NICC. Neutrophil activation, as shown by increase in CD66b, CD11b, and TF was detected only when PPP was included in the reaction. Data are representative of 6 independent experiments (the data are represented as the fold-change compared to the MFI of the nonstimulated neutrophils).

IBMIR Induces Loss of Function in NICC Through Mitochondrial Damage

To determine the effect of IBMIR on NICC viability, the metabolic function of NICC was measured on an XF analyzer using several bioenergetic parameters associated with cellular functionality.²⁸ Extracellular flux analyses were performed on untreated NICC (control) or after exposure to PPP for 1 hour. We found that the overall respiration rate of NICC was reduced, compared with control, after PPP treatment (Figure 8A). In particular, the basal oxygen consumption rate (OCR) of PPP-treated NICC was significantly lower than that of control (98.25 pmol/min per μg DNA \pm 7.60 SEM vs 153 pmol/min per μg DNA \pm 2.78 SEM, $P < 0.005$). We observe that PPP-induced damage reduced basal NICC function by about 36% (Figure 8B), which was consistent with the degree of IBMIR-associated loss of function seen after transplantation.²⁹

Analyses of the cumulative XF data from 4 independent experiments demonstrated that PPP-treated NICC have significantly reduced coupling efficiency or oxidative phosphorylation ($P = 0.004$) as well as maximum capacity ($P = 0.022$), but a modest significant reduction in proton leak ($P = 0.049$) (Table 2). There was no significant difference in either glucose stimulated OCR or the spare capacity of the NICC.

DISCUSSION

In this study, we have developed a series of novel assays to separate the various immunologic components responsible for IBMIR to independently characterize IBMIR-associated pathways of coagulation, complement activation, and neutrophil infiltration. Although platelets strongly exacerbate

