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The differential effects of extracellular high mobility group box 1 on pancreatic beta cells as a conventional alarmin or as a potential beta cell protector

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또는 잠재적 보호 인자로서
미치는 영향

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A thesis of the Degree of Doctor of Philosophy

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ABSTRACT

Introduction: Type 1 diabetes (T1D) is a disorder of the endocrine system due to the loss of pancreatic beta cells by autoimmune attacks. T1D patients require lifetime treatment of exogenous insulin and are destined to suffer from many diabetic complications. Despite technological and medical advancements, T1D still remains incurable. However, since the success of the Edmonton Protocol in 2000, islet transplantation has been considered as one of the best options for the treatment of T1D, especially in patients with hypoglycemic unawareness and glycemic lability. Besides the allo-immune responses against the donor islets, the oxidative injury to the graft has been another big hurdle for the researchers and clinicians to overcome for successful transplantation. The injury to the islet graft generates what is known as damage-associated molecular patterns (DAMPs) which can elicit innate immune responses and can further destroy the graft. Among the DAMPs are high mobility group box 1 (HMGB1), a well-known, evolutionarily-conserved protein known for its close association with the islet graft damage. Many literatures to date point to the fact that HMGB1 is a highly

destructive molecule especially to the islet and beta cells. Notably, there have also been reports of HMGB1's protective actions in various tissues. Thus, more in-depth studies on the influences of HMGB1 on islet beta cells under various conditions are warranted.

Methods: For this study, sandwich ELISA was developed and optimized for murine HMGB1 detection in culture supernatant and murine serum. After the development, the assay was validated on cell culture supernatants and murine serum. Additionally, the assay was tested with human rHMGB1. Along the way, the FBS's consistent interference with ELISA signals and its implications were further investigated. Pre-existing HMGB1 from the FBS was removed by immunoprecipitation and its subsequent effects on the beta cell viability and function were assessed via modified MTT assay plus flow cytometry and glucose-stimulated insulin secretion assay, respectively. Also, rHMGB1 was re-added to the culture media and the cultured cells were tested for viability and function with the same methods. The effects of HMGB1 as alarmin on pancreatic beta cells were inspected using a small molecule inhibitor of HMGB1, inflachromene (ICM). Murine primary islets were isolated and

incubated with or without ICM treatment and then checked for HMGB1 secretion and viability via ELISA and modified MTT assay, respectively. *In vivo,* ICM was treated on STZ-induced diabetic mice and diabetic, syngeneic islet recipients. The impacts of systemic HMGB1 blockade were analyzed in the serum by ELISA and in the graft by immunohistochemistry. The survival of the pancreatic islet and the islet graft was monitored by the blood glucose level.

Results: Sandwich ELISA was developed with a matching pair of anti-HMGB1 antibodies. The ELISA was able to detect murine HMGB1 in cell culture supernatants and serum, but it was discovered that FBS had been influencing the assay's specificity and sensitivity. When primary islets and MIN6 cells were incubated with pre-existing HMGB1-depleted FBS, significant decreases in both the viability and function were observed. The re-addition of rHMGB1 reversed the effect. Nonetheless, an excessive amount of rHMGB1 or the treatment of rHMGB1 by itself could not rescue the viability of MIN6 cells. Treatment of MIN6 cells and murine primary islets with ICM significantly reduced the level of intracellular and extracellular HMGB1 *in vitro,* and the viability of murine islets also increased.

In vivo, ICM treatment significantly reduced the serum and peri－graft level of HMGB1, and the islet graft at 6 hours post－transplantation showed increased viability. Systemic ICM injection prolonged the survival of syngeneic islet grafts in diabetic mice. Moreover, ICM treatment delayed the experimental induction of hyperglycemia.

Conclusions: Previous studies on HMGB1 regarding type 1 diabetes and pancreatic islet transplantation have established the consensus that HMGB1 is undoubtedly harmful to the pancreatic beta cells and its blockade would naturally result in improvements in the survival of beta cells and islet grafts. In this study, I have also shown that a small molecule inhibitor of HMGB1 could provide the islet graft with a mass－sparing effect, presumably by reducing the HMGB1's function as an alarmin. Nevertheless, increasing evidence has indicated that the seemingly destructive HMGB1 could sometimes be beneficial to cells and tissues, and it was demonstrated in this study that a certain level of HMGB1 in the cell culture is required for optimal beta cell growth *in vitro*, even though the underlying mechanisms remain to be elucidated. Since HMGB1 could demonstrate highly diverse functions depending on its redox state, cellular location,

relative amount to other proteins, and etc., we must not assume that the effect of HMGB1 on pancreatic beta cells will be absolutely harmful, and I have indeed witnessed the beta cell-protective side of HMGB1 in this study. In the meantime, the function of cytosolic HMGB1 should be scrutinized to fully understand the role of HMGB1 on pancreatic beta cells.

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LIST OF ABBREVIATIONS

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

IHC: immunohistochemistry

IEQ: islet equivalent

STZ: streptozotocin

rHMGB1: recombinant high mobility group box 1

SEM: standard error of mean

qRT-PCR: quantitative real-time PCR

LPS: lipopolysaccharide

HRP: horseradish peroxidase

PBS: phosphate buffered saline

BSA: bovine serum albumin

OD: optical density

TNF: tumor necrosis factor

IL: interleukin

IFN: interferon

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPF: specific pathogen-free

H&E: hematoxylin and eosin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

GENERAL INTRODUCTION

T1D is a chronic autoimmune disease characterized by insulin deficiency and hyperglycemia (1). The symptoms occur due to pancreatic islet beta cell loss, the majority of which are caused by autoimmunity (type 1a) and the rest induced by unknown causes (type 1b) (2). By 2016, about 23 million adults in the U.S. have been diagnosed with diabetes and among them 1.3 million were T1D patients, which amounts to 0.55% of the adult population in the U.S (3). In addition, T1D among youths has also been causing serious clinical and health burdens in the U.S., and the incidence of T1D has been increasing worldwide (4).

It is well known that patients suffering from T1D need intensive treatments to delay the micro- and macro-vascular complications (5). Luckily, the development of novel treatment methods such as advanced insulin analogs, improved insulin pumps and blood glucose meters, continuous glucose-monitoring devices, and integrated sensor-augmented insulin pump systems have greatly helped the clinicians and patients (5), though they could only result in a substantial reduction in the incidences of

mortalities and non-fatal complications but not the complete cure of the disease (6).

Nevertheless, the landmark success of the clinical islet transplantation in 1999 by the Edmonton Group (7) and the later improvements in the efficacy and safety of the treatment have established islet transplantation as one of the best options for T1D patients with severe complications including hypoglycemia unawareness, hypoglycemic episodes, and glycemic liability (8-10). Still, many issues have to be addressed and taken care of in islet transplantation besides the life-long immunosuppressive therapy (11). One of the hurdles that clinicians and researchers have to conquer is hypoxia and the related oxidative injury to the islets which subsequently cause significant loss of islet graft in the early periods of transplantation (12). Unfortunately, because of the low oxygen tension of the implantation sites (13) and the low revascularization rate of islet grafts (14), hypoxic stress against islet grafts is inevitable after the transplantation procedure. It is well known that many DAMPs are released following hypoxic damage, and they again accelerate the destruction of transplanted islets (15). Particularly, hypoxic

injury to the islet graft results in the generation of an infamous DAMP and an alarmin, HMGB1 (16).

HMGB1 is a nuclear protein involved in the chromatin stabilization and transcription process, but it has also been discovered to act as a DAMP when released to the extracellular environment at times of immune activation or cell death (17), hence acquiring the title 'alarmin' (18). These proteins as DAMPs bind to their specific receptors such as toll-like receptors (TLRs) or the receptor for advanced glycation end products (RAGE) and activate the downstream signaling pathways that may elicit pro-inflammatory responses or cell migration (19). The mode of HMGB1's action is dictated by its redox state (17), where its oxidized form acts like a cytokine and its reduced form acts like a chemokine. It is noteworthy that HMGB1 frequently binds to various pathogen-associated molecular patterns and deliver or amplify their signals (20). Also, it should be kept in mind that HMGB1 is prone to post-translational modifications (PTMs), and these PTMs are indications of its active secretion to the extracellular environment (20).

HMGB1 has been studied extensively in the islet transplantation field because its expression in pancreatic islets was very high compared to other tissues and could be easily released (21), and it could destroy the islets directly by receptor engagement (22) or indirectly by triggering a cascade of innate immune responses (21, 23). However, recent reports in muscle and liver (24), and even in blood (25) have indicated that HMGB1 does not always act in a destructive manner. Moreover, the pancreas-protective function of the A-box domain of HMGB1 has been well-known (26), and recently Lee *et al.* reported that modified HMGB1 could be utilized in favor of islet grafts (27, 28). Thus, I believe further investigations on HMGB1's effect on pancreatic beta cells are required for the appropriate management of HMGB1 in islet transplantation settings.

CHAPTER 1

Development of a sandwich ELISA system

for the optimal detection of murine HMGB1

in culture supernatants and sera

INTRODUCTION

Ever since the discovery of the negative—correlation between the released HMGB1 levels and the islet graft survival (29), it has been deemed crucial that we thoroughly monitor and effectively control the secretion of HMGB1 pre— and post— transplantation. To date, many attempts have been made to detect HMGB1 in culture media or murine serum. I have tried using commercial ELISA kits to detect HMGB1 as others had done (30). To my disappointment, the inconsistent and undesirable results made it difficult to obtain accurate measurements of HMGB1 in both culture media and murine sera, as previously reported by another group (31). In addition, its high cost was another major drawback of using commercial kits.

Therefore, I have devised a sandwich ELISA method that can detect murine HMGB1 in culture supernatants and sera simply and cost—effectively. Along the way, I have examined the variables that can affect the detection of the target molecule in the sandwich ELISA system, which may have further physiological implications. The optimized protocol of this ELISA was used throughout my research on HMGB1.

MATERIALS AND METHODS

1. Sandwich ELISA basic protocol

Sandwich ELISA protocol for HMGB1 detection was devised based on a previous study (32). Nunc MaxiSorp™ plate (Thermo Fisher Scientific, Waltham, MA, USA) was first coated with 50-100 μl of an antibody (1 μg/ml) of a choice diluted in sterile PBS (pH 7.4). The plate was incubated at room temperature (RT) for 2 hours, and it was washed three times with 200 μl of 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in PBS (PBST) for 5 minutes. After blocking with 300 μl of 2% BSA (VWR, Radnor, PA, USA) in sterile PBS, 50-100 μl of the murine rHMGB1 standards (Cusabio Technology LLC, Houston, TX, USA) serially diluted in sample dilution buffer (0.5% BSA in PBST) and culture supernatant or serum samples were put in each well. Specifically, the culture supernatants and serum samples were diluted 1:1 and 1:4 respectively with sample dilution buffer. Particularly, in the case of culture supernatant ELISA, rHMGB1 standards were constituted in 1:1 solution of sample dilution buffer and the culture media. After overnight incubation at 4℃, the plate was washed three times with 200 μ

l PBST, and 50-100 μl of detection antibody (1 μg/ml) diluted in the sample dilution buffer were applied. The plates were washed three times with 200 μl PBST after a 2-hour incubation at RT, and it was incubated for 1 hour at RT with 50-100 μl of HRP-conjugated secondary antibody or streptavidin (1:2500) diluted in the sample dilution buffer. After a thorough six-time wash with 200 μl PBST, 50-100 μl of 3,3',5,5'-tetramethylbenzidine substrate (TMB; Sigma-Aldrich) were put in each well, and the plate was incubated at RT for 5 minutes or more. After sufficient color development, 50-100 μl of stop solution (0.2 M sulfuric acid) were applied to each well. The results were read at 450 nm with 540 nm as reference wavelength using Sunrise absorbance microplate reader (Tecan Life Sciences, Zurich, Switzerland). All the procedures were performed at RT unless otherwise stated.

2. Antibodies

Various commercial anti-HMGB1 monoclonal and polyclonal antibodies and HRP-conjugated antibodies (**Table 1-1**) were purchased and tested to select the best antibody pair.

Capture & Detection Antibodies

Provider	Reactivity	Clonality	Immunogen	Source
R&D Systems	human	monoclonal	E.coli−derived human rHMGB1/HMG−1. Gly2−Glu215	Mouse
R&D Systems	human, mouse	polyclonal	Mouse myeloma cell line NS0−derived human rHMGB1/HMG−1. Met1−Glu215	Chicken
Cusabio	human, mouse	polyclonal	Human rHMGB1 protein (8−179AA)	Rabbit
Chondrex	human, rat, mouse, bovine	monoclonal	Human HMGB1 peptide GKGDPKKORG (2−11)	Mouse
Chondrex	Bovine, human, rat, mouse	Monoclonal	Human HMGB1 peptide KPDAAKKGVV (166−176)	Mouse

HRP−conjugated Secondary Antibodies

Provider	Reactivity	Clonality	Immunogen	Source
Thermo Fisher Scientific	mouse	polyclonal	Gamma immunoglobins heavy and light chains	Goat
Thermo Fisher Scientific	chicken	polyclonal	Chicken IgY	Goat
Jackson ImmunoResearch	rabbit	polyclonal	whole molecule rabbit IgG	Goat
Thermo Fisher Scientific	Biotin	−	−	*S. avidinii*

Table 1-1. The list of antibodies used for sandwich ELISA development.

Each non-conjugated antibody was used either as capture or detection antibody with a conventional sandwich ELISA protocol (used at 1 μg/ml concentration) for the final, matching-antibody pair selection. The biotinylated monoclonal antibody was only used as the detection antibody. A total of 15 combinations were used as the capture-detection antibody pair and their signals were evaluated.

3. Sandwich ELISA grid experiment

Grid experiments were performed after determination of the four prospective capture-detection antibody pairs to determine their optimal concentrations for signal production. The 96 wells of ELISA plates were designated with various combinations of capture antibody, detection antibody, and rHMGB1 standards of different concentrations (**Table 1-2**). The rest of the sandwich ELISA protocol remained the same as described above.

4. Sandwich ELISA protocol optimization

Several experiments on the components other than the antibody pair were conducted for further refinement of ELISA. First, three sets of coating antibody incubation time and temperature were tested for better signal production: The coating antibody was incubated for either overnight at 4℃, 2 hours at RT (20-25℃) or 1 hour at 37℃, and five-fold dilutions of rHMGB1 were used to generate the standard curve. Next, two types of BSA solutions were tested as the sample dilution buffer: 0.5% BSA in PBS and 0.5% BSA in PBST were used in the ELISA as dilution buffers while all the other components remained identical to the basic protocol. The optimal concentration of secondary antibody was

1. Chicken IgY (poly, 1-215AA) capture & mouse IgG (mono, 2-215AA) detection											
	0.5 μg/ml Det						1.0 μg/ml Det					
	1	2	3	4	5	6	7	8	9	10	11	12
	0.5 μg/ml Cap		1.0 μg/ml Cap		2.0 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap		2.0 μg/ml Cap	
A	0	0	0	0	0	0	0	0	0	0	0	0
B	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1	1
C	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10	10
D	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100	100
E	0	0	0	0	0	0	0	0	0	0	0	0
F	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1	1
G	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10	10
H	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100	100
	2.0 μg/ml Det						4.0 μg/ml Det					

2. Rabbit IgG capture (poly, 2-11AA) & chicken IgY (poly, 1-215AA) detection											
	0.125 μg/ml Det						0.25 μg/ml Det					
	1	2	3	4	5	6	7	8	9	10	11	12
	0.25 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap		0.25 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap	
A	0	0	0	0	0	0	0	0	0	0	0	0
B	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1	1
C	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10	10
D	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100	100
E	0	0	0	0	0	0	0	0	0	0	0	0
F	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1	1
G	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10	10
H	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100	100
	0.5 μg/ml Det						1.0 μg/ml Det					

3. Rabbit IgG capture (poly, 8-179AA) & chicken IgY (poly, 1-215AA) detection											
	0.125 μg/ml Det						0.25 μg/ml Det					
	1	2	3	4	5	6	7	8	9	10	11	12
	0.25 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap		0.25 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap	
A	0	0	0	0	0	0	0	0	0	0	0	0
B	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1	1
C	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10	10
D	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100	100

E	0	0	0	0	0	0	0	0	0	0	0	0
F	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1
G	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10
H	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100
	0.5 μg/ml Det						1.0 μg/ml Det					

4. Mouse IgG (mono, 2−215AA) capture & chicken IgY (poly, 1−215AA) detection											
	0.125 μg/ml Det						0.25 μg/ml Det					
	1	2	3	4	5	6	7	8	9	10	11	12
	0.5 μg/ml Cap		1.0 μg/ml Cap		2.0 μg/ml Cap		0.5 μg/ml Cap		1.0 μg/ml Cap		2.0 μg/ml Cap	
A	0	0	0	0	0	0	0	0	0	0	0	0
B	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1
C	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10
D	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100
E	0	0	0	0	0	0	0	0	0	0	0	0
F	1 ng/ml Stn	1	1	1	1	1	1	1	1	1	1
G	10 ng/ml Stn	10	10	10	10	10	10	10	10	10	10
H	100 ng/ml Stn	100	100	100	100	100	100	100	100	100	100
	0.5 μg/ml Det						1.0 μg/ml Det					

Table 1−2. The grid design for grid experiments.

