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THE CARDIO-RENAL EFFECTS OF IL-33 TREATMENT IN MYOCARDIAL INFARCTION-INDUCED KIDNEY DAMAGE

by
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ABSTRACT
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Myocardial infarction (MI) is a major public health concern and a leading cause of acute kidney injury through the cardio-renal interrelationship, otherwise known as type I cardio-renal syndrome. Interleukin (IL)-33, a member of the IL-1 superfamily, is a nuclear alarmin released upon tissue damage and necrosis, binding thereafter to ST2 receptors, eliciting consequently an inflammatory response. Strong published evidence reported an emerging role of the IL-33/ST2 axis in different models of kidney diseases including drug-induced nephrotoxicity and kidney ischemia-reperfusion. Although the IL-33/ST2 axis was shown to be involved in the pathogenesis of kidney diseases, pre-clinical investigations indicated potential protective roles for the IL-33 signaling pathway. In this study, we aimed at investigating the effect of IL-33 administration on kidney damage at 4 and 7 days post-MI in C57BL6/J male mice. MI was induced by ligating the left anterior descending artery, followed by IL-33 (1μg/day) /vehicle (PBS) treatment for 4, 7 days post-MI. Cardiac systolic function and systemic inflammation were assessed, and kidneys were subjected to histological and molecular analysis. Cardiac hemodynamic assessment revealed exacerbated left ventricular systolic function characterized by a more significant decrease in EF and FS in the IL-33 treated group day 7 post-MI when compared to vehicle treated MI group. In agreement, cardiac sST2 mRNA expression levels markedly increased at day 7 in the IL-33 treated MI when compared to the vehicle treated MI group. Systemic inflammation assessment revealed a significant decrease in plasma TNF-α levels at 4 days but a substantial increase in plasma IL-1β at 7 days in the IL-33 treated MI group compared to the vehicle treated MI group. At the level of the kidneys, IL-33 treatment post-MI induced morphological alterations characterized by a significant decrease in Bowman's capsule area and glomerular retraction at day 4 only when compared to vehicle treated MI group. Nevertheless, a significant decrease in renal fibrosis with IL-33 treatment 4 days post-MI relative to vehicle treated MI group was faced by a significant increase at day 7. Molecularly, total renal ROS score significantly increased in IL-33 treated MI group when compared to vehicle treated MI mice, while remained unchanged at day 7. Conversely, IL-33 treatment significantly decreased renal pro-fibrotic markers including α-smooth muscle actin (α-SMA) and collagen 3 (COL3) and the apoptotic regulatory genes such as Bcl-2-associated X protein/B-cell lymphoma 2 ratio 4 days post-MI. At 7 days post-MI however, renal α-SMA and COL3 significantly increased in the IL-33 treated MI group while no

significant change in BAX/BCL2 ratio was observed. Metabolically, IL-33 treatment significantly increased NAD and SIRT3 mRNA expression levels at day 4, but not at day 7 post-MI. Collectively, our findings reveal that although IL-33 treatment seems to improve renal homeostasis as early as 4 days post-MI, this protection seems to be offset as early as day 7 post-MI which correlates with aggravating adverse LV remodeling. Further investigation is required to conclude whether the adverse impact of IL-33 treatment on the kidneys is direct or fueled by the exacerbation of cardiac remodeling at 7 days post-MI.

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INTRODUCTION

A. Cardio-Renal Physiology

Cardio-renal integrative physiology is of utmost importance for hemodynamic stability and normal body function [1]. The kidney, specifically, denotes a special significance, as its relation with the heart is fine-tuned and bidirectional. For instance, while the heart affect kidney function by governing perfusion key parameters (i.e cardiac output), cardiac performance is directly affected by the fluid and electrolyte homeostasis that is directly adjusted by the kidneys [1]. This crosstalk is controlled by multiple physiological systems incorporated to regulate the cardiac output, volume status, and vascular tone. The renin-angiotensin system (RAS), the autonomic nervous system (ANS), and the natriuretic peptide (NP) system are recognized as major conservatives of this cardio-renal function assisted together in a delicate homeostasis [2]. Stretch receptors in the carotid sinus and the aortic arch referred to as the baroreceptors are responsible for the short-term regulation of the blood pressure eliciting in seconds to minutes either the sympathetic or the parasympathetic modulatory effects [3]. A long-term recovery, however, importantly involves renal mechanisms including the SNS and RAAS. Low blood pressure and sympathetic nerve cells acting on the β1-adrenoreceptors can directly stimulate renin release from the juxtaglomerular apparatus, or indirectly by prostaglandins released from neighboring macula densa cells when sensing low sodium [4]. Renin, a distant acting enzyme, chops off the precursor hormone angiotensinogen secreted by the liver into