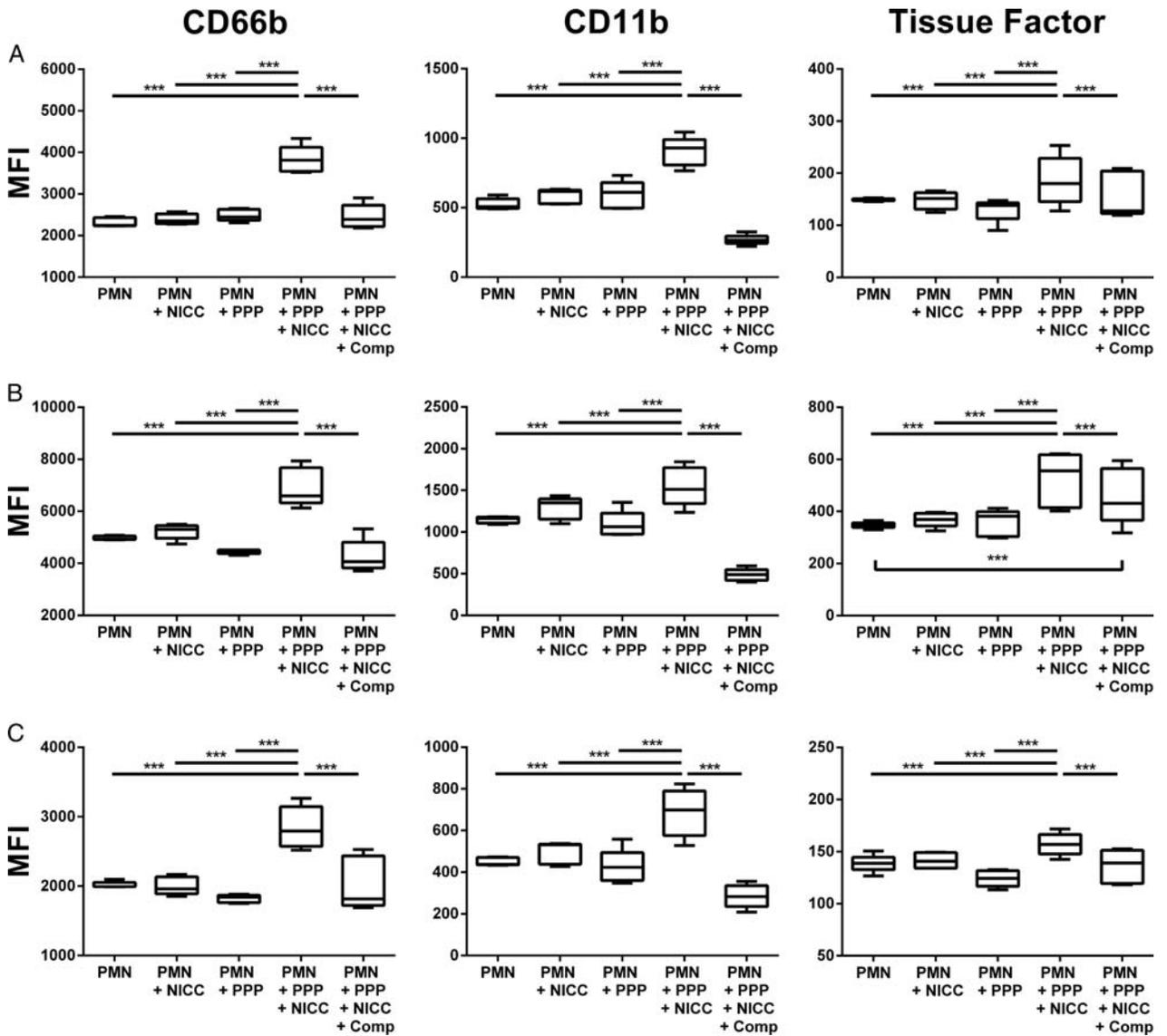


FIGURE 6. Neutrophil activation after exposure to NICC with PPP was inhibited by compstatin. Representative MFI quantification of neutrophil activation markers, CD66b, CD11b, and surface tissue factor after exposure to NICC with or without PPP and/or 200 μ M compstatin from (A) all cells, (B) doublet cells, or (C) single cells. Data are representative of 6 to 7 independent experiments. Whiskers represent 5% to 95% spread of the data. *** $P < 0.0005$.

coagulation, xenogeneic NICC induce thrombin generation even in the absence of platelets. Similar to other reports,^{15,18,30} complement activation was an important early

event in IBMIR. However, complement activation remained robust even in the absence of platelets. Additionally, we have described for the first time that complement was necessary

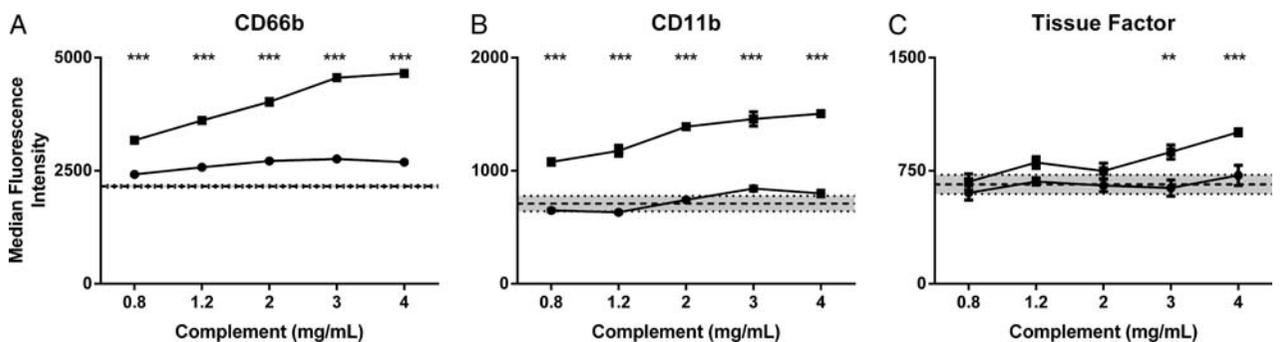


FIGURE 7. Complement is necessary for neutrophil activation during IBMIR. Mean fluorescence intensity of (A) CD66b, (B) CD11b, and (C) surface tissue factor from purified neutrophils (5×10^5) after exposure to rabbit complement sera at the indicated concentrations with (square) or without NICC (circle). Black line is the MFI of unstimulated neutrophils \pm SD in grey shaded area. Data are representative of 3 independent experiments performed in quadruplicates. Comparing squares to circles: *** $P < 0.001$, ** $P < 0.01$.