To assess the optimal concentration of both capture and detection antibodies of the four combinations, grid experiments were performed on 96−well plates. Each plate could generate 12 standard curves with differing concentrations of capture and detection antibodies. The standard curves were later evaluated by the modified ROC dots. Det, detection; Cap, capture; Stn, standard

also a subject under scrutiny: After the routine sandwich ELISA procedure, 1:2500, 1:5000, and 1:10000 dilutions of HRP-conjugated anti-chicken IgY were applied on the ELISA plate, and the plate was incubated for 30 minutes at RT ready for color development. Lastly, rHMGB1-containing RPMI 1640 media (GE Healthcare Life Sciences, Logan, UT, USA) were mixed 1:1 with PBST immediately before the sample incubation step, and the remaining ELISA procedures were performed in the same fashion. All the resultant signals of above experiments were checked for specificity and sensitivity.

5. ELISA specificity and sensitivity assessment

To better assess the specificity and sensitivity of ELISA signals after grid experiments and signal optimization, I performed a process which was a modified version of receiver operating characteristic (ROC) curve generation. Briefly, each known concentration of rHMGB1 served as cut-off points, absorbance / rHMGB1 concentration served as the sensitivity, and 1 / background absorbance (OD at 0 ng/ml of rHMGB1) served as the specificity. For the sake of comparison, the sensitivity and specificity were all normalized to the highest and lowest

absorbance, respectively, for a given cut-off point. Plotted dots, instead of curves, were evaluated with reference to their relative positions on the graph. Dots closer to the coordinate (0,1) were referred to as more optimal experiment conditions.

6. Animal experiments

Female BALB/cAnHsd (BALB/c) and C57BL/6N (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed in the Institute for Experimental Animals at Seoul National University (SNU) College of Medicine. Murine islet isolation and syngeneic islet transplantation were performed as previously described (33), but BALB/c mice of 8-10 weeks were used instead as both donor and recipient. Isolated islets were sustained in RPMI 1640 media supplemented with 10% FBS (Young In Frontier, Seoul, Korea) and Antibiotic-Antimycotic (Anti-Anti; Thermo Fisher Scientific) in a 37°C, 5% CO_2 incubator. STZ (150 mg/kg; Sigma-Aldrich) injection for two consecutive days was carried out in B6 mice to measure their serum HMGB1 levels after gradual pancreatic beta cell destruction. Blood was collected from isoflurane-anesthetized mice by retro-orbital bleeding. For incubation experiments with

cytokine cocktail, the cells were seeded at 300 IEQs/well in 24-well plates (Corning, Durham, NC, USA) with recombinant murine TNF-α, IL-1β, and IFN-γ (all at 20 ng/ml; Biolegend, San Diego, CA, USA). All the animal experiments were approved by the SNU Institutional Animal Care and Use Committee (IACUC; IACUC no. SNU-170518-3-2).

7. Human HMGB1 detection by sandwich ELISA

For testing the efficiency of human HMGB1 detection by my ELISA, HMG-1 (Sigma-Aldrich) was serially diluted in the sample dilution buffer, CMRL 1066 (Corning) supplemented with 10% FBS, or 1:1 mixture of sample dilution buffer and 10% FBS-supplemented CMRL 1066, and the rest of the protocol was carried out.

8. Statistical analyses

All statistical analyses were performed using Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA). Values were expressed as mean ± SEM. Differences between groups were compared by unpaired t-test.

RESULTS

Selection of candidate antibody pairs for sandwich ELISA

Fifteen combinations of capture-detection antibodies were tested by being incorporated into standard sandwich ELISA for the detection of murine rHMGB1 in the range of 0-1000 ng/ml (**Figure 1-1**). Four out of the fifteen pairs of antibodies were selected based on their signal sensitivity and linearity. Since the antibodies' concentrations were fixed to 1 μg/ml regardless of their clonality, and the specificity and sensitivity of the signals could be improved, I next proceeded to the grid experiments.

Grid experiments with candidate antibody pairs

Grid experiments were conducted with four of the antibody pairs chosen previously to determine the optimal concentrations of capture and detection antibodies (**Table 1-2**). It could be observed that the capture antibody significantly dictated the ELISA signals in general (**Figure 1-2A**). The results of grid experiments were assessed based on their signal sensitivity and specificity through modified ROC dot generation (**Figure 1-2B**). The sensitivity and specificity of these antibodies were specified
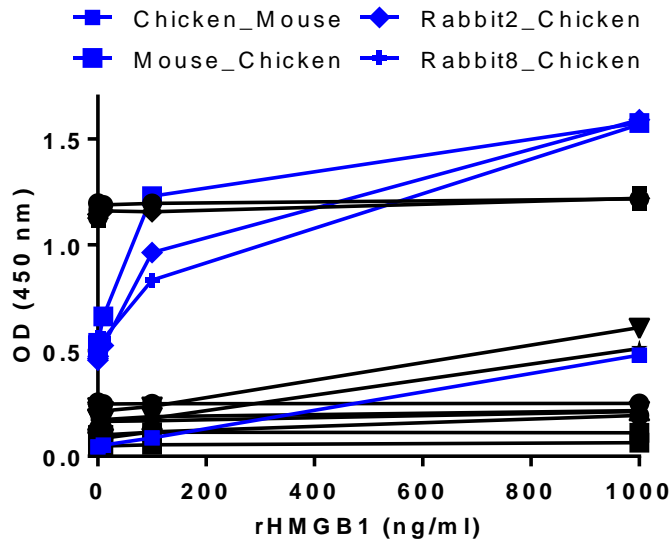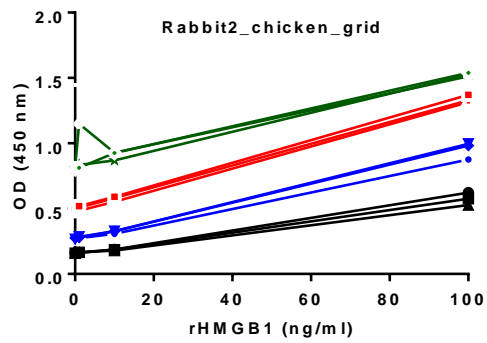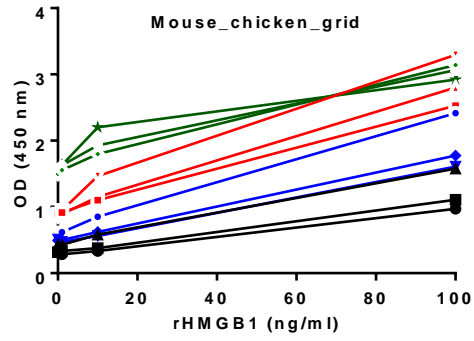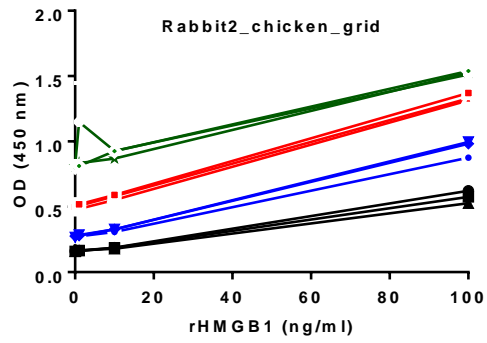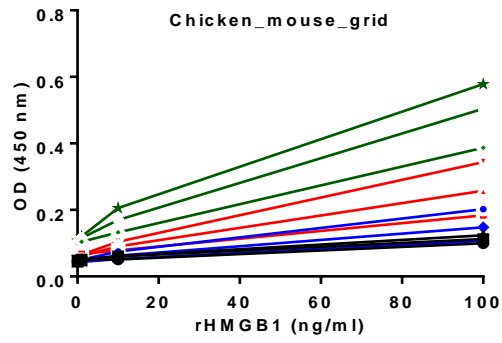
Figure 1-1. Antibody pair selection for the grid experiments.

Fifteen pairs of antibody combinations were tested for sandwich ELISA signal generation of murine rHMGB1 at 0-1000 ng/ml range at 1 μg/ml concentration. The four standard curves in blue color represent the selected antibody pairs based on signal linearity.
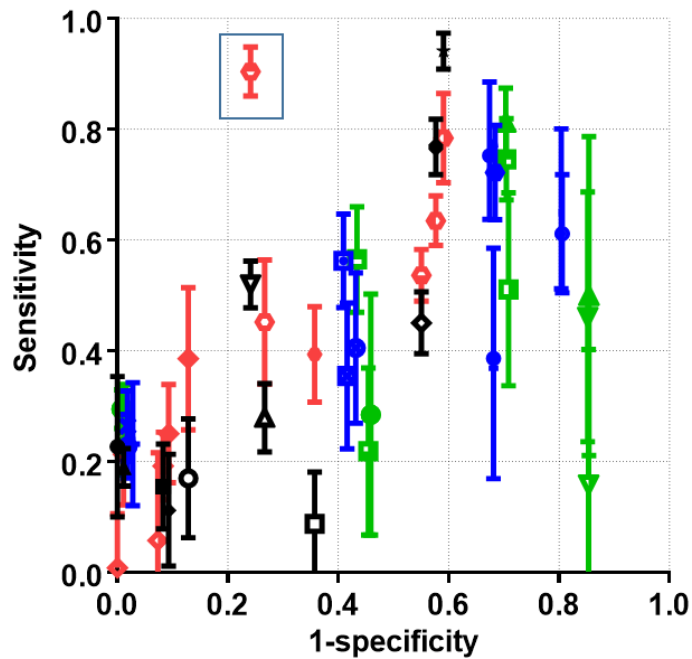
A

B

Figure 1-2. Evaluation of sandwich ELISA grid experiment signals by modified ROC dots.

(A) Grid experiment results of the four candidate antibody pairs are shown. Each color represents a set of combinations with the same concentration of detection antibody. (B) Modified ROC dots were constructed based on the sensitivity and specificity of the grid experiments. Each color represents a specific set of capture and detection antibody, and each point represents a set of specific concentrations of the antibody pair.

according to the standard that I established based on the relative ELISA signals among the combinations. I assumed that the background signal at 0 ng/ml of rHMGB1 is inversely correlated with the specificity, which meant that the one with the lowest background noise at zero concentration had the highest specificity for murine HMGB1. Also, the sensitivity of the antibody pair was determined by the slope of the standard curve that was constructed based on HMGB1 concentration on the x-axis and its absorbance on the y-axis. Ultimately, the combination showing the highest relative specificity and sensitivity with the lowest concentrations was selected: mouse anti-HMGB1 IgG (MAB1690) at 2.0 μg/ml as capture antibody and chicken anti-HMGB1 IgY (MAF1690) at 0.5 μg/ml as detection antibody.
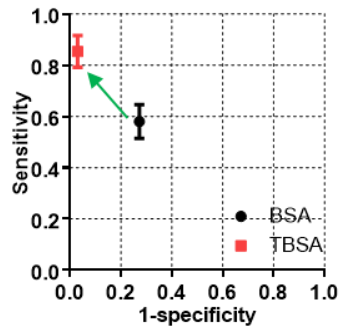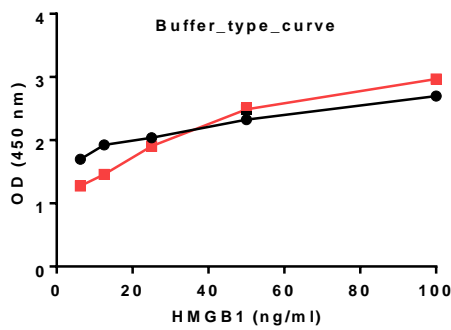

Optimization of sandwich ELISA signals

Several other factors that could affect the specificity and sensitivity of detected HMGB1 signal were scrutinized. The effect of different ambient temperatures during capture antibody incubation on the HMGB1 signal was tested as shown in **Figure 1-3A**. The standard curve and also the modified ROC dots
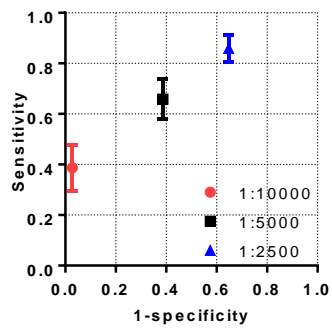
A

B

C

D

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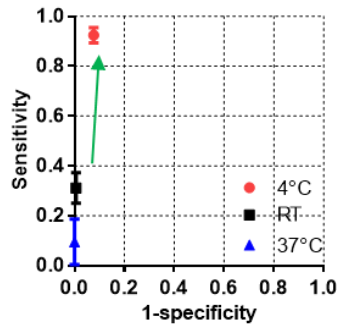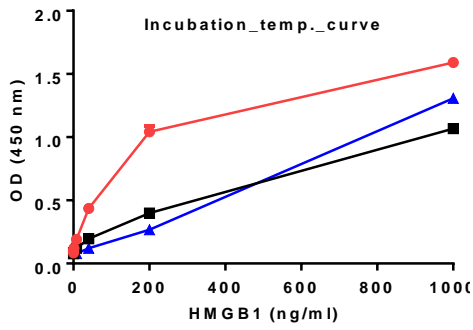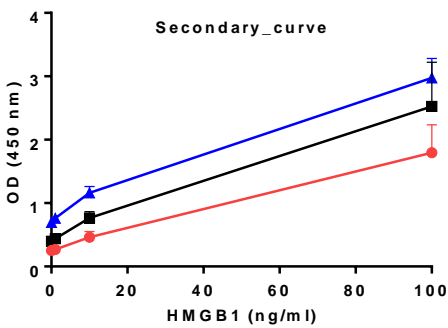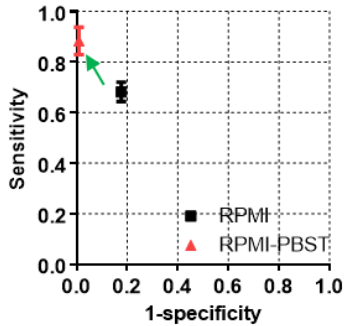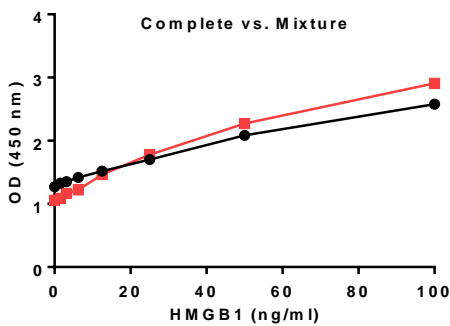
Figure 1-3. Signal optimization for the sandwich ELISA

(A) Different temperature conditions for the incubation of capture antibody were tested and analyzed with modified ROC dots. Green arrow denotes the increase in sensitivity and specificity. The reaction volume was 50 μl. (B) Two types of sample dilution buffers were tested and analyzed with modified ROC dots. Green arrow denotes the increase in sensitivity and specificity. The reaction volume was 100 μl. BSA, 0.5% BSA in PBS; TBSA, 0.5% BSA in PBST (C) Different concentrations of the HRP-conjugated secondary antibody were tested and analyzed with modified ROC dots. The reaction volume was 100 μl. (D) Murine rHMGB1 signals in 10% FBS-supplemented cell culture media and 1:1 mixture of sample dilution buffer and 10% FBS-supplemented cell culture media. The standard curves were analyzed with modified ROC dots. Green arrow denotes the increase in sensitivity and specificity. The reaction volume was 100 μl.

showed that the specificities were similar in the three groups (overnight incubation at 4℃, 2 hours at RT, or 1 hour at 37℃), but overnight incubation at 4℃ resulted in the highest sensitivity. Two types of sample dilution buffer were also tested for the specificity and sensitivity of the generated HMGB1 signals (**Figure 1-3B**). The group which used 0.5% BSA in PBST as the sample dilution buffer showed higher specificity and sensitivity than 0.5% BSA in PBS as shown by the modified ROC dots. The concentrations of secondary antibody was another variable that could affect the detection of HMGB1 signals (**Figure 1-3C**). As the concentration of the secondary antibody increased, the specificity illustrated by each of the standard curve decreased while the sensitivity increased. Because minimizing the amount of antibody being used was also the goal of this study, 1:10000 dilution of the secondary antibody seemed most appropriate. Lastly, since the standard curves generated with rHMGB1 in the cell culture media seemed to show decreased specificity and sensitivity, the cell culture medium was diluted 1:1 with the sample dilution buffer, and the specificity and sensitivity were rescued to some extent (**Figure 1-3D**).

ELISA experiments with culture supernatants and murine sera

After completion of the optimization process, sandwich ELISA was performed with actual islet cell culture supernatants and sera from STZ-injected mice and murine syngeneic islet recipients (**Figure 1-4**). First, I conducted a well-known islet incubation experiment (21), where the treatment with harmful cytokine cocktail would cause islet cell death and subsequently increase the level of HMGB1 within the culture supernatant (**Figure 1-4A**). I could detect a significantly higher amount of HMGB1 in cytokine cocktail-treated islet culture supernatants. Next, I performed murine syngeneic islet transplantation to diabetic BALB/c mice and collected their serum at 0, 6, 24, and 48 hours post-transplantation. I observed an increase in HMGB1 signal in the murine sera at 6 and 48 hours post-transplantation (**Figure 1-4B**), a phenomenon similar to the existing reference (21). I also observed a gradual increase in serum HMGB1 signal in B6 mice injected with STZ (**Figure 1-4C**), which should coincide with the gradual destruction of pancreatic beta cells by the drug.