angiotensinI. As angiotensin I passes through the bloodstream, it is further metabolized by the angiotensin-converting enzyme (ACE) generated by endothelial cells into angiotensin II, the very active hormone inducing constriction of blood vessels via AT1 receptors and water and sodium retention by the kidneys [5] [6]. These strategies of raising blood pressure are further sustained by aldosterone and the antidiuretic hormone (ADH) released through angiotensin II action on the adrenal and pituitary glands, respectively [7] [8]. The NP, however, serves as a counter-regulatory system by inducing vasodilation, suppressing renin, aldosterone and norepinephrine release, and exerting diuretic and natriuretic effects on the kidneys [9].

B. Myocardial Infarction

1. Definition

Myocardial infarction (MI) is a major public health concern and a leading cause of acute kidney injury. Although it may go undetected, it may also severely deteriorate cardiac function and result in sudden death. MI is characterized by the necrosis of cardiac muscle secondary to prolonged ischemia initiated by a decrease in blood flow [10]. Atherosclerosis represents a major substrate of MI. When a plaque ruptures it leads to platelets aggregation and the formation of a life-threatening thrombus narrowing the artery [11]. The starving muscle eventually dies and a primary intervention would be salvaging the infarcted myocardium as soon as possible within a critical time frame [12].

2. Epidemiology

Acute coronary syndrome (ACS) represents a leading cause of mortality and morbidity. It is a major precipitant of HF, accounting for almost 42% [13]. On top, MI represents the prominent form of ACS [14]. The 2019 report of the American Heart Association (AHA) computed that approximately every 40 seconds, one American will have an MI [15]. The average age of the first incidence of MI is 65.6 years old for men and 72 years old for women and the gender-based disparity shows that MI is more prevalent in men than in women [15] [16] [17]. Nevertheless, several studies consistently indicated a higher mortality rate in females compared to males most often about 40% higher, in which the paucity of MI occurrence in young females seems highly relatable [18]. This recognition was shown to be restricted to younger ages and is invariable at older ages which indicates for a possible sex-age interaction [19].

3. Adverse Ventricular Remodeling

Although a pronounced success in reducing short term mortality after myocardial infarction accompanied the use of the PPCI and advanced pharmacological therapies, adverse cardiac remodeling still occurs in a significant portion of the surviving patients[20, 21] Besides affecting 30% of MI cases, it highlights the increased risk of heart failure and poor prognosis [22]. The mammalian heart possesses a poor regenerative potential projecting the importance of efficient remodeling in limiting the concomitant vulnerability to heart failure. Clinically, adverse remodeling manifests as increased ventricular dilatation and thinning, myocardial hypertrophy and cardiac dysfunction [23]. The process involves

profound modifications on the geometry, structural, and the functional level, not restricted to the infarct region but also extends to the viable area [23]. The fundamental determinants of the extent of infarct expansion are the infarct size, as well as the efficiency of the healing process that if uncontrolled or influenced by comorbidities, will end up in further complications such as cardiac rupture and aneurysm formation [24]. On that end, an emphasis was placed on the settlement of the inflammatory and reparative phase and the transition between them as this is critically decisive. With a prolonged inflammatory response, excessive loss of myocytes in the infarcted area loads an intensive function on the remote myocardium which adapts by undergoing hypertrophy. Despite the increase in the contractile units, the systolic function is depressed due to reduced systolic sarcoplasmic calcium release and desensitization of myofilaments to calcium [25]. Furthermore, augmented matrix-degradation facilitates chamber dilatation and infarct expansion to the non-infarcted myocardium, on the other side excessive matrix-deposition leads to wall stiffness and impaired diastolic function [26].

C. Cardio-renal Syndrome (CRS)

1. CRS definition

Cardio-renal interdependency imposes a deranged relationship, whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other organ, a pathology referred to as the ‘Cardio-renal Syndrome’ [27]. A systematic review revealed that around 30% of 80,098 hospitalized and non-hospitalized HF patients developed moderate to severe kidney impairment [28]. Besides, it is well noted that patients

with chronic kidney disease are at high risk for cardiovascular complications including MI and HF [29] with mortality mostly attributed to cardiac causes rather than the renal disease itself [30]. Being clinically a common manifestation comprising a wide spectrum of disorders, CRS is phenotypically streamlined into 5 subtypes based on the primary failing organ and the severity of the disease [31].