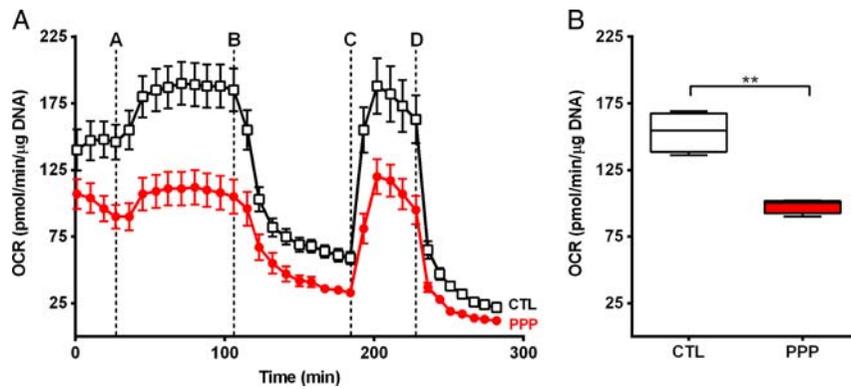


FIGURE 8. Extracellular Flux analyses demonstrated mitochondrial damage in NICC exposed to PPP. A, OCR data of NICC after exposure to PPP (red box) compared with untreated control NICC (white box). B, Cumulative average mean baseline OCR of control NICC versus PPP treated NICC. Data represent mean \pm SEM of 4 independent experiments, each performed in 10 replicates. Whiskers represent 5% to 95% spread of the data. ** $P < 0.01$.

for neutrophil activation in xenogeneic IBMIR against NICC. Despite this, NICC could induce TF release from neutrophils in a complement-independent manner. This activation of complement and neutrophils, even in the absence of platelets has now been clearly shown to lead to an estimated 40% loss of function in NICC, with severe mitochondrial damage seen in the surviving cells. This realization allows us to further focus our strategy of genetically manipulation of donor pigs.

Instant blood-mediated inflammatory reaction was first identified as a major cause of islet loss when islets were evaluated after exposure to human blood in an in vitro closed loop system.⁶ Thrombin generation and complement activation were identified as important initiating events and provided a platform for testing potential interventions.³¹⁻³⁵ Later studies focused on the importance of neutrophil activation and infiltration within islets and demonstrated that inhibition of platelet activation had additive protective effects over anticoagulation.³⁵ However, differentiating primary from secondary effects remained a challenge because of the simultaneous activation of several immunological and coagulation pathways.^{7,20-22}

In this study, NICC-mediated thrombin generation in the presence of platelets reached peak thrombin concentration within 5 minutes of NICC exposure. This was much faster than previously reported thrombin antithrombin (TAT) or factor VII-antithrombin formation from closed loop assays^{21,33} because TAT assays are measured by an enzyme-linked immunosorbent assay taken at a cross-sectional time point, whereas using a fluorometric assay, thrombin generation is measured as a continuous variable. Hence our analysis allows a “real-time” measurement of thrombin kinetics, which more closely models physiological responses.³⁶ This new information suggests that the rapid response should be accounted for when designing interventions targeting IBMIR.

Our analysis of the complement activation pathway demonstrated a robust C3a response, with modest C4a and C5a response in our xenogeneic model. Similar work performed in an allogeneic model (exposing human islets to human PPP) demonstrated a less severe complement activation response (data now shown), indicating that xenogeneic complement activation is more robust compare with that of the allogeneic response. Activation of both the classical and alternative pathways of complement has been implicated in the activation of IBMIR.¹⁷ Recently, Kang et al¹⁸ described the alternative

pathway as having a pivotal role in xenogeneic IBMIR. Our observation of strong complement C3a formation alongside modest C4a expression supports this finding, and a smaller albeit significant involvement of the classical pathway was probably the result of preformed antibody binding to NICC. Indeed, despite high Gal expression on NICC,³⁷ strong activation of the classical pathway was not observed in our model. Even when PRP was added, high levels of C4a expression were not seen (data not shown), indicating that absence of platelets was not the cause of modest activation of the classical pathway. Therefore, although anti- α -Gal antibodies activated the classical complement pathway, it remained less important than the alternative pathway activation.

Interaction of activated neutrophils with components of the coagulation pathway plays a pivotal role in the early loss of islet xenograft function both in vivo and in vitro models of IBMIR.^{20,35} Activated neutrophils have multiple effector mechanisms for destroying foreign cells including phagocytosis and secretion of oxidants and proteinases.²⁷ Neonatal islet cell cluster-mediated neutrophil activation occurs both in the presence and absence of platelets, but not when plasma was completely absent. By inhibiting complement activation in plasma using compstatin, we showed abrogation of neutrophil activation, demonstrating that NICC-mediated neutrophil activation was a complement-dependent mechanism. This was confirmed by activating neutrophils with NICC and purified rabbit complement sera, instead of human plasma. We believe this is not a xenospecific response as in preliminary studies a similar result was found when neutrophils were exposed to PPP and human islets (manuscript in preparation). The data presented here highlight the importance of early complement activation in the activation of neutrophils. Their activation and recruitment to islets represent an important target for prevention of IBMIR. In fact, the neutrophil activation levels presented here may be an underestimate because this study only detects free neutrophils and does not account for neutrophils potentially bound to NICC after activation.