In addition, ELISA was also performed with human rHMGB1 protein, as to explore the possibility whether this

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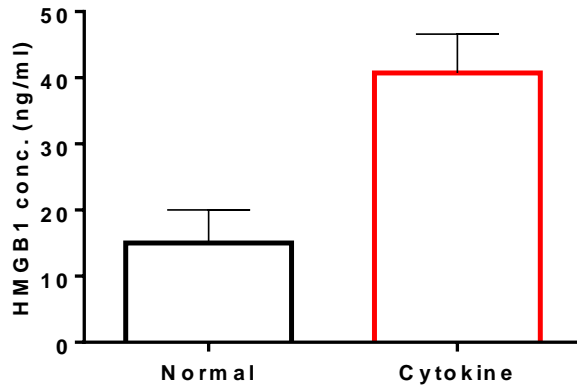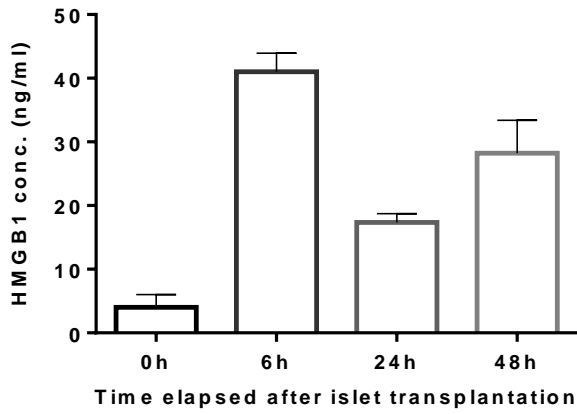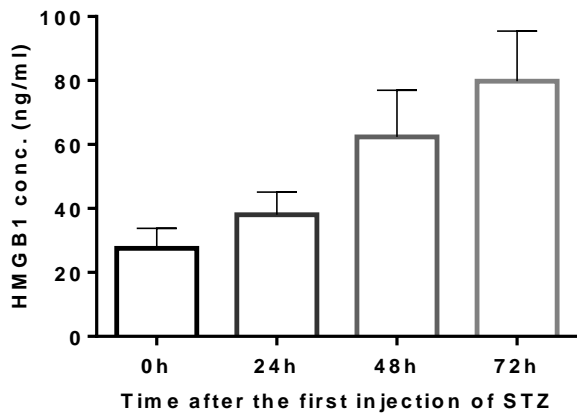
Figure 1-4. Sandwich ELISA experiment results with primary islet culture supernatant and murine sera.

(A) HMGB1 level in the culture supernatants of murine islets with or without the cytokine cocktail (20 ng/ml of recombinant murine TNF-α, IL-1β, and IFN-γ). ***, p<0.001. (B) HMGB1 level in the sera of murine syngeneic islet recipients (diabetic BALB/c mice; n=4) at different time points post-transplantation. The syngeneic islets (300 IEQs) were transplanted under the left kidney capsule of the recipients. Oh denotes the time of the surgical procedure. (C) HMGB1 level in the serum of B6 mice (n=5) injected with STZ (150 mg/kg) at 0- and 24-hour. Oh denotes the time of the drug injection.

sandwich ELISA system could be utilized in the clinics (**Figure 1-5**). Fortunately, I could detect human rHMGB1 in sample dilution buffer with acceptable sensitivity and specificity. The human rHMGB1 signal in conventional human islet culture media (CMRL 1066) decreased in both specificity and sensitivity, and as opposed to the murine rHMGB1 signal it was not rescued when diluted 1:1 with the sample dilution buffer.
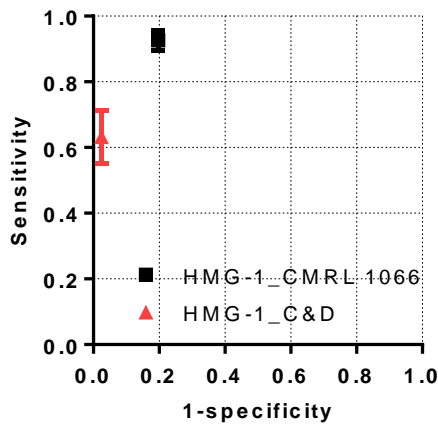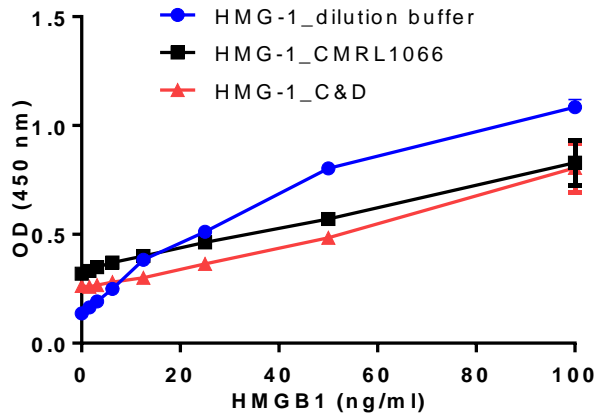
Figure 1-5. Sandwich ELISA experiment results with recombinant human HMGB1 protein.

The detection of human rHMGB1 in the sample dilution buffer, (Top) CMRL1066 media and 1:1 mixture of sample dilution buffer and CMRL1066 was assessed by the developed sandwich ELISA. (Bottom) Modified ROC dots showing the degree of ELISA signal sensitivity and specificity were generated.

DISCUSSION

Here, I have shown the history of designing a sandwich ELISA system which could detect murine HMGB1 in islet cell culture media and sera. Identification of the best matching pair of antibodies and their concentrations was the key step in this development. It was noteworthy that the discrepancy of immune-specificity of the antibodies did not guarantee higher ELISA signals (**Figure 1-1 & 1-2**). Additional optimization process refined my ELISA system's sensitivity and specificity (**Figure 1-3**). Due to its inherent characteristics, such as the nonspecific binding to other proteins (34), I presumed it would be difficult to quantify HMGB1. Although my current sandwich ELISA protocol was successful in detecting the physiological range of HMGB1 (**Figure 1-4**), further adjustments could be made to improve the sensitivity and specificity, such as pretreatment of samples with perchloric acid (31). Moreover, if possible, I want to develop a redox state-specific HMGB1 assay system which would be of great use when studying the function of the detected HMGB1.

  The aim of this study was to develop an ELISA that could

be easily implemented in various murine islet transplantation−related *in vitro* and *in vivo* experiments. Nevertheless, the development process would be more worthwhile if this assay was applicable to HMGB1 from larger animals, namely humans and pigs. It is especially important to check the level of HMGB1 in islet transplant patients, because its level has been known to inversely correlate with the transplantation outcomes (35). Fortunately, I was able to detect human rHMGB1 in standard dilution buffer and human islet culture media CMRL 1066 with comparable sensitivity and specificity (**Figure 1−5**). It would be my next task to test the efficacy of my ELISA with human sera in the future. Moreover, although relatively little is known about the role of porcine HMGB1 in pig−to−mouse or pig−to−NHP islet xenotranplantation, it would be comparably important given the conservedness of HMGB1 (36). Thus, my ELISA could be utilized in porcine islet xenotransplantation−related studies once its competence is approved.

It could have been especially difficult to detect this protein in murine serum since there might be components that directly mask HMGB1 from detection via conventional assays (37). However, it was notable in my results that supplementation

of the culture media with FBS generated high background signals and hindered specific and sensitive detection of HMGB1 in the culture media, possibly because FBS naturally contains HMGB1 (**Figure 1-6**). Accordingly, I added a modification to the protocol to lower the background signal and potential signal interference by the FBS, which was effective as shown by the modified ROC dots (**Figure 1-3D**). Still, the fact that HMGB1 is detected in complete cell culture media raises concerns that the pre-existing HMGB1 could actually affect the cultured murine, porcine, or human cells as murine HMGB1, porcine HMGB1, or human HMGB1 would do, respectively.

I believe there are a few noteworthy implications to this notion: 1) the 'background ELISA signals' we had always gotten with FBS might have had 'background effects' to the cultured cells, 2) the basal level of HMGB1 may have influenced cultured cells all along, and 3) the knowledge and insights we had gathered via *in vitro* experiments could have had HMGB1's hidden effect to it. It would be meaningful to scrutinize this issue to unveil the pre-existing HMGB1's effect on cultured cells, and more importantly to question our general assumptions on common *in vitro* experiments.

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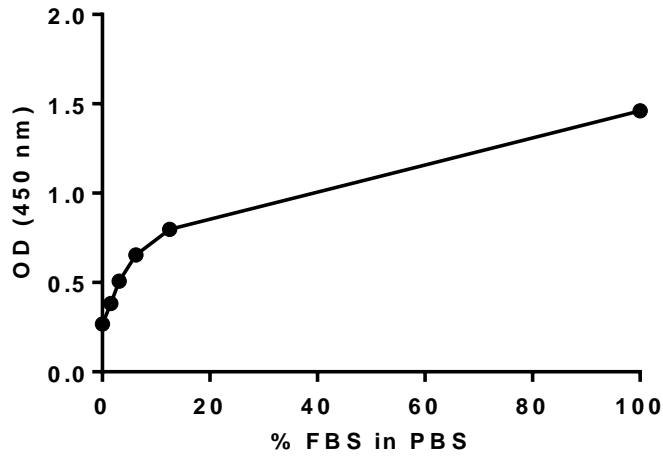
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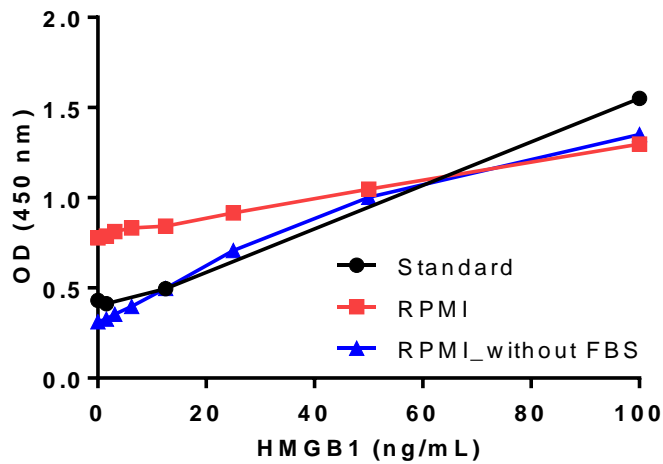
Figure 1-6. Tests on FBS interference against ELISA signals.

(A) Signals generated with dilutions of commercial FBS by sandwich ELISA. (B) Murine rHMGB1 signals in sample dilution buffer, 10% FBS-supplemented RPMI 1640 media, and RPMI 1640 without FBS. The reaction volume was 50 μl.

CHAPTER 2

The effect of pre-existing HMGB1 within

FBS on murine pancreatic beta cell biology

INTRODUCTION

In islet transplantation, both clinical and pre-clinical, the healthiness of the donor islets would be one of the most crucial factors for the successful engraftment (10). In addition to the means for appropriate procurement and isolation of the tissue (38), optimal culture condition for the isolated islets prior to implantation would be vital, and FBS has been often included in the culture medium of murine and human islet culture media to reach the goal (39, 40). Even though some literatures point out that FBS might be harmful to cell cultures due to unknown, xenogeneic substances (41), its specific effect has not been studied thoroughly in pre-transplantation islet cultures.

Previously, I realized that the HMGB1 signal could be generated from commercial FBS. Considering the production process of FBS where hypoxic death of bovine fetuses occur (42), I hypothesized that FBS must contain certain amounts of HMGB1, possibly at a high level. Therefore, the effects of pre-existing HMGB1 in FBS on pancreatic islet beta cell biology were investigated.

MATERIALS AND METHODS

1. Removal of pre-existing HMGB1 in the FBS

For the elimination of pre-existing HMGB1 in FBS, Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific) was used with mouse anti-human HMGB1 IgG (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Half of the FBS samples were processed without the antibodies to serve as the negative control, and after centrifugation, only the supernatant (FBS) was used for the culture experiments.

2. Western blot analysis

Total protein amounts of FBS and elutes obtained from the immunoprecipitation experiments were analyzed by BCA assay (Thermo Fisher Scientific). Intact or HMGB1-removed FBS (500 μg of total protein) were diluted in 4X Laemmli sample buffer (277.8 mM Tris-HCl, pH 6.8, 44.4% (v/v) glycerol, 4.4% SDS, 0.02% bromophenol blue, and 10% 2-mercaptoethanol; Bio-Rad, Hercules, CA, USA) and the final volume was adjusted with PBS and then heated at 100℃ for 5 minutes. Next, protein

samples were loaded into 12% SDS-PAGE gel and transferred to poly-vinylidene fluoride membranes (Bio-Rad) followed by blocking with 5% skim milk and 5% BSA in PBST at RT for 2 hours. Membranes were then incubated with anti-HMGB1 chicken IgY (1:200; R&D Systems) overnight in blocking buffer at 4℃. After washing with PBST for 30 minutes, membranes were incubated with polyclonal goat anti-chicken IgY conjugated with HRP (1:4000; Thermo Fisher Scientific) for 40 minutes at RT. Enhanced chemiluminescent HRP substrate (Thermo Fisher Scientific) was added to the blot and the signal was detected by Amersham Imager 600 (GE Healthcare Life Sciences).

3. *In vitro* pancreatic islet viability test

Mouse insulinoma cell line (MIN6) as well as murine islets were used to test the viability change after pre-existing HMGB1 removal. MIN6 cells were sustained in Dulbecco's Modified Eagle Medium (DMEM; GE Healthcare Life Sciences) supplemented with Anti-Anti and with 15% of either normal FBS or HMGB1-removed FBS for 48 hours prior to the experiments.

A modified MTT assay was carried out using a Cell Counting Kit 8 (CCK8; Dojindo Molecular Technologies, Inc.,

Rockville, MD, USA) according to the manufacturer's protocols. 1 x 10^4 MIN6 cells or 300 IEQs of murine islets were incubated with the CCK8 reagent for 2 hours in a CO_2 incubator. The absorbance of the culture supernatant was read at 450 nm with 650 nm as reference using a Sunrise absorbance microplate reader.

Cell apoptosis was measured using the Annexin V−APC Apoptosis Detection Kit with Propidium Iodide (PI; Biolegend) according to the manufacturer's protocols. MIN6 cells or murine islets were collected and re−suspended in 1X Annexin V binding buffer at a concentration of 1 x 10^6 cells/ml or 3 x 10^4 cells/ml (single cells), respectively. Murine islets were dissociated into single cells by incubating with TrypLE™ Express (Thermo Fisher Scientific) for 2−5 minutes at 37℃ and vigorous vortexing. Then, the cells were stained with Annexin V−APC and PI for flow cytometry analysis with FACSII Canto (BD Biosciences, San Jose, CA, USA). Cells that were stained positively for both Annexin V−APC and PI were considered necrotic. 7−AAD assay was also performed as previously reported (43). 7−AAD (1 μg/ml; Biolegend) was treated on 1 x 10^6 MIN6 cells, incubated for 30 minutes on ice in the dark, and

analyzed on the flow cytometer. The apoptotic states of MIN6 cells were also determined by tetramethylrhodamine ethyl ester (TMRE) staining method (44), as reported previously (45). 5 x 10^5 MIN6 cells were treated with 200 nM of TMRE (Thermo Fisher Scientific) and incubated for 5 minutes at RT in the dark. The cells were analyzed on the flow cytometer.

4. qRT-PCR and cytometric bead assay (CBA)

qRT-PCR was performed on MIN6 cells as described by others (45, 46). After the culture experiments, MIN6 cells were washed with PBS twice and total RNA was extracted with TRIZOL (Thermo Fisher Scientific) as the manufacturer's recommendations. The primers used were: *gapdh* F, 5′-GGA GAG TGT TTC CTC GTC CC-3′ and R, 5′-ATG AAG GGG TCG TTG ATG GC-3′; *bcl2* F, 5′-TTC GCA GAG ATG TCC AGT CA-3′ and R, 5′-TTC AGA GAC AGC CAG GAG AA-3′; *bag1* F, 5′-GAA ACA CCG TTG TCA GCA CT-3′ and R, 5′-GCT CCA CTG TGT CAC ACT C-3′; *bax* F, 5′-GGC TGG ACA CTG GAC TTC CT-3′ and R, 5′-GGT GAG GAC TCC AGC CAC AA-3′; *casp2* F, 5′-GGC TAC AAT GTC CAT GTG CT-3′ and R, 5′-CCA CTA CGC AGG AGT CTG TG-

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3′; *casp3* F, 5′-CAA GTC AGT GGA CTC TGG GA-3′ and
R, 5′-CGA GAT GAC ATT CCA GTG CT-3′; *casp6* F, 5′-
TCA GGG CTA GGA CAC CG-3′ and R, 5′-TTG AAG ATG
AGG GCA ACT CC-3′.