2. CRS Phenotypes

- Type I: acute cardiorenal syndrome

Acute heart failure (e.g. acute decompensated heart failure, acute coronary syndrome (MI), acute cardiogenic shock) resulting in acute kidney injury

- Type II: chronic cardiorenal syndrome

Chronic cardiac dysfunction (e.g. chronic congestive heart failure) causing progressive and potentially permanent chronic kidney disease

- Type III: acute renocardiac syndrome

Primary worsening of renal function (e.g. acute kidney ischemia or glomerulonephritis) causing acute cardiac dysfunction (e.g. heart failure, arrhythmia, ischemia)

- Type IV: chronic renocardiac syndrome

Primary chronic kidney disease (e.g. chronic glomerular or interstitial disease) contributing to decreased cardiac function, cardiac hypertrophy and/or increased risk of adverse cardiovascular events

- Type V: secondary cardiorenal syndrome

Acute or chronic systemic disorders (e.g. diabetes mellitus, sepsis) causing both cardiac and renal dysfunction [32].

3. Epidemiology

Cardio-renal syndrome type 1 (CRS1) is prominently observed in the clinical setting. Although the cardiac etiologies were clarified, the disparity in defining AKI, the timeframe of investigation, and the heterogeneity of selected populations led to inconsistency in the incidence of CRS1, and therefore the epidemiological view lacks the precision. For instance, the incidence of CRS1 in acute coronary syndrome and acute decompensated heart failure ranges from 9-19% and 20-45% respectively [33-36]. Consequently, the Acute Dialysis Quality Initiative (ADQI) group established a consensus definition for AKI called RIFLE criteria [37], modified thereafter by the Acute Kidney Injury Network (AKIN) [38] and KDIGO group[39]. Based on the primary causes of CRS1, a systematic review done by Vandenberghe and colleagues reported a higher prevalence in ADHF (47.4%) relative to ACS (14.9%) and cardiac surgery (22.1%) [39]. Despite this frequent occurrence in ADHF, CRS1 in ACS is associated with in higher in-hospital stay and mortality[40]. In MI particularly, renal dysfunction denotes clinical and prognostic relevance. Approximately 20% of patients hospitalized with acute MI suffer from AKI which increases to more than 50% when complicated with a cardiogenic shock [40] [41]. AKI represents an independent predictor of mortality and morbidity in the short and long term[33] [42] [43] and patients are highly susceptible to adverse cardiovascular outcomes

and readmission[44]. Even minor changes in serum creatinine associate with increased long term mortality, longer in-hospital stay, high cost, and end-stage renal disease progression [44, 45].

D. CRS1 Pathophysiology

The substantial burden of acute kidney injury is very high in the setting of MI [44] [33]. In addition to the increasing rates of risk factors on a global scale including, obesity, diabetes, and hypertension [46], the poor insight into AKI pathogenesis impedes the development of highly reflective diagnostic and targeted-treatment approaches. Traditionally, renal hypo-perfusion from reduced cardiac output deemed to map this pathogenesis with a lot of circulating studies targeting this concern [47] [48] [49]. At the onset of MI, cardiac systolic dysfunction leading to a reduced cardiac output (CO) result in poor renal perfusion, and consequently in decreased glomerular filtration rate (GFR) [50]. When renal afferent arterial flow decreases, the RAAS, the SNS, and vasopressin secretion become activated for compensation [51, 52] . However, in this case, the activation perpetuates and overwhelms the endogenous vasodilating factors, and the kidneys become resistant to the NP system, breaking the physiological balance [53] [54]. Consequently, vasoconstriction with extensive fluid retention results in venous congestion and kidney damage. Several clinical studies demonstrated that elevated central venous pressure increases renal backward pressure reducing therefore renal blood flow and GFR, making venous pressure a critical player in CRS1 pathophysiology [50] [55] [56]. In MI, left ventricular ejection fraction (LVEF) can independently predict AKI [57]. Additionally, a

retrospective study showed that the concomitant elevation of CVP and decreased LVEF is a high-risk factor that results in a 10-fold increase in the incidence of AKI in patients with ST-elevated MI (STEMI) [58]. In the past, contrast media administration has been considered a major cause of AKI that significantly correlates with contrast volume [58]. Though, recent studies showed controversial results pointing out a more complex multifactorial pathophysiology [57, 59]. Investigations have uncovered the critical contribution of systemic inflammation and endothelial dysfunction in AKI [60]. For instance, Cosentino et al. detected that the admission high-sensitivity C-reactive protein (CRP) in patients with MI strongly associates with AKI development and severity [61]. In support, Fujii et al. showed that CRP promotes the expression of adhesion molecules, depletes vasodilators (NO), and impairs the antioxidant defense provoking endothelial dysfunction, a well-reported mechanism in AKI development [62]. In addition to the enhanced inflammatory response, STEMI patients with AKI have increased oxidative stress and sympathetic nervous system activation suggesting a synergistic act in enhancing AKI development [63].