Neutrophils producing TF have been reported to have enhanced procoagulant activity³⁸ and generate neutrophil extracellular traps, which further exacerbate thrombosis and sepsis.^{39,40} We reported previously that NICC-bound TF was important in initiating IBMIR.¹² Others have shown that platelets produce de novo TF,⁴¹ potentially exacerbating

IBMIR. In this study, neutrophils produced TF upon NICC exposure, with a significant increase in surface TFs after treatment with plasma and NICC. Similar to expression of CD66b and CD11b, neutrophil expression of TF was completely plasma-dependent and was not observed in the presence of NICC alone. However, complement inhibition with compstatin did not completely inhibit TF expression on neutrophil doublets, suggesting that although complement-mediated activation plays a major role in neutrophil TF expression, additional complement-independent pathways exist in IBMIR-associated neutrophil TF expression, particularly in the context of highly activated neutrophils of seen as aggregates in vitro.⁴² The observation that neutrophils express TF upon activation identifies an additional pathway for amplifying IBMIR and confirms the importance of neutrophil activation in the response.

Using the XF analyzer, the viability and function of NICC after IBMIR was evaluated. Approximately 40% of islet function was lost after plasma-induced IBMIR, corresponding to previous reports demonstrating a loss of up to two thirds of islets after transplantation.⁹ Although the OCR in response to high glucose was not significantly different between control and plasma-treated NICC, our data indicated that PPP-treated islets suffered from a severe loss of metabolic function, which manifested primarily as reduction of mitochondrial coupling efficiency (aerobic respiration) and proton leak, as well as reduction in maximum capacity. Recent studies suggest that, unlike other cells, islets have a naturally higher proton leak associated with insulin secretion.⁴³ As such, reduction in proton leak provides additional evidence that IBMIR adversely impacts islet function. These findings were similar to previous reports demonstrating decreased maximum capacity in adult pig islets after IBMIR.⁴⁴

By compartmentalizing the mechanisms of IBMIR into its distinct components, we have identified the importance of plasma-derived factors, such as complement, thrombin, and platelets, in xenogeneic NICC-induced IBMIR. Neonatal islet cell clusters express TF,¹² which results in thrombin production, which in turn initiates coagulation, a mechanism further amplified by platelets. Although thrombin production may be an important complement activator,⁴⁵ exposure of NICC to plasma leads to thrombin-independent complement activation (manuscript in preparation). Additionally, complement was a key driver of neutrophil activation, an important effector arm in NICC destruction. Furthermore, complement caused direct lysis of NICC via production of the C5b-9 complex. Once activated, this complex pathway was very difficult to stop, because several mechanisms exist that amplify the response. In particular, TF production was not only initiated by NICC¹² as well as platelets⁴¹ but was further amplified by neutrophils. Additionally, platelets can amplify thrombin production, which leads to multiple drivers of the clotting process.

Because of the complexity of IBMIR, it is likely that multiple therapeutic or genetic strategies targeting multiple pathways will be required to successfully inhibit it. Prevention of islet xenotransplant-associated IBMIR via genetic modification has advantages over therapeutic intervention as multiple pathways can be blocked without potential systemic side effects. We have previously shown that many of the in vivo effects of IBMIR were avoided when α -Gal^{-/-} CD55/59 transgenic NICC were transplanted into NHP.¹⁹ Other studies

have shown that expression of complement regulatory molecules on pig islets prolonged graft survival for up to 1 year posttransplant in NHP.⁴⁶ Addition of thrombomodulatory genes is also expected to increase protection against IBMIR. By investigating specific components of IBMIR induction in an independent manner, we have characterized multiple mechanisms by which IBMIR is initiated in a xenogeneic setting. Strategies directed at inhibiting IBMIR can therefore be further refined by targeting specific points in IBMIR induction to block multiple pathways, improving graft protection against IBMIR and prolonging graft survival and function in the xenogeneic setting.

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