The protein levels of inflammatory markers in the culture
supernatants of primary islets were assessed via Mouse
Th1/Th2/Th17 Cytokine Kit (BD Biosciences) as recommended
by the manufacturer. The results were analyzed with FACSII
Canto.

5. Sequential-static measurement of islet insulin secretion by ELISA

Static glucose-stimulated insulin secretion (GSIS) assay for
murine islets was conducted using a 96-well plate islet insulin
secretion assay protocol essentially as in Truchan *et al.*'s study
(47). Briefly, after incubation for 48 hours in a 60-mm petri dish,
five islets of equal size were chosen and added to each well of a
96-well V-bottom plate (Sarstedt, Numbrecht, Germany), and
then the protocol was carried out. Lastly, 150 μl of lysis buffer
(1.5% HCl in 70% ethanol) was added to each well, collected with
the islets, and then kept overnight at -20℃ in order to obtain

total insulin. The 2 mM- and 20 mM-glucose Krebs Ringer Bicarbonate-HEPES buffer (KRBH) replaced from the wells in each stimulation step were also taken and kept at $-20℃$ until being used for insulin ELISA. Mouse insulin ELISA (ALPCO, Salem, NH, USA) was run with the KRBH supernatants and the lysis buffers diluted to 1/10 according to the manufacturer's protocol (the dilution factors were pre-determined by candidate dilution factor test). To account for differences in islet size, the total insulin content was used to normalize the amounts of insulin secreted to 2 mM- and 20 mM-glucose solutions.

6. Endotoxin detection assay

To eliminate any confounding factor, the culture media were 0.2 μm-filtered before culture experiments and also the endotoxin level was tested with ToxinSensor^{TM} Chromogenic LAL Endotoxin Assay Kit (Genscript, Piscataway, NJ, USA) according to the manufacturer's protocols to exclude the possibility of FBS and rHMGB1 contaminations.

7. Statistical analyses

All statistical analyses were performed using Prism 6.01. Values were expressed as mean $\pm$ SEM. The differences between values were compared by unpaired t-test.

RESULTS

Removal of pre-existing HMGB1 from FBS and its effect on ELISA signals

Previously, the possible existence of HMGB1 in commercial FBS was revealed by ELISA (**Figure 1-6**). To my expectation, the HMGB1 signal in FBS rose dose-dependently following a semi-log curve (**Figure 1-6A**). Moreover, the rHMGB1 signals in FBS-free RPMI 1640 showed almost an identical trend to that in a standard dilution buffer (**Figure 1-6B**), indicating that the FBS must be the sole factor that had affected the rHMGB1 signal detected by ELISA. Nonetheless, at this point it could not be guaranteed that other confounding factors in the FBS may have interfered with the ELISA signals.

To explore further into the effects of pre-existing HMGB1 in FBS, the HMGB1 was depleted from the FBS via immunoprecipitation, and its depletion was confirmed by SDS-PAGE and western blot analysis. The remaining HMGB1 in the FBS was checked by 12% SDS-PAGE and western blot (**Figure 2-1**). HMGB1 was not detected in the FBS fraction at the first trial (**Figure 2-1A**) and only a slight band was detected in the
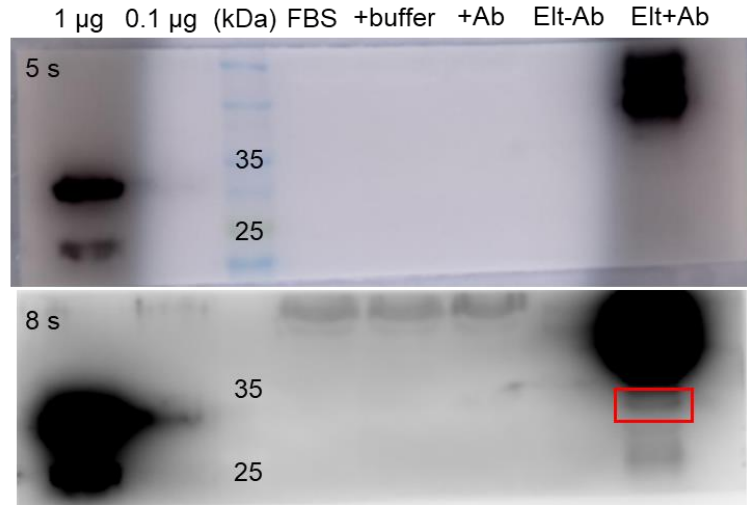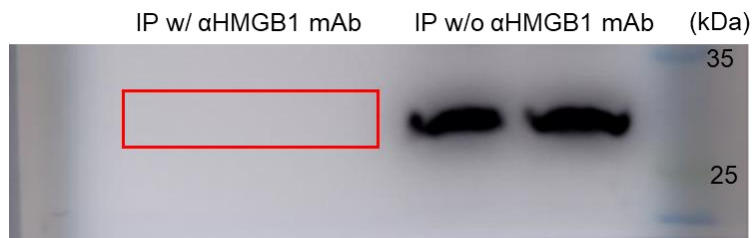
Figure 2-1. Western blot of rHMGB1 and HMGB1-depleted FBS.

(A) Western blot of rHMGB1 standards, FBS samples harvested during the immunoprecipitation experiment, and the eluted fractions after immunoprecipitation. Longer exposure (5 seconds vs. 8 seconds) revealed a slight band of HMGB1 in the eluted fraction (red box). (B) Immunoprecipitation resulted in the depletion of pre-existing HMGB1 from FBS. Two separate samples of FBS were run by SDS-PAGE.

eluted fraction. At the second trial, clear bands of HMGB1 were observed in the FBS in which immunoprecipitation was performed without the anti-HMGB1 monoclonal antibody (**Figure 2-1B**). The immunoprecipitation and subsequent ELISA has proven that there was a certain amount of pre-existing HMGB1 in the FBS: extrapolation from the OD of rHMGB1 in 10% FBS-supplemented RPMI 1640 produced the background HMGB1 concentration difference which was almost equal to 10 ng/ml (**Figure 2-2**; 15.28 ± 1.683 vs. 23.16 ± 1.496). It was noteworthy that in contrast to the results in **Figure 1-6B** of FBS-free medium, depletion of HMGB1 did not rescue the ELISA signal specificity and sensitivity (**Figure 2-3**), confirming that factors other than pre-existing HMGB1 had interfered with ELISA signals.

Effect of pre-existing HMGB1 removal on pancreatic beta cell viability

Since the detrimental effect of extracellular HMGB1 on pancreatic islet cells has been well known (21, 48), I sought to investigate whether this 'background' HMGB1 in FBS had physiologically influenced cultured beta cells. After the 48-hour
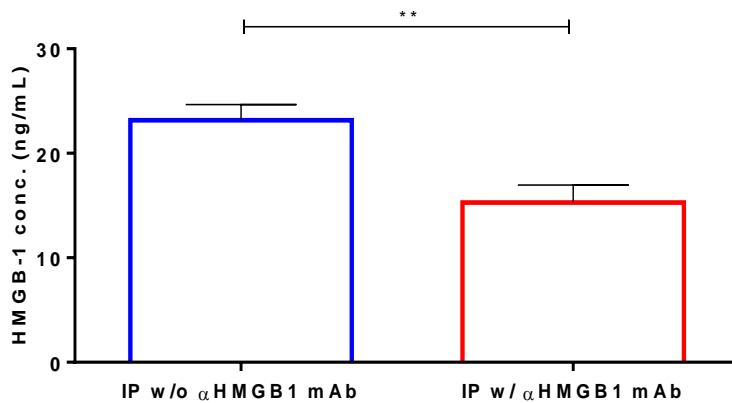
Figure 2-2. The background HMGB1 signal difference between 10% RPMI 1640 supplemented with FBS after immunoprecipitation with or without anti-HMGB1 mAb.

When standard curves were generated with rHMGB1 diluted in standard dilution buffer, FBS-supplemented RPMI 1640, and HMGB1-depleted FBS-supplemented RPMI 1640, the OD signals at 0 ng/ml rHMGB1 within FBS-supplemented RPMI 1640 and HMGB1-depleted FBS-supplemented RPMI 1640 were used to calculate the background HMGB1 signals. Data are from 4 independent experiments. **, $p<0.01$.
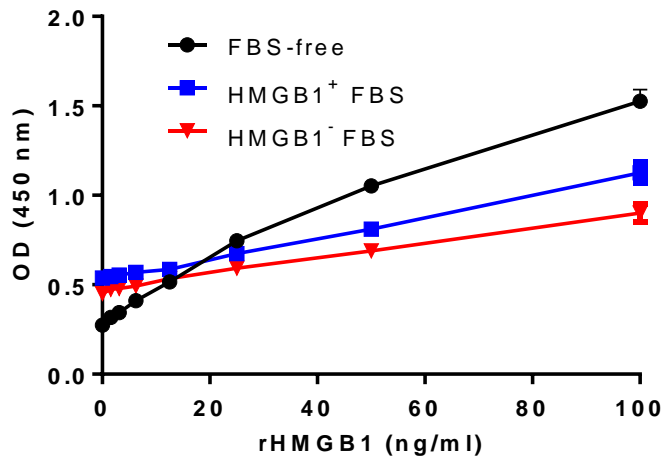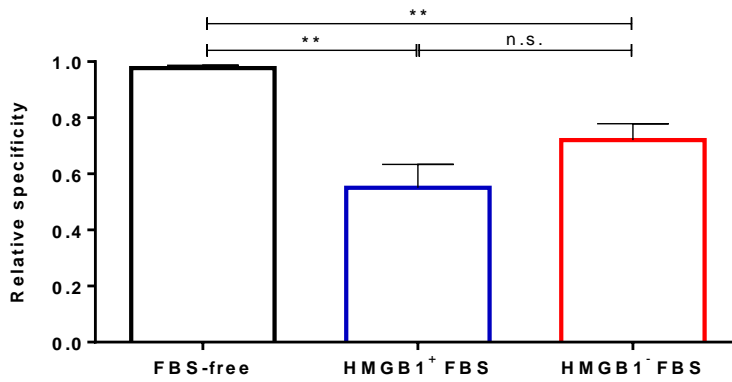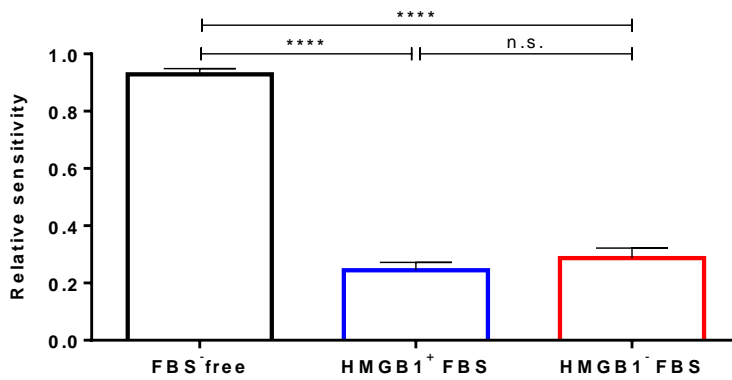
Figure 2-3. HMGB1 ELISA signal interference by FBS with or without pre-existing HMGB1 removal.

(A) Standard curves of rHMGB1 were generated with various sample matrices. The curves are representative of 3 independent experiments. (B-C) The specificity (B) and sensitivity (C) of ELISA signals generated with known amount rHMGB1 in culture media without FBS (black), with FBS (blue), and with HMGB1-depleted FBS (red). **, $p<0.01$; ****, $p<0.0001$; n.s., not significant.

culture, it was revealed that the removal of pre-existing HMGB1 resulted in a significant reduction of the viability of cultured primary islet cells ($p<0.0001$; **Figure 2-4A**). The same phenomenon could be observed in MIN6 cells ($p<0.0001$; **Figure 2-4B**). To complement the viability evaluation by CCK8, flow cytometry analysis was performed on MIN6 cells. Consistent with CCK8 data, flow cytometry analysis also indicated that more cells became non-viable with pre-existing HMGB1 removal ($p<0.05$; **Figure 2-4C**). Invariably, culturing with or without pre-existing HMGB1 resulted in significant difference in TMRE-positive MIN6 cells ($p<0.001$; **Figure 2-4D**). TMRE-positivity indicates general mitochondria healthiness. Though the flow cytometry analysis of islet single cells did not demonstrate a significant difference in viability (**Figure 2-5**), much of these data uniformly indicated that the depletion of pre-existing HMGB1 from FBS affected the cultured pancreatic beta cell viability negatively.

Transcriptional and translational changes to the cultured beta cell with pre-existing HMGB1 removal

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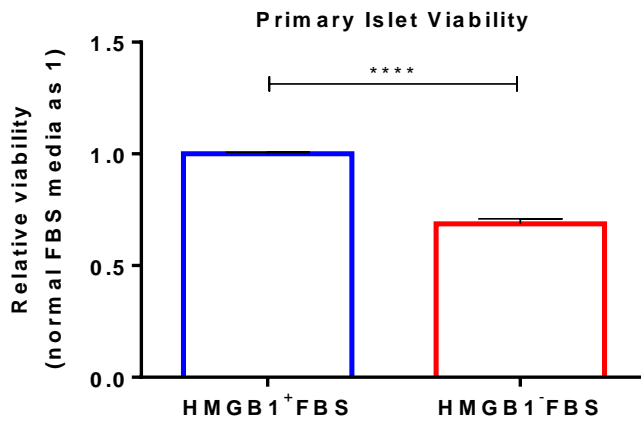
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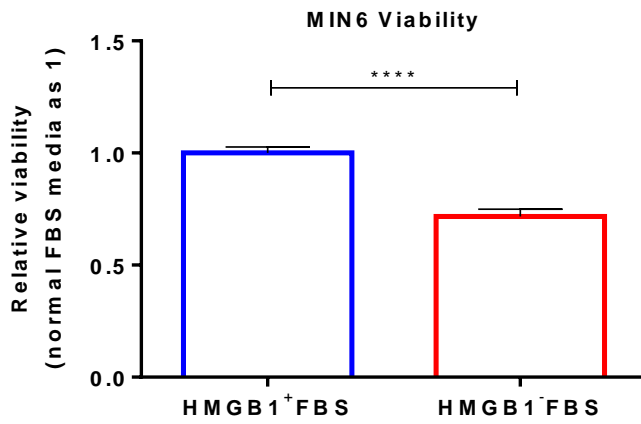
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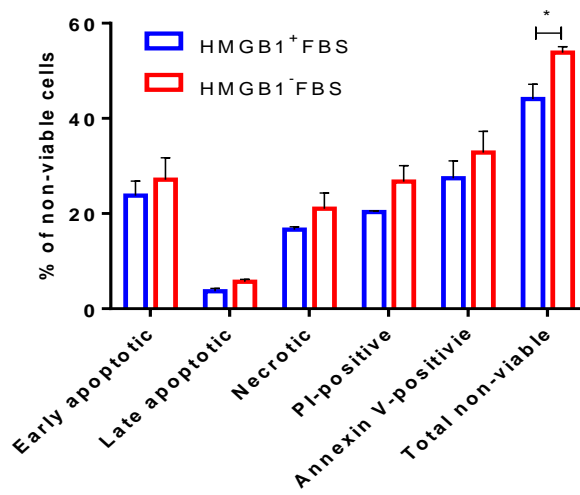
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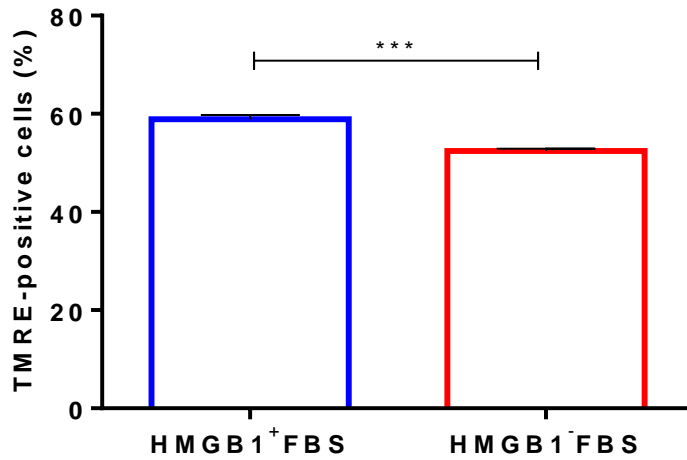
Figure 2-4. The effect of pre-existing HMGB1 removal on the viability of cultured beta cells.