E. AKI Manifestation in MI

1. Functional Alterations

The deterioration in kidney function among hospitalized patients may refer to multiple factors that include hemodynamic alterations, the use of contrast media, and drug toxicity such as loop diuretics [59]. Clinical studies have based their assessment of kidney function on serum creatinine and GFR. As mild changes in serum creatinine showed high

prognostic relevance, early detection remains a challenge for timely effective treatment and risk stratification [64]. Cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) have emerged as promising markers of renal dysfunction with prognostic significance in cardiac diseases and were more sensitive than serum creatinine [65, 66]. Moreover, microalbuminuria, a measure of kidney damage, has been demonstrated to define high-risk populations in addition to kidney function[67] [68]. In line with the clinical findings, MI induction in rats significantly reduced the EF and FS after week-1, during which GFR significantly decreased [69]. The classical compensatory mechanisms for hemodynamic impairment (SNS, RAAS) by potently reducing the overall kidney blood flow may attribute to the early decline in GFR. Additionally, Dokkum et al. reported more severe proteinuria in unilaterally-nephrectomized rats with mild MI relative to those with moderate MI [70].

2. Structural Alterations

The kidney's vulnerability to injury is in part related to the tubule-blood vessel structural association. The proximal tubule incessant demand to generate ATP via oxidative phosphorylation makes it the most sensitive cellular entity prone to hypoxia and cellular death. Moreover, a glomerular tuft subjected to extensive vasoconstriction due to hypoperfusion would expectedly collapse [71]. In agreement, Lu Et al. reported the presence of renal dysfunction with marked tubular and glomerular swelling and fibrosis 3-days after permanent ligation of the left coronary artery [72]. An increase in renal fibrosis also was observed at week-1 and reached maximal at week 16 in MI rats. Although hemodynamic recovery was detected post-week 1, GFR recovered at the 8-week time point

and re-established at 16-week. Nevertheless, transient renal dysfunction is frequent in MI [73]. Moreover, fibrosis represents a pivotal hallmark that can directly affect kidney function irrespective of the primary insult that developed kidney disease, which may explain the re-strike of GFR deterioration [74]. Additionally, tubular kidney injury molecule-1 (KIM-1), a biomarker upregulated in proximal tubular injury, was significantly increased in tubules of the MI rats [69]. Interestingly, KIM-1 peaked at week-1 and showed a similar biphasic pattern with GFR with opposite direction at most time points, which indicates KIM-1 potential use as an early biomarker for detecting kidney injury and following up the disease progression.

3. Molecular Alterations

a. Inflammation

Systemic inflammation predicts mortality in patients with AKI [75]. Acute MI induces renal inflammation evidenced by the upregulation of the inflammatory cytokines (IL-1β, TNF-α, and IL-6), VCAM-1 expression in glomerular endothelial cells, and leukocytes recruitment in the kidneys of MI mice after 24hr of induction [76]. VCAM-1 upregulation reflects endothelial activation that enhances endothelial-leukocyte interaction and increases local inflammation. Endothelial dysfunction represents a target and a provoker of inflammation [77]. Therefore, MI acute inflammatory response may impair endothelial function and initiate renal damage[61] [78]. Anzai et al. reported a significant increase in WBC count and systemic inflammation (CRP, IL-6) in STEMI patients with AKI relative to non-AKI [63]. Moreover, compelling evidence presents an association

between local and systemic inflammation in MI (such as peripheral monocytosis, C-reactive protein, IL-1β, TNF-α, and IL-6) and adverse outcomes and remodeling [79]. These findings suggest the implication of inflammation in a positive feedback loop.

b. Oxidative stress

While small amounts of ROS are required for basic biological processes such as growth, proliferation, and cell survival, excessive ROS can initiate or aggravate kidney damage. In the vasculature, ROS causes endothelial dysfunction and promotes inflammation and leukocytes recruitment [79]. By inhibiting the endothelial cells' ability to synthesize NO, ROS increases the vasoreactivity response to angiotensin II, endothelin-1 (ET-1), and sympathetic nervous system. Reciprocally, as proinflammatory cytokines, angiotensin II, and aldosterone levels culminate, ROS generation gets further stimulated [79, 80]. This Excess ROS can reduce tubules Na reabsorption, alter gene expression, increase glomerular basement membrane permeability, and induce cellular death [81]. Moreover, accumulating evidence shows ROS contribution in renal fibrosis by enhancing the TGF-β/SMAD pathway [82] [82]. Anzai et al revealed the presence of significantly higher levels of serum MDA-LDL in patients with AKI compared with non-AKI suggesting an essential contribution of oxidative stress in kidney injury development [63]. In support, LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1) knockout mice subjected to LAD ligation showed significantly improved cardiac and renal function with a marked reduction in renal inflammation, histopathology, and fibrosis compared with the wild type [72].