(A) CCK8 assay results of cultured primary islets. ****, $p<0.0001$. (B) CCK8 assay results of culture MIN6 cells. ****, $p<0.0001$. (C) Viability of MIN6 cells determined by Annexin V and PI staining. A representative scatter plot is shown. *, $p<0.05$. (D) Cell viability of MIN6 cells were also determined by TMRE staining. ***, $p<0.0001$.
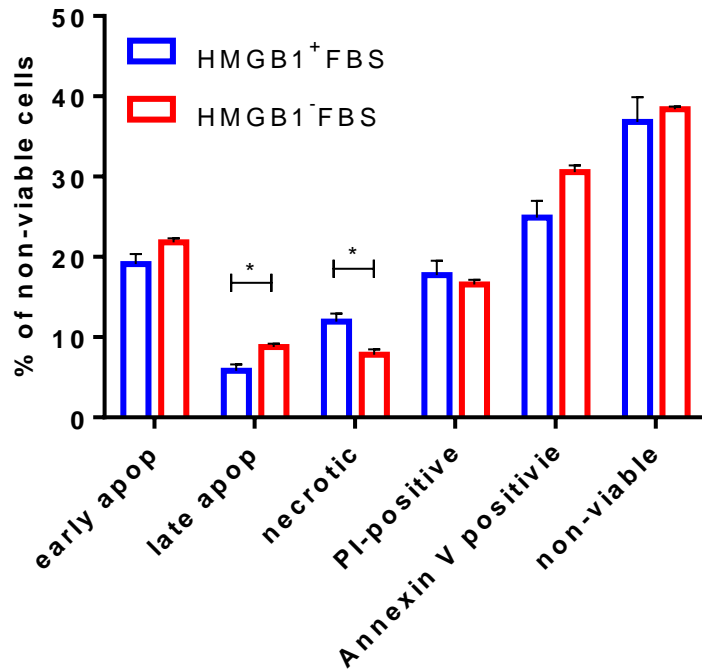
Figure 2-5. Viability of primary islet single cells determined by flow cytometry.

To perform flow cytometry, the murine primary islets were first dissociated into single cells by trypsinization and strong vortexing. 3×10^4 islet single cells were then stained with annexin V-APC and PI kit to be analyzed by a flow cytometer. *, $p < 0.05$.

To characterize the changed viability of culture beta cells, qRT-PCR was performed on MIN6 cells to measure the differences in pro- and anti-apoptotic gene expression. Unfortunately, it was revealed after the normalization of the results with *gapdh* expression that HMGB1 removal from FBS did not result in up-regulation of pro-apoptotic factors or down-regulation of anti-apoptotic factors in cultured islet beta cells (**Figure 2-6**). Meanwhile, the CBA demonstrated that the IL-6 levels in the cell culture supernatants differed significantly depending on the pre-existing HMGB1 in the FBS ($p<0.01$; **Figure 2-7**). The significant difference in the level of other cytokines was not detected.

Effect of pre-existing HMGB1 removal on pancreatic beta cell function

Subsequently, the effect of pre-existing removal of HMGB1 from FBS on pancreatic islets was functionally assessed via *ex vivo* static GSIS assay. Notably, the results of GSIS indicated that HMGB1 removal from FBS was unfavorable to the islet function: islets cultured with HMGB1-depleted FBS secreted significantly less insulin upon stimulation with glucose at 20 mM ($p<0.0001$;
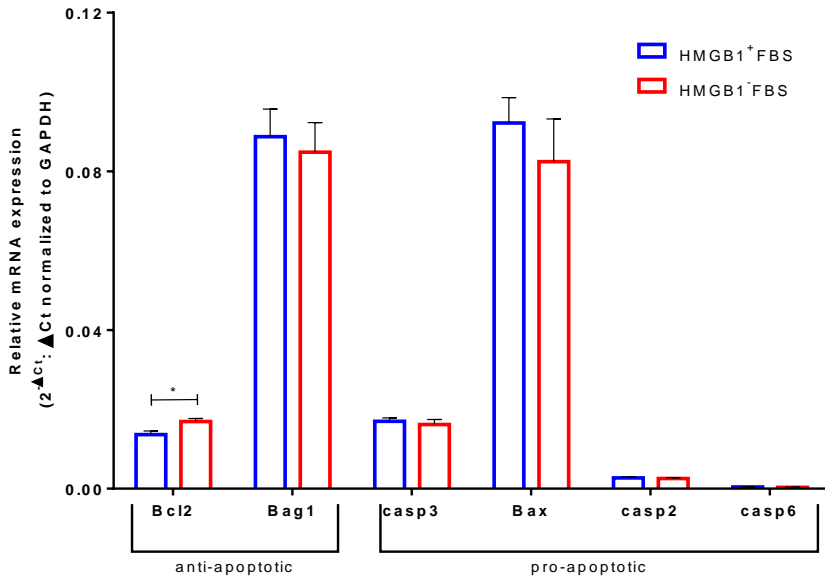
Figure 2-6. qRT-PCR analysis of apoptosis-related genes in MIN6 cells.

The mRNA transcripts of apoptosis-related genes were quantified by qRT-PCR. The results were normalized to the housekeeping gene *gapdh* expression. ∗, $p<0.05$.
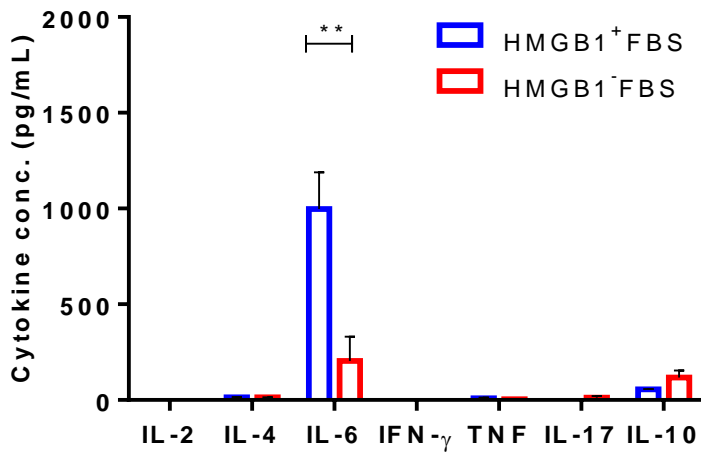
Figure 2-7. The effect of HMGB1-depletion from the FBS on cytokine level in pancreatic islet cell culture.

The amount of cytokines within culture supernatants of primary islets (150 IEQs) incubated 48 hours with or without pre-existing HMGB1 removal, measured via CBA. **, $p<0.01$.

Figure 2-8). In the meantime, there was no significant difference in the steady-state insulin secretion (2 mM) although the mean insulin secretion was reduced (p=0.4237).

Changes in pancreatic beta cell viability and function after the addition of rHMGB1

In sum, it was discovered that the depletion of HMGB1 from FBS resulted in reduced viability and function of cultured islet beta cells. Consequently, it was inevitable to test whether re-addition of a complementary amount of HMGB1 could restore the viability and function of cultured islet beta cells. For every islet beta cell culture supplemented with HMGB1-depleted FBS, 10 ng/mL of rHMGB1 was introduced to investigate the effect. To eliminate any confounding factor, the media were filtered and tested for endotoxin contamination before culture experiments, which were far under 0.1 EU/μg (Figure 2-9).

Interestingly, the addition of rHMGB1 enhanced the viability of primary islets significantly (p<0.05; Figure 2-10A), nearly to the level before the depletion of pre-existing HMGB1 within the FBS. However, the addition of an excessive amount of rHMGB1 (100 ng/mL) could not enhance the viability to a higher
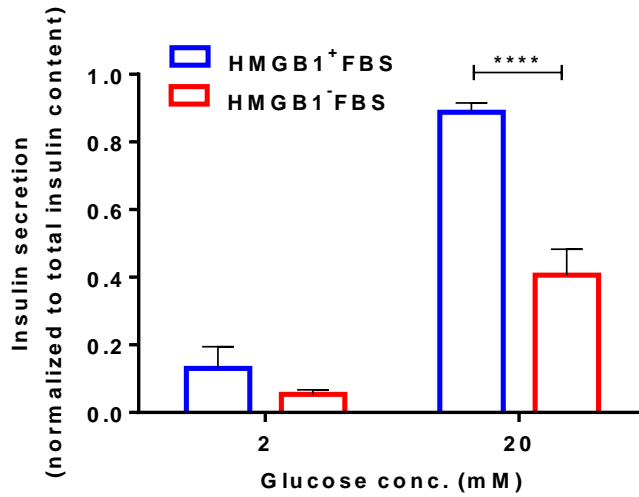
Figure 2-8. GSIS of pancreatic islet cells cultured in media with or without pre-existing HMGB1 removal.

The islets were first equilibrated for 1 hour in 2 mM glucose solution, and then incubated sequentially for 1 hour each in 2 mM and 20 mM glucose solutions. ****, p<0.0001.
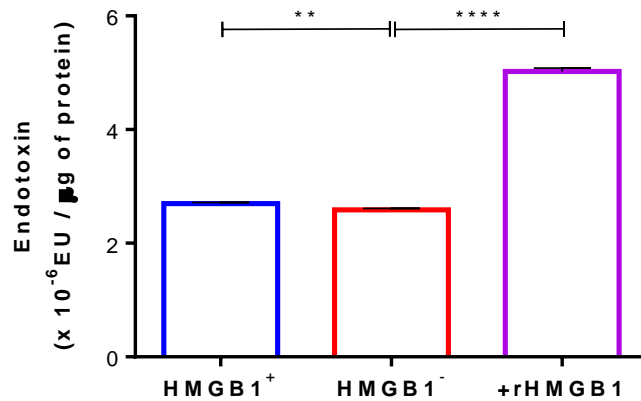
Figure 2-9. Endotoxin level in various types of 10% FBS-supplemented RPMI 1640 media.

Endotoxin levels of normal FBS-supplemented media, HMGB1-depleted FBS-supplemented media, and HMGB1-depleted FBS plus rHMGB1-supplemented media (10 ng/ml of rHMGB1) were tested. **, $p < 0.01$; ****, $p < 0.0001$; n.s., not significant.

A

Primary Islet Viability

B
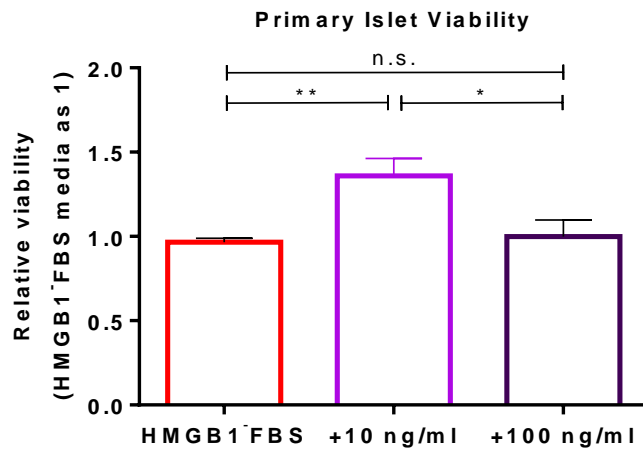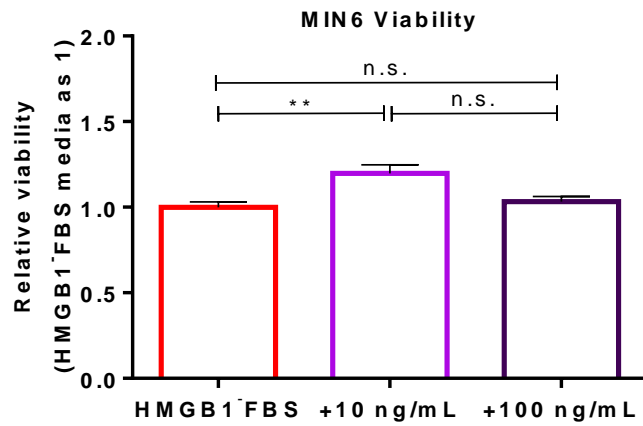
MIN6 Viability

C
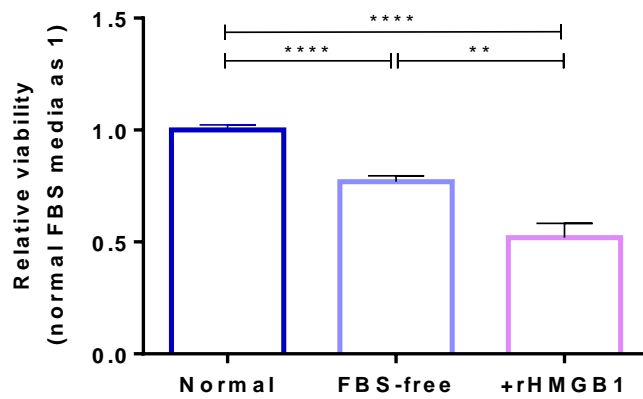
D
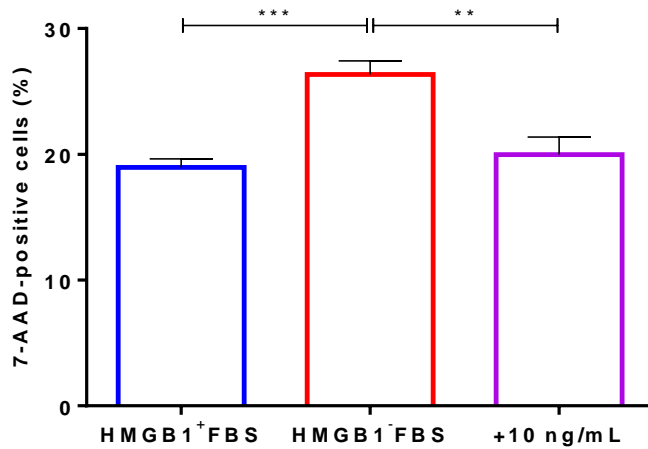
Figure 2-10. The effects of rHMGB1 re-addition on beta cell viability.

(A) CCK8 assay results of cultured primary islets. **, $p<0.01$; *, $p<0.05$; n.s., not significant. (B) CCK8 assay results of cultured MIN6 cells (1×10^5). **, $p<0.01$; n.s., not significant. (C) MIN6 cells were cultured in FBS-supplemented DMEM, FBS-free DMEM, or FBS-free DMEM plus 10 ng/mL of rHMGB1 for 48 hours, and then CCK8 assay was performed. ***, $p<0.001$; **, $p<0.01$. (D) Viability of MIN6 cells determined by 7-AAD staining. 7-AAD-positive cells are non-viable cells. ***, $p<0.001$; **, $p<0.01$.

degree. The change in viability of MIN6 cells determined by CCK8 assay also showed a consistent pattern ($p<0.05$; **Figure 2-10B**). Nonetheless, just as in primary islets, excessive amount of rHMGB1 (100 ng/mL) could not raise the viability of MIN6 cells. Also, it was investigated whether rHMGB1 addition alone (10 ng/mL) to the FBS-free media could sustain MIN6 cells. As shown in **Figure 2-10C**, serum starvation decreased MIN6 cell viability ($p<0.0001$) and rHMGB1 addition exacerbated the effect ($p<0.01$), indicating that pre-exsiting HMGB1 was probably not the dominant factor in FBS for optimal beta cell culture. In addition, the analysis after 7-AAD staining similarly indicated improved viability after rHMGB1 addition in MIN6 cells (**Figure 2-10D**). The function of primary islets also demonstrated difference after the addition of rHMGB1 (**Figure 2-11**). Contrary to the results of culture experiments with HMGB1-depleted FBS (**Figure 2-8**), the steady-state insulin secretion increased significantly ($p<0.05$) but the stimulated-state insulin secretion did not ($p=0.3170$). Altogether, the results showed that re-addition of rHMGB1 to the media could rescue the viability and function of islet beta cells.
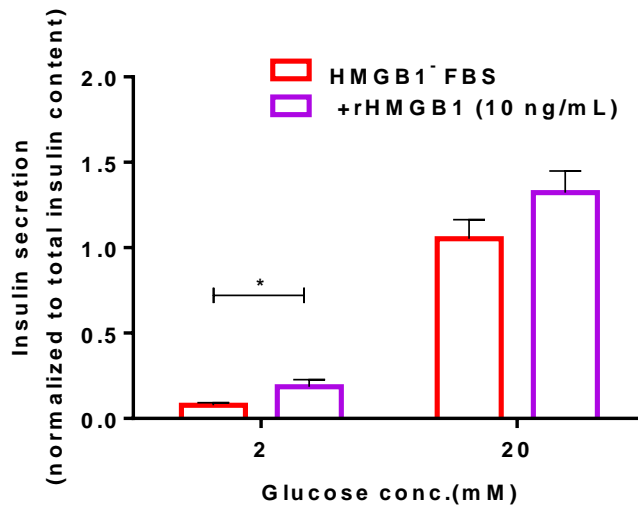
Figure 2-11. The effects of rHMGB11 re-addition on beta cell function.

GSIS of pancreatic islet cells cultured in HMGB1-depleted FBS-supplemented media with or without the addition of rHMGB1 (10 ng/ml). The islets were equilibrated for 1 hour in 2 mM glucose solution, and then incubated sequentially for 1 hour each in 2 mM and 20 mM glucose solutions. *, $p < 0.05$.

DISCUSSION

Previously, the presence of HMGB1 in commercial FBS was checked by sandwich ELISA experiments. The pre-existing HMGB1 in the FBS was the alleged factor that hindered the specific and sensitive detection of HMGB1 in the cell culture supernatant (**Figure 1-6**). However, immunoprecipitation and subsequent ELISA revealed that pre-existing HMGB1 contributed only partly to the deterioration of signal specificity and sensitivity (**Figure 2-3**), which should simply make the other components in FBS the culprit of ELISA signal disturbance. It has been known that quantification of HMGB1 is difficult due to its inherent characteristics, such as its nonspecific binding to other proteins (34). This phenomenon seems very natural considering the promiscuity of HMGB1 which makes it a proficient DAMP (49). Still, I believe that a more accurate method should be formulated and standardized to circumvent the confounding factors for HMGB1 measurement in FBS-supplemented media. Mass spectrometry might be an alternative in this instance (50). Nevertheless, to my knowledge, HMGB1 ELISA signal interference by FBS has never been addressed before,

presumably because the subtle difference of HMGB1 in cell culture media has rarely been the center of scientific research.

Even though the actual amount could be arbitrary depending on the chosen measurement method, I assumed that the presence of HMGB1 in FBS could raise concerns about its potential harmful effects on cultured cells, given its high conservedness among mammals where murine HMGB1 (UniProt: P63158) and bovine HMGB1 (UniProt: P10103) were found to share 98.6% homology on BLASTP and because omitting FBS from the cell culture medium could not be a practical option in murine islet cultures. The implication to this notion is that the ‘background HMGB1’ that had been detected by ELISA might have had ‘background effects’ on the cultured cells. Intriguingly, the removal of pre-existing HMGB1 from FBS actually had a negative effect on pancreatic islet viability (**Figure 2-4**), a result opposite to what I had hypothesized. In other words, HMGB1, a seemingly harmful protein to pancreatic islets, might have been an indispensable cell culture component. Extracellular HMGB1 has been long known to be harmful to islet beta cells, but some studies have indicated that the A-box fragment of HMGB1 could increase the survival of pancreatic

islets (27, 28). Also, HMGB1 was reported to be pro-autophagic in cells other than pancreatic islets, which enhanced cell survival (51, 52). The relationship between the pre-existing HMGB1 in FBS and murine islets is associated with the A-box fragments' effect or the change in viability is linked to the autophagy-apoptosis axis would be interesting subjects for further research.

      To delineate the phenomenon of viability shift, qRT-PCR and CBA were implemented. Although the differential expression of genes that might affect the viability of beta cells was not observed (**Figure 2-6**), I went on to check the protein-level difference of mediators, particularly cytokines. It has been well known that islets can produce and respond to various cytokines (53). CBA results indicated that the elimination of HMGB1 from FBS actually resulted in increased IL-6 levels in the primary islet cell culture supernatants (**Figure 2-7**). This phenomenon seemed feasible because the production of IL-6 by pancreatic beta cells has been documented before (54) and our group previously reported that IL-6 showed islet-protective effects *in vitro* and *in vivo* (33). However, it should be noted that despite rare discussions on the relationship between HMGB1 and IL-6 concerning pancreatic islet biology, Itoh *et al.* recently reported

that anti-IL-6R antibody treatment in mice prevented the HMGB1-mediated loss of transplanted islets (23). There were also reports that indicated the potential harmful effects of IL-6 on pancreatic islets (55). Therefore, I believe that more in-depth study should be performed on the underlying mechanisms of the differing IL-6 level in culture supernatants, which might help explain the contradictory effects of IL-6 on pancreatic islet cells.

Undoubtedly, the viability and function of cells are closely linked, and my data demonstrated that pancreatic beta cells cultured in HMGB1-depleted FBS also showed decreased insulin-secretion function (**Figure 2-8**). Nevertheless, it should be noted that the reduced level of HMGB1 in the media could have impaired the secretion of insulin from beta cells. In 2013, Mera *et al*. reported that Ca^{2+} influx to beta cells cause the release of HMGB1 (16), and Guzman-Ruiz *et al*. reported that HMGB1 levels coincided with insulin release and intracellular Ca^{2+} concentrations in a rat beta cell line (56). It should be investigated whether the same phenomenon could be observed in non-pathological conditions.

I believe this study led us to question our general

assumptions on common *in vitro* experiments, where the knowledge and insights we had gathered via *in vitro* experiments could have had HMGB1's hidden effect to it. Even at what seemed a negligible amount of difference (~10 ng/ml measured by my ELISA), the absence of pre-existing HMGB1 affected the islet viability and function substantially and the re-addition restored them (**Figure 2-10 & 11**). It could be argued that omitting a factor from a well-designed culture medium would negatively affect the viability of cultured cells. Nonetheless, HMGB1 has been deemed very unfavorable to pancreatic islet cells and its depletion must have been beneficial to the cultured islet cells, yet I discovered a rather paradoxical phenomenon. Hence, this discovery alerts us to always question our previous knowledge and assumptions in the field of science.

Some literatures have pointed out that FBS might be harmful to cell cultures due to unknown, xenogeneic substances (41), and preventing the use of FBS would also be desirable in the socio-ethical context. The advent of chemically-defined media could have been the ideal alternative in this case, but the insufficient capability to sustain cells besides its unaffordable price have hampered its wide application (57). According to my

research, addition of an appropriate amount of rHMGB1 could be the solution for this dilemma. Further analyses of this phenomenon could help find the more proper culture condition for maintaining pancreatic islets prior to transplantation.

In conclusion, I investigated the effect of this FBS－derived HMGB1 on the viability and function of pancreatic islets, and uncovered that its removal was indeed detrimental to the islets. This discovery indicates the contradictory role of HMGB1 in pancreatic islet physiology, which is under active investigation in other fields of biology. Also, this notion could shed light on the optimal culture condition for pancreatic islets, especially in the clinics where maintaining the wellness of donor islets is very important.

CHAPTER 3

HMGB1 secretion blockade results in the

reduction of graft loss in the early period of

islet transplantation

INTRODUCTION

In islet transplantation, hypoxic stress inflicted upon the islet graft and the vulnerability of the pancreatic islets to the stress (38) are major hurdles towards successful engraftment. HMGB1 is highly associated with hypoxia−induced islet cell loss either through direct binding to the islets via TLR2 or TLR4 engagement (58) or indirectly through immune cells and mediators such as neutrophils and IFN−γ (59). Naturally, there have been attempts to prevent the loss of pancreatic islet graft by blocking the secretion of HMGB1 (16) or neutralizing its effect (22), many of which succeeded in protecting islet graft and reversed the diabetes in mice.

In this study, I tested whether ICM, a small−molecule inhibitor of HMGB1 previously known to block the HMGB1 secretion in neuro−inflammatory cells with great potency and little toxicity (60), could work in the same manner on pancreatic islets *in vitro* and islet transplantation *in vivo.* I discovered that ICM could block the secretion of HMGB1 in isolated pancreatic islets, and showed that the HMGB1 blockade by ICM could spare the mass of islet grafts in diabetic mice recipients.

MATERIALS AND METHODS

1. Animals

Female BALB/c and B6 mice at 8 to 12 weeks of age were purchased from Jackson Laboratories and maintained in the SNU SPF animal facilities. All experimental procedures were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (NIH Publication No. 86-23, revised 2011) and published by the National Institute of Health. This study was approved by the IACUC of SNU (IACUC no. SNU-170518-3-2 & SNU-170804-4).

2. Reagents

ICM was kindly provided by Prof. Seung Bum Park at SNU Department of Chemistry. ICM was obtained in a lyophilized state, and was reconstituted with dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 60 μg/μl (159 mM) for further use. For optimal injection, ICM or DMSO was mixed with polyethylene glycol 400 (Sigma-Aldrich) and distilled water (DW) at a ratio of 1:8:11 (ICM or DMSO:PEG:DW) (61).

3. Islet isolation and culture

Murine islet isolation was performed according to a previously described method (33) but with BALB/c mice as donors. The isolation buffer, wash buffer, and the Ficoll solutions contained either ICM (10 μM) or DMSO (1:2000) for the experiment on the ICM efficacy during the isolation process. The isolated murine islets were incubated in RPMI 1640 media supplemented with 10% FBS and Anti-Anti.

For normoxic incubation experiments, the cells were seeded at 300 IEQs/well in 24-well plates with either ICM (10 μM) or DMSO (1:2000) for 48 hours in a 37℃, 5% CO_2 incubator. For hypoxic incubation experiments, the primary islets were seeded at 300 IEQs/well in 24-well plates with either ICM (10 μM) or DMSO (1:2000) for 48 hours in a hypoxic incubator (37℃, 1% O_2, 5% CO_2, 94% N_2).

4. *In vitro* viability assay

In vitro viability assay was performed using CCK8 according to the manufacturer's protocols. 300 IEQs of primary islets, after incubation either with ICM (10 μM) or DMSO

(1:2000) for 48 hours, were incubated with the CCK8 reagent for 2 hours in a CO_2 incubator. The absorbance of the culture supernatant was read with Sunrise absorbance microplate reader at 450 nm with 650 nm as reference.

5. Diabetes induction and islet transplantation

Islet transplantations to diabetic mice were conducted (vehicle-control group, n=7; ICM group, n=7) as previously described (33). After the routine islet isolation process, the islets were incubated for 24 hours without any treatment prior to the transplantation. The conventional (300 IEQs) and marginal (200 IEQs) mass of islets were hand-picked under a dissecting microscope. Diabetes was induced in the recipient mice by injection of STZ (100 mg/kg) for two consecutive days. After transplantation, ICM (10 mg/kg) was administered intraperitoneally for seven consecutive days starting at day 0, and at day 0 the drug was injected 1 hour prior to the transplantation procedure. To study the effect of ICM on diabetes induction, ICM was administered intraperitoneally for two days, immediately after STZ injections.

6. ELISA

Briefly, 96-well ELISA plates (Thermo Fisher Scientific) were coated overnight at 4℃ with 2 μg/ml mouse anti-HMGB1 IgG (R&D Systems). Culture supernatants and murine sera were diluted 1:1 and 1:4, respectively, with the sample dilution buffer (0.5% BSA in PBST) and incubated overnight at 4℃. The captured murine HMGB1 was detected with 0.5 μg/ml of chicken anti-HMGB1 IgY (R&D Systems) and 1:10000 HRP-conjugated anti-chicken IgY polyclonal antibody (Thermo Fisher Scientific). The signal was developed by TMB substrate and the coloration was read at 450 nm OD by Sunrise absorbance microplate reader.

7. Immunocytochemistry (ICC) and IHC

ICC was performed as previously described (62) on MIN6 cells to visually confirm the ICM's effect. After growing MIN6 cells on sterilized 25-mm coverslips with 15% FBS-supplemented DMEM media, the coverslips were treated with 100 ng/ml of LPS (Sigma-Aldrich) and either ICM (10 μM) or DMSO (1:2000) at the same time for 24 hours. Rabbit anti-human HMGB1 IgG (Flarebio, College Park, MD, USA; 5 μg/ml) was used as primary antibody and Alexa488-conjugated goat anti-rabbit IgG

(Thermo Fisher Scientific; 2 μg/ml) was used as secondary antibody.

IHC was performed, as previously described (63), on the paraffin-embedded sections of islet grafts at 6 hours post-transplantation to determine the HMGB1 level differences in vehicle control- or ICM-treated groups. Biotinylated mouse anti-HMGB1 IgG (Chondrex, Redmond, WA, USA; 1 μg/ml) and guinea pig anti-insulin IgG (Abcam, Cambridge, UK; 1:100) were used as primary antibodies, and Alexa488-conjugated streptavidin (Thermo Fisher Scientific; 1 μg/ml) and Alexa647-conjugated goat anti-guinea pig IgG (Abcam; 2 μg/ml) were used as secondary antibodies, respectively. The results of ICC and IHC were observed and visualized under a fluorescence microscope (AxioCam; Carl Zeiss AG, Oberkochen, Germany). The final image processing was performed using the AxioVision software (Carl Zeiss AG).

8. TUNEL assay

H&E staining was performed as previously described (64). The assessment of islet graft cell viability was performed on the paraffin-embedded islet graft sections at 6-hour post-

transplantation via TUNEL Assay Kit-HRP-DAB (Abcam) according to the manufacturer's recommendations.

9. Statistics

All statistical analyses were performed through Prism 6.01. Values were presented as mean ± SEM. The differences between groups were compared by unpaired t-test or Mantel-Cox test.

RESULTS

ICM reduces the level of HMGB1 in islet cultures

The first step I took was to determine whether ICM could demonstrate the same potency to pancreatic islets as it did to the microglia. Since islet beta cells express the receptors for LPS and are known to express HMGB1 when engaged with LPS (65, 66), I sought to investigate the ICM's effect on LPS-treated MIN6 cells. Through ICC, it was observed that ICM treatment could reduce the expression of LPS-induced HMGB1 in MIN6 cells (**Figure 3-1**). Particularly, HMGB1 seemed to localize to the nuclear compartment of the cells after ICM treatment (white arrows). In fact, ICM was reported to directly bind to HMGB1 and inhibit its post-translational modification so that it could not be accumulated in the cytosol of microglia (60).

Since it is well known that pancreatic islets secrete HMGB1 during and after the isolation process due to the associated hypoxic stresses (48), I investigated whether ICM administration could lower the amount of secreted HMGB1 after routine islet isolation. The experiment was designed to simply assess the effect of ICM treatment during the isolation procedure
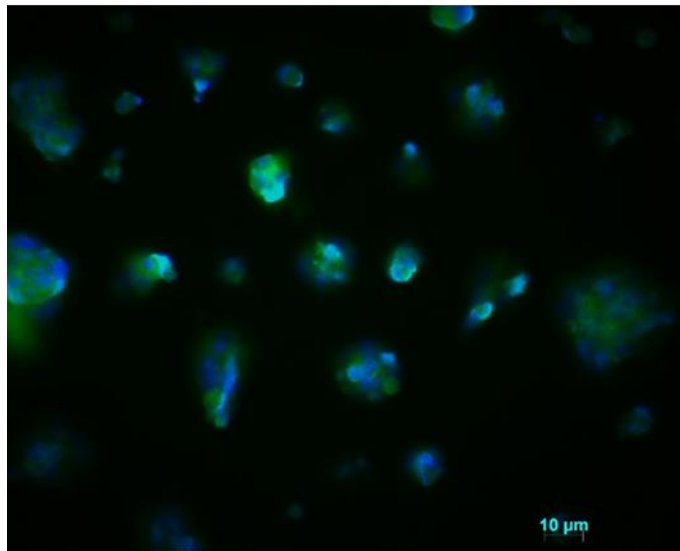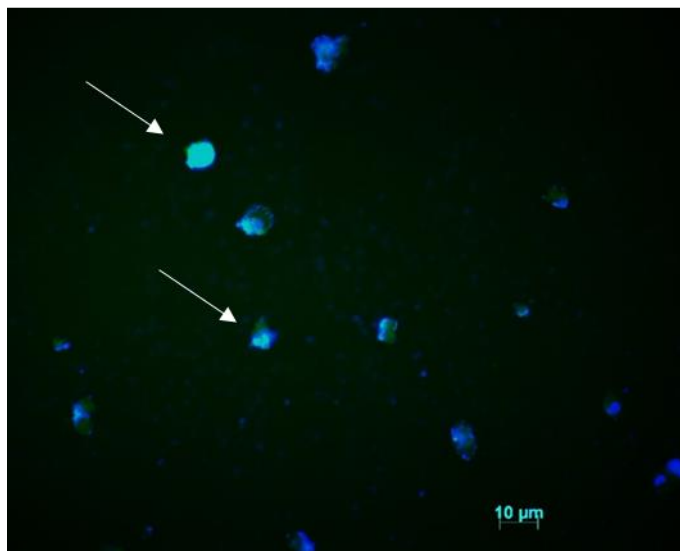
Figure 3-1. Immunocytochemistry of LPS-treated MIN6 cells with or without ICM.

MIN6 cells were treated with 100 ng/ml of LPS and ICM (10 μM) or DMSO (1:2000). White arrows indicate the presumed event of HMGB1 nuclear localization. Green, HMGB1; Blue, DAPI.

or the routine incubation prior to the actual *in vivo* islet transplantation (**Figure 3-2A**). I confirmed that ICM treatment in the post-isolation culture was effective in significantly reducing HMGB1 secretion, both with (p<0.0001) and without (p<0.0001) ICM administration during the isolation (**Figure 3-2B**). It was noteworthy that even the short-term pretreatment of ICM during the isolation procedure resulted in the reduction of HMGB1 secretion in the 48-hour incubation (p<0.05), but ICM pretreatment did not result in significanty decreased HMGB1 level unless there was ICM in the cell culture medium (p=0.2479). Taken together, it was discovered that the administration of ICM could lower the amount of HMGB1 secreted from isolated pancreatic islets.