c. Fibrosis

Fibrosis is a sequential event of increased inflammation, oxidative stress, and neurohormonal activation that causes stiffness and loss of function. Primarily, the outcome of all progressive kidney diseases is represented by interstitial fibrosis. The infiltration of inflammatory cells such as monocytes, macrophages, lymphocytes, and dendritic cells and the release of danger molecules including ROS and profibrotic cytokines create a platform for activation and expansion of matrix-producing cells, principally the fibroblasts [83]. α-SMA expression denotes the activation of fibroblasts to myofibroblasts that extensively deposits matrix-components predominantly fibronectin and collagen type I and type II. Coherently, Lekawanvijit et al. reported an increase in macrophages infiltration and the expression of the profibrotic TGF-β and SMAD proteins along with interstitial fibrosis in kidneys with deteriorated function at 1-week post-MI [69]. Angiotensin II, besides being involved in the inflammatory process, it increases the expression of TGF-β and may enhance the differentiation of fibroblasts to myofibroblasts contributing to renal interstitial fibrosis [69].

F. Interleukin (IL-33)

1. Discovery

Interleukin (IL-33) is a cytokine, which belongs to the IL-1 superfamily. The first attempt in its discovery was through the recognition of an upregulated gene in vasospastic cerebral arteries after a subarachnoid hemorrhage back into 1999, and was named “DVS27” [84]. In 2003 a protein abundantly expressed in high endothelial venules that supports the

migration of lymphocytes to secondary lymphoid organs was detected and named called nuclear factor from high endothelial venules NF-HEV [85]. Two years later, Schmitz et al determined that DVS27 and NF-HEV are the same molecule which was subsequently identified as a member of IL-1 family and a natural ligand for the orphan receptor ST2 and termed IL-33 accordingly [86]. This discovery launched an intensive research for delineating IL-33's cellular targets and mediated actions in pathogenesis [86].

2. Structure

In humans, the gene encoding IL-33 is located on chr9p24.1. The full-length protein is composed of 270 amino acids with a molar mass of ~30kDa. Whereas the murine IL-33 gene is located on the chr19qC1 region and comprises 266 amino acids corresponding to a protein of 29.9kDa. IL-33 protein has two terminal conserved domains, the N-terminal nuclear domain, and the C-terminal IL-1-like domain, separated by a divergent central part [84, 86]. The helix-turn-helix is a conserved pattern in its N-terminus (amino acids 1-65) containing a nuclear localization sequence and a DNA-binding motif responsible for tethering IL-33 in the nucleus [87]. IL-33 associates with chromatin by a protein-protein interaction between its chromatin-binding motif (amino acids 45-53) and the nucleosome acidic patch formed by the histone heterodimer H2A/H2B [88]. Like other IL-1 family members, the three‑dimensional structure of its C-terminus adopts a β-trefoil structure [89]. The crystal structure of IL-33/ST2 interaction revealed two critical binding sites on IL-33 which was shown to crucially involve charge complementarity allowing IL-33 to approach and bind the three Ig-like domains of ST2 receptor [90].

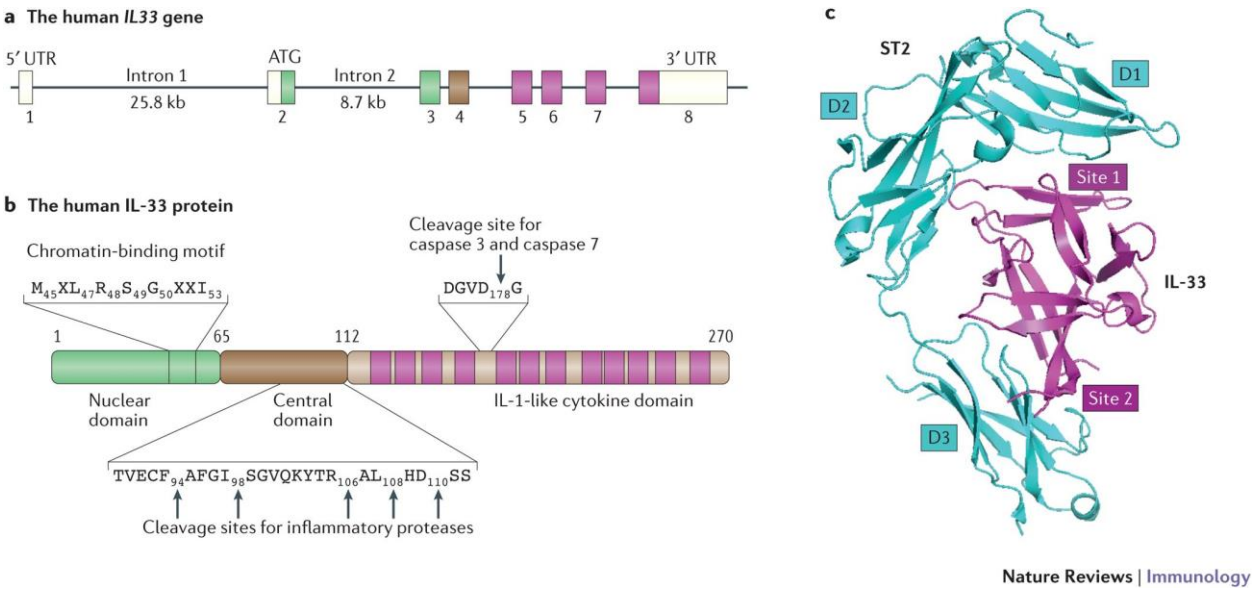

Figure 1: Interleukin-33 gene and protein structure

a | The human interleukin-33 (*IL33)* gene is located on the short arm of chromosome 9 at 9p24.1, whereas its mouse counterpart is found on the syntenic chromosome 19qC1 region [75] [76]. A large intron (>25 kb; intron 1) separates the first non-coding exon (exon 1) from the first coding exon (exon 2). **b** | Alignment of human and mouse IL-33 sequences (52% identity over 270 residues) [75] [76] revealed that the IL-33 protein is composed of two evolutionary conserved domains (the nuclear domain and the IL-1-like cytokine domain) that are separated by a highly divergent linker region in the centre (the central domain). Chromatin-binding motif and cleavage sites for caspases and inflammatory proteases are indicated. **c** | The crystal structure of the IL-33–ST2 (suppression of tumorigenicity 2) complex is shown (Protein Data Bank ID: 4KC3 [81]). Two ST2-binding sites were identified in IL-33 and acidic residues Glu148 and Asp149 at site 1 and Glu165 at site 2 were found to have a crucial role in high-affinity binding by forming specific salt bridge interactions with basic residues of ST2 [81]. UTR, untranslated region. [91]

3. Expression and Release

In steady-state IL-33 is widely expressed in several organs including the heart, kidney, liver, spleen, and lungs. Unlike conventional cytokines, the analysis of IL-33 sources at the cellular level revealed predominant restriction to the nuclei of endothelial cells, epithelial cells, and fibroblasts of normal human tissues and thus mostly in tissue

barriers [92] in addition to few immune cells mainly activated dendritic cells and macrophages [93]. Unlike humans, IL-33 is only detected in the endothelial cells of the vascular tree of inflamed, but not, normal mouse tissues [94]. Under pathological conditions, Il-33 expression increases in multiple diseases including skin diseases, inflammatory bowel diseases, asthma, and rheumatoid arthritis [95]. Unlike the known classical pathway (ER-Golgi secretory pathway) of cytokines release, IL-33 is released in full length to the extracellular space as a nuclear alarmin in response to tissue damage, necrosis, or mechanical stress [96].

4. Bioactivity Regulation

IL-33 is modulated by several endogenous mechanisms. The sequestration of this protein in the nucleus is maintained by its N-terminus and is crucial for regulating IL-33 mediated inflammatory response and tissue homeostasis [97]. Additionally, in the settings of cellular apoptosis, caspases 3 and 7 cleave IL-33 at a consensus site of cleavage in the IL-1 cytokine domain, hence rendering IL-33 inactive [98].

Once released into the extracellular space, full-length IL-33 exert a modest biological activity that is potentiated after cleavage by inflammatory proteases (cathepsin G, neutrophil elastase, chymase, or tryptase) released from neutrophils or mast cells invading the inflamed tissue [99] [100]. IL-33 activition can be hindered by oxidation of cysteine residues and the formation of two disulfide bridges in the IL‑1‑like cytokine domain disrupting the ST2 binding site shortly after its release [101].