It is common to utilize hypoxic incubation to mimic the physiological stress on islets after the implantation (12). In this sense, the routinely isolated murine islets were incubated for 24 hours in normoxia, then they were transferred to a hypoxic incubator and incubated for 48 hours with or without ICM. This experimental scheme will simulate the first 48 hours of syngeneic islet transplantation (**Figure 3-2A**), and I found that ICM treatment during the 48-hour incubation significantly

A
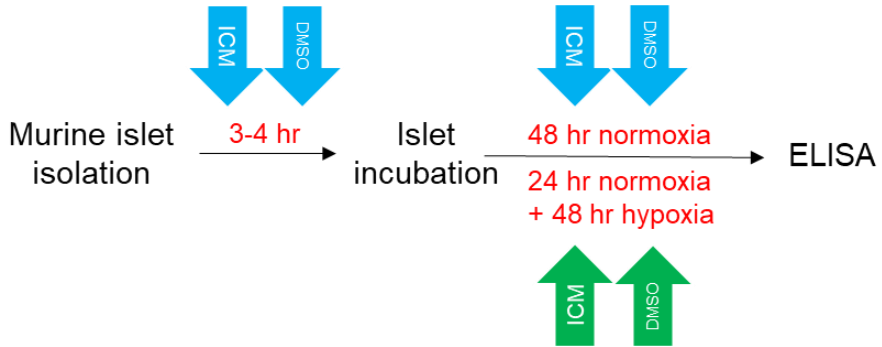
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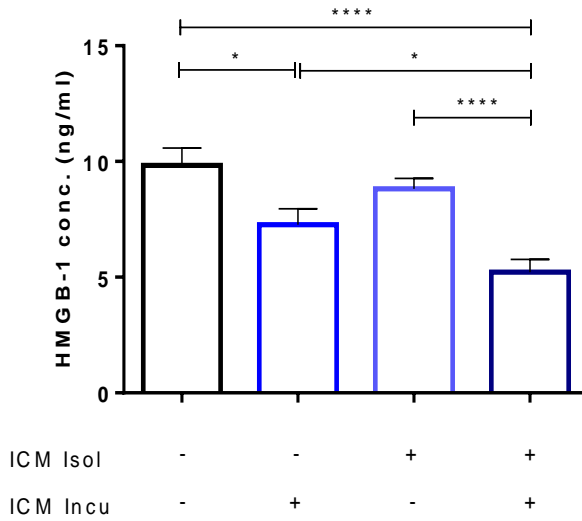
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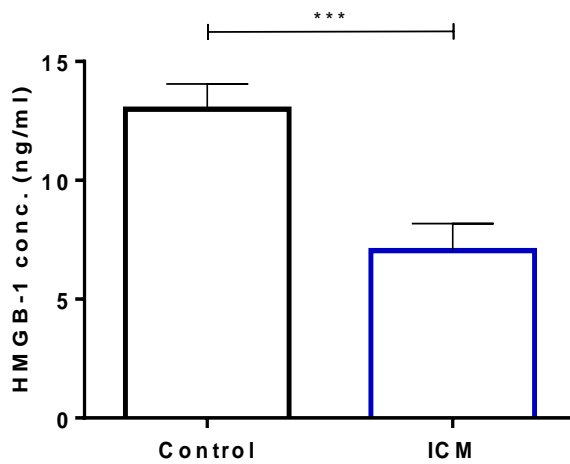
Figure 3-2. ICM effect on isolated murine islets.

(A) A schematic of islet culture conditions for the ELISA experiments. Blue and green arrows denote treatments for separate experiments. (B) Culture supernatant HMGB1 levels in different conditions. ICM was administered during either isolation or subsequent incubation steps, or both. *, $p<0.05$; ****, $p<0.0001$; Isol, treatment during isolation; Incu, treatment during incubation. (C) Culture supernatant HMGB1 level after routine islet isolation, routine 24-hour normoxic incubation, and 48-hour hypoxic incubation, mimicking early periods of syngeneic transplantation. ***, $p<0.001$.

decreased the amount of HMGB1 in the culture supernatant ($p<0.001$; **Figure 3-2C**). Collectively, ICM was potent in reducing the amount of secreted HMGB1 significantly after isolation and incubation processes where islets begin to experience the hypoxic stress.

ICM reduces the level of HMGB1 in transplanted islets and recipients' sera

Subsequent *in vivo* experiments were performed to recapitulate the *in vitro* results. After the routine islet isolation procedure without any drug treatment, murine syngeneic islet transplantation was performed with the conventional amount of pancreatic islets (300 IEQs). The serum (control group, $n=8$; ICM group, $n=8$) and graft (control group, $n=4$; ICM group, $n=6$) HMGB1 level of syngeneic islet recipients were assessed at different time points by ELISA and IHC, respectively. In correspondence with *in vitro* data, the HMGB1 levels in the post-transplantation serum of islet recipients were significantly decreased by ICM treatment (**Figure 3-3**). Also, at 6-hour post-transplantation, IHC demonstrated that ICM treatment had significantly reduced the HMGB1 level (green fluorescence)
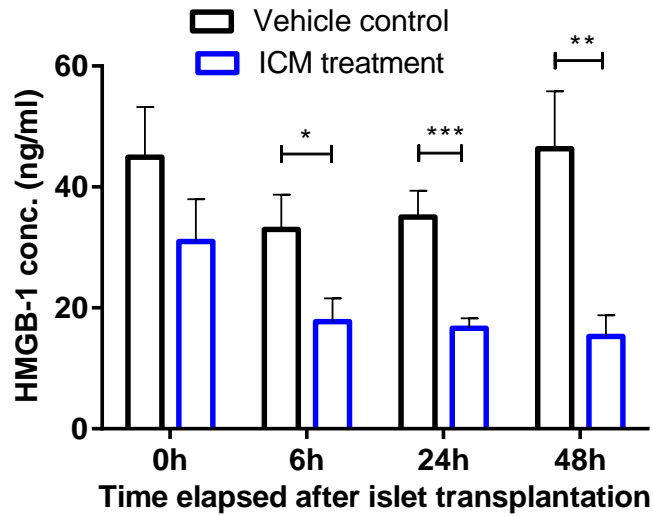
Figure 3-3. *In vivo* ICM effect on pancreatic islet recipient serum.
Serum HMGB1 level of diabetic BALB/c islet recipients. 300
IEQs of murine syngeneic islets were transplanted to diabetic
BALB/c mice, and DMSO or ICM was treated 1 hour before the
transplantation procedure and at 24 hours post-transplantation.
∗, $p<0.05$; ∗∗, $p<0.01$; ∗∗∗, $p<0.001$.

within the implanted islets as well as in the renal parenchyma (**Figure 3-4**). Insulin was simultaneously stained (red fluorescence) to verify the mass and the location of pancreatic beta cells. Altogether, it seemed that ICM was also efficacious *in vivo* to decrease the HMGB1 level in the islet transplantation model.

HMGB1 blockade by ICM results in enhanced viability *in vitro* and *in vivo*

As mentioned earlier, dying islets release HMGB1 into the extracellular milieu and it again could harm neighboring islet cells. Since ICM was successful in decreasing the HMGB1 level *in vitro* and *in vivo*, I further investigated the viability of islets under the effect of ICM. Incubation with ICM after routine islet isolation revealed that the ICM-treated group of islets were more viable than the control group (**Figure 3-5A**; $p<0.01$). Furthermore, TUNEL assays on the paraffin-embedded sections of implanted islets under the renal capsule demonstrated the prevalence of apoptotic islet cells (white arrows) in the vehicle-control group compared to the ICM-treated group (**Figure 3-5B**). In addition,
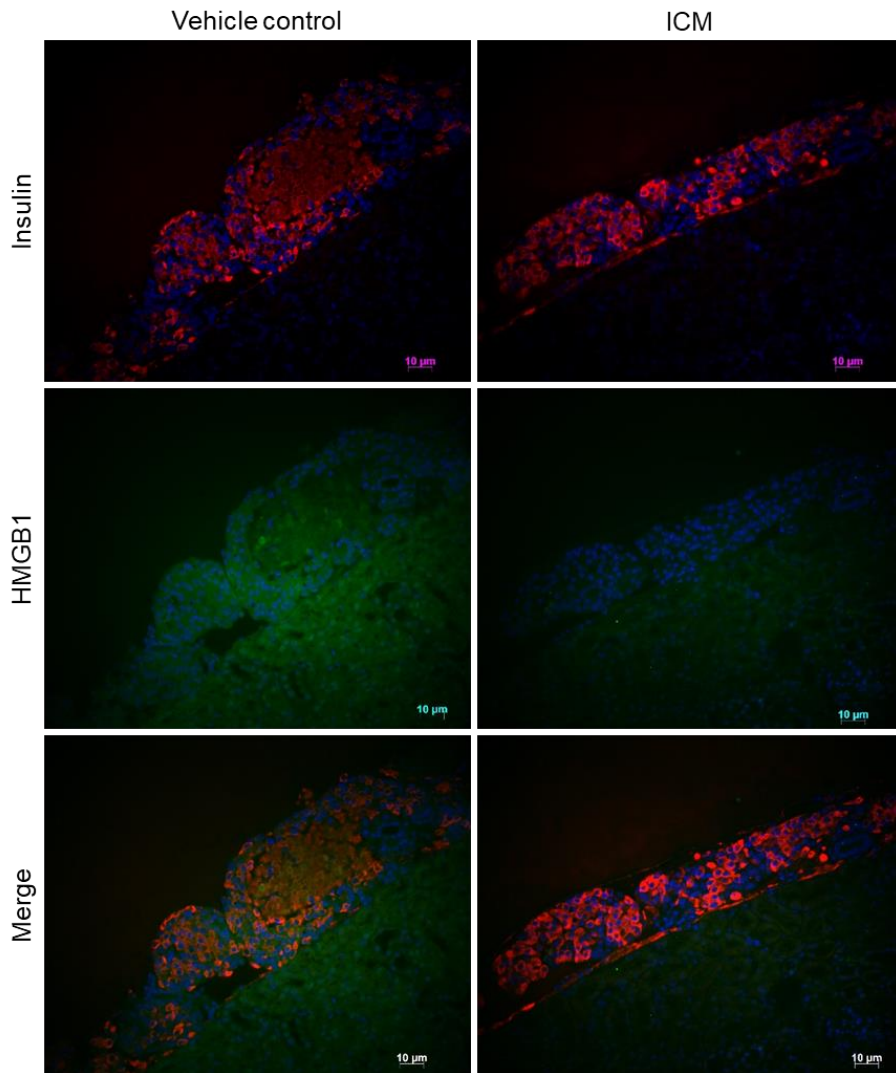
Figure 3-4. *In vivo* ICM effect on pancreatic islet recipient graft site.

HMGB1 (green) and insulin (red) levels in the islet graft at the renal subcapsular region at 6 hours post-transplantation.

A

B

	Vehicle control	ICM
H&E		
TUNEL		
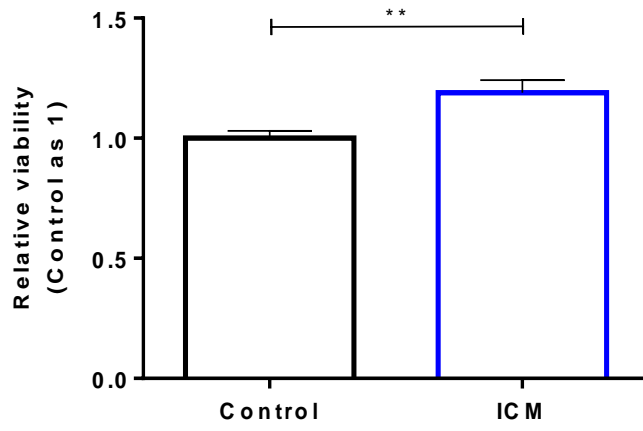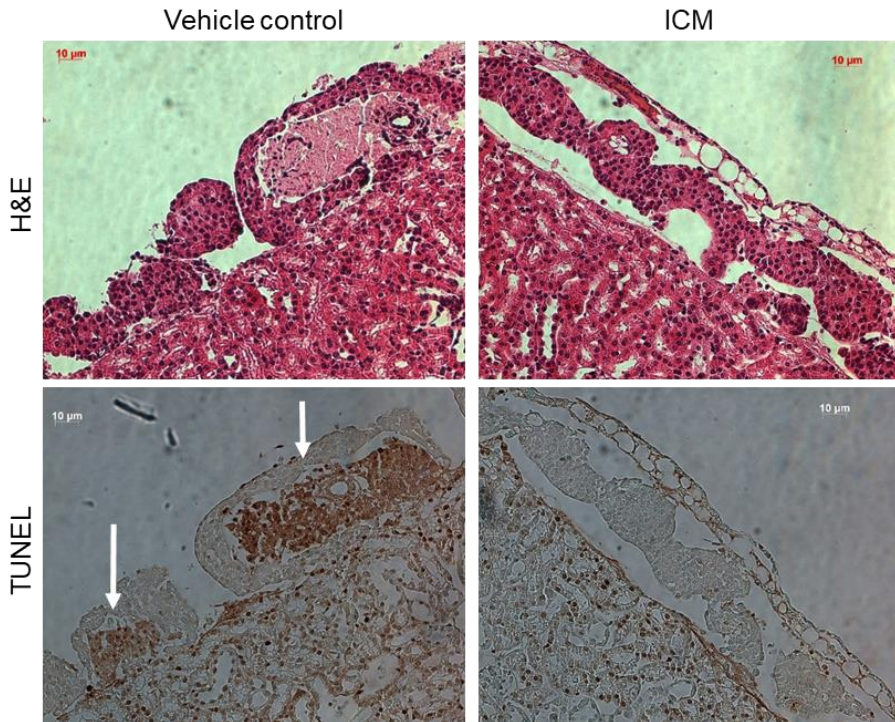
Figure 3-5. Effects of HMGB1 blockade by ICM on pancreatic islet viability.

(A) *In vitro* pancreatic islet viability demonstrated with CCK8 assay. Murine islets were isolated and treated with ICM (10 μM) or DMSO (1:2000) for 48 hours. Mean OD of the control group was used for normalization. **, *p*<0.01. (B) *In vivo* islet graft viability shown by TUNEL assay. Apoptotic islet grafts are in brown color (indicated by white arrows). Grafts were procured at 6 hours post-transplantation.

ICM treatment impeded hyperglycemia induction after STZ injection (**Figure 3-6**). This result confirmed HMGB1's role in beta cell destruction by STZ (67), and suggested that timely management of HMGB1 release might be effective in preventing the onset of T1D.

ICM administration has mass-sparing effect on syngeneic islet grafts

Since ICM was effective in HMGB1 blockade and islet cell viability enhancement, I investigated whether HMGB1 blockade by ICM could have the mass-sparing effect during the early period of islet transplantation. Thus, marginal mass murine syngeneic islet transplantation, a standard model to test an intervention's mass-sparing effect on the pancreatic islet graft, was performed. If ICM treatment significantly raised the ratio of euglycemic recipients, I could assume that ICM prevented the sub-optimal mass of pancreatic islets from destruction, and the HMGB1 blockade would be held responsible for the success. Certainly, the ICM treatment during the early periods of islet transplantation resulted in the complete cure of diabetes in the recipients compared to the vehicle control group which received
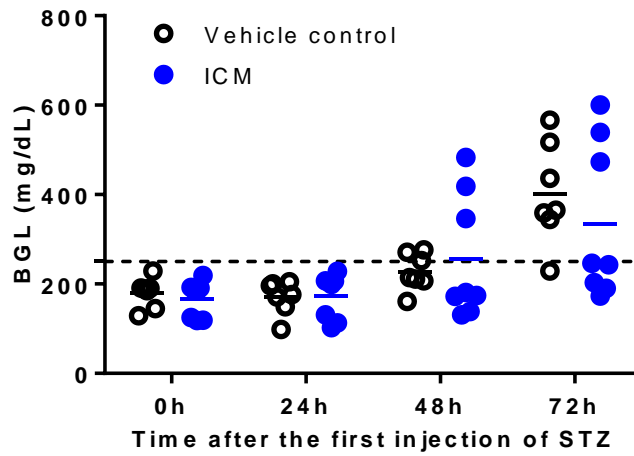
Figure 3-6. Evaluation of HMGB1 blockade on beta cell destruction by STZ.

T1D was experimentally induced in B6 mice with intraperitoneal injections of STZ (100 mg/kg, twice at hour 0 and hour 24). ICM (10 mg/kg, twice at hour 0 and hour 24) or DMSO was also injected intraperitoneally to the mice, just before STZ administration. The dashed line indicates 250 mg/dl, a threshold non-fasting blood glucose level to indicate hyperglycemia. BGL, blood glucose level.

only DMSO (p<0.001; **Figure 3-7**). Most of the ICM-treated recipients showed stable non-fasting blood glucose levels, and nephrectomy on 36 days post-transplantation proved that the islet grafts under the renal capsule were accountable for the glycemic control (**Figure 3-7A**). The marginal mass of islets cured only 2 out of 7 diabetic recipients which underwent the identical procedures without ICM treatment (**Figure 3-7B**). All in all, these data indicated that the ICM-treatment's effect resulted in the mass-preservation of the transplanted islets.