G. IL-33 Receptors and Novel Signaling

ST2 is encoded by the IL1RL1 gene. In human, it is localized on chromosome 2 and spans ~40kb. Homologous were found in the genomes of mice, rats and fruit flies. It produces two major variants by alternative promoter activation and splicing from the same primary transcript. [102]

1. ST2L

ST2L is the full length transmembrane isoform of ST2 made up of an extracellular domain of three linked immunoglobulin-like motifs, a transmembrane domain, and a Toll/IL-1 receptor (TIR) cytoplasmic domain [103]. It is considered the biological receptor of IL-33 involved in innate and adaptive immunity through its wide expression in variety of hematopoietic cells. Among the major targets of IL-33 are the constitutively ST2-expressing tissue resident mast cells, regulatory T cells (Tregs) and innate lymphoid cells (ILC2s). In addition to other cell types including Th2 cells, M2 polarized macrophages, basophils, eosinophils, natural killer T (NKT), and invariant natural killer (iNKT) cells [104].Thus, IL33 is portrayed as a pleiotropic immunomodulator that mediates both inflammatory and repair responses, suggesting a janus-like effects that varies within the disease context and progression [105] [106].

2. sST2

sST2 is the truncated soluble form of ST2 receptor lacking the transmembrane domain and the cytoplasmic domain with a distinctive C-terminal [89]. sST2 functions as a decoy receptor of IL-33, limiting therefore Il-33 bioactivity through ST2L receptor [107]. In humans, sST2 is produced spontaneously by cells in the kidneys, heart, and lungs [108]. In vitro, sST2 synthesis and release are amplified in mast cells following activation with IL-33 [109]. Moreover, mechanical strain and proinflammatory cytokines such as TNF-α and IL-1β were shown to cause enhanced sST2 expression in the lungs of epithelial cells and cardiac myocytes [108].

3. Mode of action and signaling

IL33 is a dual function molecule that can act as a tissue-derived nuclear alarmin and as a chromatin-binding transcription regulator. Although IL-33 transcriptional regulatory properties are not fully elucidated, the overexpression of Il-33 is linked to transcriptional repression [87] [110]. As a cytokine, IL-33 signals the presence of damage to local immune cells. Once released, IL33 binds to ST2 receptor inducing therefore a conformational change and the formation of a heterodimeric complex between ST2 and the co-receptor IL-1 receptor accessory protein (IL1RAcP), leading to the dimerization of the TIR domain [111], Myeloid differentiation primary-response protein 88 (MyD88 adaptor), IL-1R-associated kinase 1 (IRAK1) and IRAK4, and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) are then recruited. This complex, in turn, induces the

activation of the downstream NF-κB and MAP kinases (ERK, P38, and JNK) signaling pathways triggering pleotropic responses. [91]

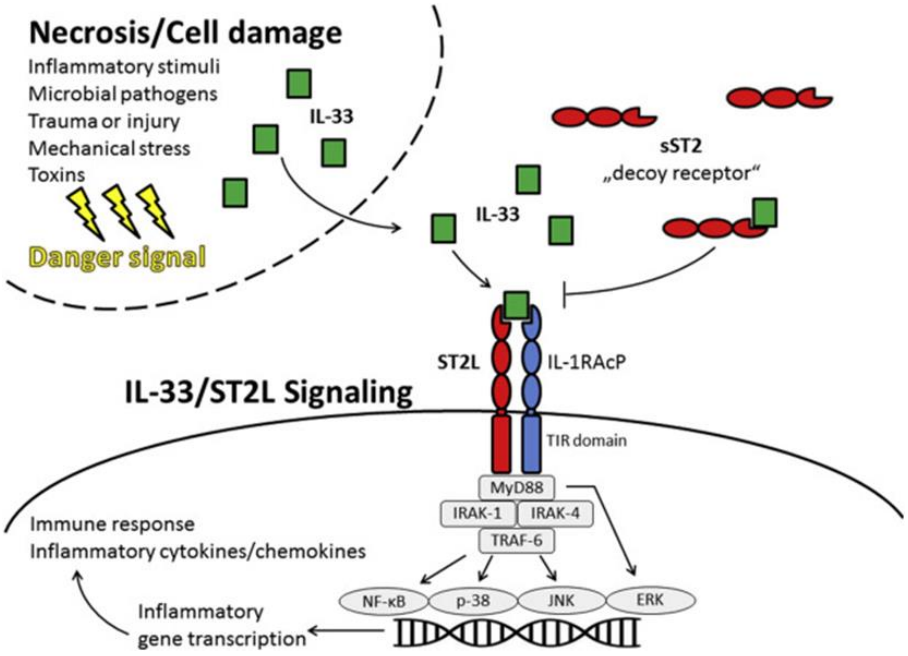

Figure 2: Interleukin-33/ST2L signaling

Damage to stromal cells can induce necrosis and release of full-length IL-33 (active IL-33) which can activate the heterodimeric ST2L/IL-1RAcP complex on a variety of immune cells or can be neutralized by sST2, which acts as a "decoy" receptor for IL-33. On activation of the ST2L/IL-1RAcP complex, signaling through the Toll-IL-1 receptor domain is induced. By activation of diverse intracellular kinases and factors, this leads to an inflammatory gene transcription and ultimately to the production of inflammatory cytokines/chemokines and an adequate immune response. [107]

H. IL-33 and MI

IL-33 biological relevance in the context of myocardial infarction gained insight after its decoy receptor, sST2, emerged as a potential novel cardiovascular biomarker, with accumulating evidence linking sST2 levels to adverse ventricular remodeling, worsened prognosis, and increased mortality on the short and long term post-MI [112] [113] [114].