A
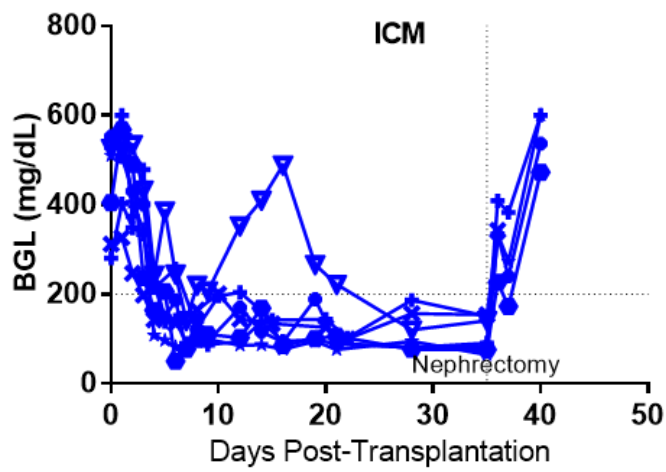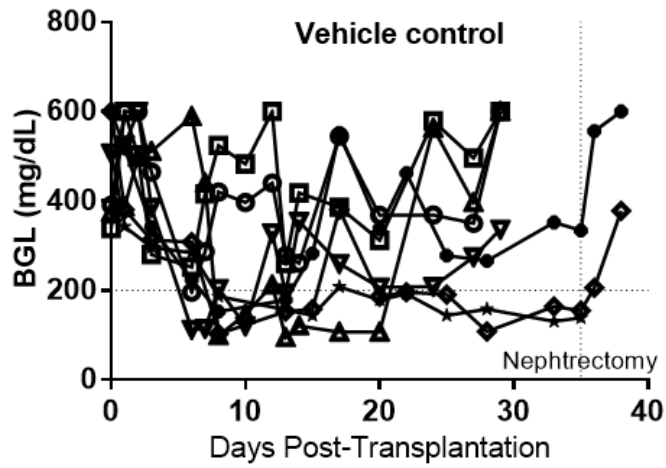
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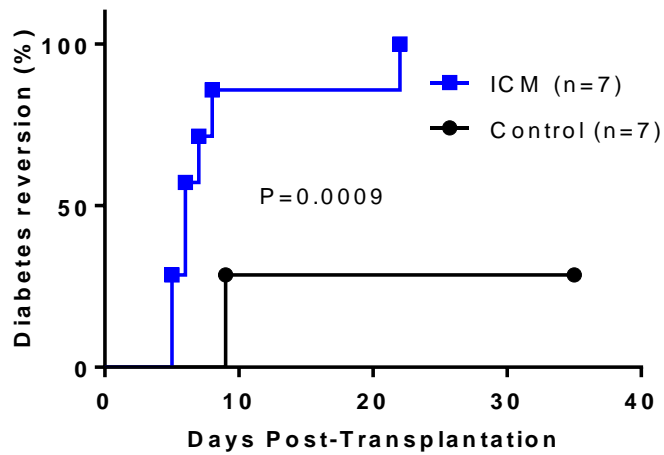
Figure 3-7. Effects of HMGB1 blockade by ICM on marginal-mass syngeneic islet transplantation.

(A) Blood glucose level follow-up of diabetic recipients implanted with a marginal mass of syngeneic islets with or without ICM treatment (10 μM). Nephrectomies were performed on 36 days post-transplantation to confirm that the euglycemia was attributed by the graft under the left kidney capsule. BGL, blood-glucose level. (B) Diabetes reversion rate of diabetic recipients represented as Kaplan-Meier curves.

DISCUSSION

Here, I tried to show that usage of ICM, a small-molecule inhibitor of HMGB1, has certain gains in murine islet transplantation. Recently, ICM was proven to be effective in the sepsis treatment (61), indicating that it could be used on the broader spectrum of HMGB1-secreting cells. I speculated that ICM could be used in pancreatic islet transplantation settings, where pancreatic islets act as a substantial reservoir of HMGB1 protein (21, 48) and increased serum level of HMGB1 is negatively correlated with the survival of islet grafts (29, 35). To my expectation, ICM could lessen the secretion of HMGB1 in stressful pancreatic islets *in vitro* (**Figure 3-2**), and lower serum and graft HMGB1 levels *in vivo* (**Figure 3-3 & 3-4**). The reduced level of HMGB1 correlated with enhanced viability of pancreatic islets (**Figure 3-5**) and HMGB1 blockade also ameliorated the collateral damage by STZ on pancreatic beta cells (**Figure 3-6**). Ultimately, ICM treatment significantly improved the outcome of marginal mass islet transplantation (**Figure 3-7**), suggesting its mass-sparing effect.

It has been reported that HMGB1 could activate the

dendritic cell-natural killer T cell-neutrophil axis, which ends up in IFN-γ-mediated islet cell death (21). In cardiac transplantation models, HMGB1 was shown to stimulate macrophage TLR4-IL 23-IL 17A axis and contribute to the neutrophil accumulation and ischemia-reperfusion injury (68). Thus, I decided to control HMGB1 because I wanted to prevent the vicious cycle that stems from this alarmin. Although I did not comprehensively observe the downstream effects of HMGB1 blockade, HMGB1 blockade by ICM treatment was fortunately effective in increasing the viability of islets and protected the islet grafts.

It would be interesting to study if, in syngeneic islet transplantation, the pretreatment of islets with ICM alone could produce the same outcome. The short-term administration (~3 hours) of ICM during the islet isolation procedure resulted in decreased levels of HMGB1 in the culture supernatant 48 hours later (**Figure 3-2B**), although the effect could have been attributed more to the ICM in the cell culture media. Mera *et al.* previously reported that the pretreatment of isolated murine islets with Na^+/Ca^{2+} exchanger inhibitor (SEA0400) resulted in lower level of serum HMGB1 and euglycemia in murine

syngeneic islet recipients (16). Considering that ICM exhibited a prolonged anti-inflammatory effect on LPS-stimulated a microglial cell line (60), I presume that it could function for the same duration on pancreatic islets and be as similarly effective as the SEA0400. The drawback would be that it would not stop cells other than islets from releasing HMGB1 to the extracellular environment as an intraperitoneal injection would do. Indeed, I observed that systemic ICM administration resulted in the reduced HMGB1 expression from the renal tissue as well (**Figure 3-4**).

In this sense, I think it would be meaningful to further examine the consequences of ICM treatment on islets, especially regarding the HMGB1 blockade mechanisms of ICM. ICM's acting mechanism depends on its ability to directly bind to HMGB1's NLS and stop it from cytosol accumulation (60). Interestingly, there have been reports on the cell-protective effects of cytoplasmic HMGB1 as a modulator of autophagy (69) albeit in limited tissues until now. Further research on this issue would be required to get a grasp of the feasibility of the hypothesis, the beta cell-protective role of cytoplasmic HMGB1. If cytosolic HMGB1 were indeed protective of beta cells and ICM

prevented it from conferring protection against cellular stress, then what I have witnessed would further emphasize HMGB1's role as an alarmin in islet transplantation. In other words, HMGB1's destructive effect as an alarmin would be predominant in syngeneic islet transplantation settings compared to its cell-protective effect.

I performed tests of ICM's effect by following the *in vitro* (10 μM) and *in vivo* (10 mg/kg) scheme which have already been validated by the previous studies (60, 61). Fortunately, the concentrations and dosages were also effective on islet beta cells. Still, it would be meaningful to assess the impact of differential concentrations of ICM, especially when higher concentrations of ICM could induce cell death via autophagy inhibition (70).

In conclusion, this study demonstrated that a small molecule inhibitor of HMGB1, ICM was successful in blocking the HMGB1 secretion from murine pancreatic islets *in vitro*, and it showed *in vivo* the islet mass-sparing effect in a transplantation model. This discovery calls for additional studies on the safe and efficient measures to preserve the mass of islet grafts in the

early period of transplantation, and I suggest that HMGB1 should

not be missed out on any one of them.

GENERAL DISCUSSION

Here, I have seen paradoxical actions of extracellular HMGB1 on murine islets and beta cells. As mentioned multiple times, HMGB1's detrimental actions on islet beta cells have been recognized well in the islet transplantation field where HMGB1 would almost always function as an alarmin. The destructive effect on beta cells was regardless of HMGB1's redox state or post-translational modifications. Surprisingly, the role of HMGB1 against islet beta cells in basal, homeostatic conditions remains vaguely known. Any rational researcher, including myself, would have guessed that extracellular HMGB1 would be unequivocally harmful to islet beta cells under any circumstance, based on previous knowledge. However, the results of my research indicate that HMGB1 could function differently in standard *in vitro* cultures. Actually, the concept that HMGB1 could serve protective roles to cells has been gaining recognition recently.

I speculate that these paradoxical results could be shown because of HMGB1's 'promiscuous' behavior, a term coined by Dr. Taniguchi's team to describe HMGB1's propensity to

bind to other molecules (49). In 2016, Son *et al.,* reported that HMGB1 could show anti-inflammatory effects when complexed with the complement component 1q (C1q), and suggested that this could be one of the major mechanisms in the immune system to terminate the inflammation (25). They suggested that the distinct effects of extracellular HMGB1 on monocytes were dictated by the HMGB1's relative ratio to its heteromeric counterpart, C1q: if there was a sufficient amount of C1q in a high-HMGB1 environment, then the HMGB1/C1q complex would bring forth anti-inflammatory responses. Correspondingly, the phenomenon I witnessed in the study on pre-existing HMGB1 within FBS could be explained: even though there was a certain amount of HMGB1 in FBS, the various factors (e.g. growth factors or nucleic acids) in the environment could work together with HMGB1 to deliver beta cell-favorable signals. On the other hand, when the HMGB1 levels were relatively low or high compared to the factors due to depletion or cellular release, respectively, the results could be unfavorable. In my case, a glimpse of the former phenomenon (relatively low HMGB1) is shown in Chapter 2, and the latter (relatively high HMGB1) in Chapter 3. Consequently, later analyses of the HMGB1's actions

on pancreatic beta cells should always include the possible heteromeric existence of HMGB1.

The location-specific role of HMGB1, though in a slightly different context, would be another factor to delineate the HMGB1's cell-protective function. In 2010, Tang *et al*. reported that HMGB1 was indeed a critical regulator of autophagy, where nucleus-to-cytoplasmic translocation of HMGB1 was strongly associated with autophagy promotion in cell lines under stress (51). In 2015, Zhu *et al*. confirmed similar effects of intracellular, cytoplasmic HMGB1 in colitis model (71). In their study, Zhu *et al*. demonstrated that cytoplasmic HMGB1 could act as a switch in intestinal epithelial cells which dictated their pro-autophagic or pro-apoptotic fate during inflammation-induced injuries. Interestingly, there have been multiple studies on the occurrences of autophagy-apoptosis transition in stressed beta cells (72-75). As these recent reports suggest the possibility of cell-protective actions of cytosolic HMGB1 within beta cells via autophagy modulation, the next study on this subject should be focused on the discovery of a similar mechanism in stressed beta cells.

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국문 초록

서론: 1 형 당뇨병은 자가면역 반응으로 인하여 췌도 베타세포가 파괴됨으로써 발생하는 내분비계 질환이다. 1 형 당뇨병 환자들은 외인성 인슐린 치료를 지속적으로 받아야하며 여러가지 합병증으로 인해 평생 고생한다. 의학 기술의 눈부신 발전에도 불구하고 1 형 당뇨병의 완치는 불가능한 상태이다. 다행히 2000 년 에드먼턴 그룹의 성공적인 췌도 이식은 저혈당무감작증과 같은 치명적인 합병증을 치료할 수 있는 가능성을 열어주었다. 그런데 장기이식 시 피할 수 없는 동종이식거부반응과는 별개로, 이식한 조직이 노출되는 산화스트레스도 극복해야 할 중요한 요소로 여겨진다. 그리고 이러한 스트레스로 유도되는 췌도 이식편의 손상은 선천면역반응을 유발하는 손상 연관 분자 패턴(damage-associated molecular pattern, DAMP)의 생성을 야기하며, 이는 다시금 이식편의 손상을 유발하는 것으로 알려져 있다. 다양한 DAMP 중 가장 잘 연구된 것은 진화적으로 잘 보존된 high mobility group box 1 (HMGB1)으로서, 췌도 이식편의 손상과 깊은 관련이 있는 것으로 알려져 있다. 현재까지의 수많은 연구 결과들로 HMGB1 이 췌도와 베타세포에 해로운 염증성 분자임이 입증되었으나, 여타 조직들에서는 HMGB1 이 조직을 보호하는 역할을 할 수도 있다는 사실도 보고된 바 있다. 따라서

HMGB1 이 췌도 베타세포의 생명 작용에 어떤 영향을 끼치는지 다양한 모델에서 연구해 볼 필요성이 있다.

방법: 본 연구를 위해 먼저 ELISA 기법이 개발되었으며, 세포배양액과 마우스 혈청에서 HMGB1 을 측정하기 위한 최적화 작업을 거쳤다. 이후 실제 세포배양 상층액과 마우스의 혈청에서 개발된 ELISA 측정법을 검수하였다. 또한 이를 활용하여 인간 HMGB1 을 측정할 수 있는지의 여부도 알아보았다. 한편, ELISA 개발 과정에서 소태아혈청이 ELISA 신호를 교란시킨다는 사실이 관찰되어, 이러한 현상이 측정기법과 세포배양에 있어서 어떤 의미를 가질 수 있는지 분석하였다. 소태아혈청에 기본적으로 존재하는 HMGB1 을 면역침강반응으로 제거하였고, CCK8 기법과 유세포 분석으로 생존력을 평가하였으며, glucose-stimulated insulin secretion 기법으로 기능상 차이를 분석하였다. 또한 재조합 HMGB1 을 배지에 보충하고 췌도 베타세포의 생존력과 기능도 알아보았다.

췌도 베타세포에 HMGB1 이 alarmin 으로서 주는 영향을 더 자세히 규명하고자, 저분자 HMGB1 억제제인 inflachromene (ICM) 을 처리하는 실험을 수행하였다. 마우스 췌도를 분리하고 배양하는 과정 중에 ICM 을 처리하고 HMGB1 의 분비량은 ELISA 로, 세포의 생존력은 CCK8 기법으로 분석했다. 인위적 당뇨의 유발과정 중 ICM 처리를 하고 당뇨의 유발 정도를 분석하였으며, 당뇨가 유발된 마우스에 동계 췌도 이식하는 과정 중에 ICM 처리를 하고 ELISA

와 면역조직화학 등으로 HMGB1 의 억제와 이에 따른 이식편의 생존을 평가하였다.

결과: 최적의 항 HMGB1항체의 조합으로 효율적이고 정확한 sandwich ELISA가 개발되었다. 본 ELISA 기법을 통해 세포배양 상청액과 마우스 혈청에서 HMGB1이 예상대로 측정되었으나, 소태아혈청이 본 측정법의 특이도와 민감도에 영향을 줄 수 있음이 확인되었다. 소태아혈청에 기본적으로 존재하는 HMGB1을 제거하고, 이를 이용해 마우스 췌도와 마우스 베타세포주(MIN6)를 배양했을 때, 생존력과 기능의 유의적인 저하가 관찰되었다. 또한 재조합 HMGB1으로 제거된 HMGB1을 보충했을 때(10 ng/ml) 이러한 현상이 회복되었다. 하지만 과량의 재조합 HMGB1으로 보충하거나(100 ng/ml), 혹은 HMGB1만을 첨가하는 것으로는 MIN6 세포의 생존력이 증가하지 않았다. 생체외 실험에서 마우스췌도와 MIN6 세포에 HMGB1 억제제인 ICM을 처리하였을 때 세포 내•외부의 HMGB1 수준이 저하되는 것을 관찰하였고, 마우스 췌도의 생존력도 증가되었다. 동물실험에서는, ICM 처리가 동계 췌도 이식 수혜 마우스의 혈청과 이식편에서 HMGB1 수준을 현저히 낮추고, 이식편의 생존력을 증대시킴을 확인했다. 동계 췌도 이식이 실행된 당뇨 마우스에 ICM을 처리하면, HMGB1 억제 효과가 모든 이식편의 생존에 크게 기여함이 관찰되었다. 또한 streptozotocin과 ICM이 동시에 처리된 마우스에서는, 고혈당증의

발생빈도가 저하됨을 관찰하였다.

결론: HMGB1 에 대한 1 형 당뇨병과 췌도 이식 모델에서의 기존 연구를 종합하면, HMGB1 은 췌도 베타세포에 해로운 역할을 하며, 따라서 HMGB1 을 억제하면 베타세포와 췌도 이식편 생존력이 향상할 것임을 예상할 수 있다. 본 연구를 통해, 저분자 HMGB1 억제제의 투여로 HMGB1 이 alarmin 으로서 작용하는 것을 예방하여 이식 초기에 췌도 이식편을 보호할 수 있음이 확인되었다. 하지만 최근의 일부 연구결과들은 유해하다고 알려진 HMGB1 이 세포와 조직에 이로울 수 있다는 증거들을 제시하고 있다. 본 연구에서도, 비록 그 상세 기전에 대한 연구가 더 필요하지만, 베타세포의 최적의 생존을 위해서는 배양액 내에 일정량의 HMGB1 이 필요함이 제시되었다. HMGB1 은 산화•환원상태, 위치, 다른 분자들과의 상대적 비율 등에 따라 다양한 기능을 할 수 있으므로, 췌도 베타세포에게 HMGB1 이 주는 영향도 다양하게 나타날 수 있다고 생각된다. 본 연구에서는 HMGB1 이 베타세포의 생존에 미치는 새로운 일면을 관찰하였는데, HMGB1이 췌도 베타세포에 미칠 영향을 명백히 이해하기 위해서는 세포질 내 HMGB1 의 기능과 역할에 대한 연구가 진행되어야 할 것이다.

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fetal bovine serum on murine pancreatic beta cell biology. 2020 Jan 14:1−8.)와 *Biochemical Biophysical Research Communications* (Chung H, Hong SJ, Choi SW, Koo JY, Kim M, Kim HJ, Park SB, Park CG. High mobility group box 1 secretion blockade results in the reduction of early pancreatic islet graft loss. 2019 Jul 5;514(4):1081−1086)에 출판 완료되었습니다.

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