These observations raised the hypothesis that IL-33/ST2 signaling mediates a cardioprotective role in the heart by the virtue of its canonical model. In the human adult heart, IL-33 is constitutively expressed in the nuclei of cardiac endothelial cells, cardiac fibroblasts, cardiomyocytes, and coronary artery smooth muscle cells and is released during cellular injury [115]. IL-33 expression by these cells is also inducible by inflammatory cytokines including IL-1b, TNF-α, and IFN-γ [115]. Furthermore, IL-33 and sST2 are induced in cardiomyocytes and to a higher extent in fibroblasts following biomechanical strain [116] [117] [118]. In vitro studies have addressed some beneficial effects of the IL-33/ST2 system on cardiac myocytes. For instance, IL-33 was found to inhibit the hypertrophic effect induced by angiotensin and phenylephrine on rat neonatal cardiomyocytes, which was reversed by the blockade of the ST2L receptor or the addition of sST2 [117]. In Another study, IL-33 showed up an antiapoptotic effect on neonatal rat cardiomyocytes induced with hypoxia associated with a reduction in cleaved caspase-3 and an upregulation in the expression of antiapoptotic cytokines (cIAP1, XIAP, survivin, Bcl-2, and Bcl-xL) [119]. In the setting of MI, however, findings are controversial. While some animal studies revealed a protective role, others showed that IL-33 might be detrimental to the heart. In a rat ischemic/reperfusion model a reduction in fibrosis, infarct size, and apoptosis was observed with the administration of IL-33 [119]. In an MI mice model, IL-33 treatment reduced ventricular dilatation, improved contractile function, and increased survival in wild-type mice but not in ST2−/− mice [119]. In addition to providing structural and functional protection, IL-33 has demonstrated also to cause a reduction in macrophages infiltration and proinflammatory cytokines and

chemokines production including IL-1β, IL-6, and TNF-α, and to mediate the inhibition of p38 MAPK phosphorylation and NF-κB activation [120]. Although the aforementioned studies suggest that IL-33 attenuates adverse ventricular remodeling post-MI, a recent study documented an aggravation of ventricular remodeling with IL-33 treatment post-MI. This was evidenced by the detection of severe cardiac dysfunction, ventricular dilatation, and infarct expansion, and increased apoptosis and mortality rate by cardiac rupture [121]. Multiple reasons could stand behind these controversial findings, including the model studied, the animal strain, the timing of intervention, the dose of IL-33, as well as the quality of the techniques in use. Our laboratory was among the first labs to address this controversy and shed the light on the potential detrimental effects of 1ug/day of IL-33 on early stages of cardiac remodeling following MI [121].

H. IL-33 in Acute Kidney Injury

Very few studies investigated IL33 cellular expression in the kidneys. In humans, IL-33 constitutive expression was documented in the endothelial nuclei of renal large and small vessels [92]. In mice, one study revealed IL-33 constitutive and predominant localization in microvascular endothelial cells (CD31+ cells) from which it was immediately released following renal ischemia-reperfusion injury (IRI) [122]. Another study indicated that IL-33 protein is expressed in renal tubulointerstitial myofibroblasts in which it gets upregulated following unilateral ureteral obstruction [123]. The upregulation of IL-33 expression was recognized in other different kidney injury models as well. For instance, cisplatin-induced kidney injury resulted in increased IL-33 serum and renal protein expression levels [124].

Furthermore, following renal ischemic-reperfusion injury, renal mRNA and protein expression levels of IL-33 in addition to serum IL-33 were markedly increased [122]. This have arisen the awareness toward the role served by this natural intensive expression induced by the injury and thus multiple studies were directed towards investigating how IL-33 interferes in the featured pathological mechanisms of kidney damage. To further portray IL-33 therapeutic potentials, experimental studies revealed the involvement of IL-33 treatments in the pathogenesis and reparative responses of kidney injury [125].

1. IL-33 in Renal Ischemic-Reperfusion injury (IRI)

Renal ischemia is a major cause of AKI that can be manifested by several clinical conditions including MI. In the IRI mice model, Ferhat et al. demonstrated that IL-33 deficient mice (IL-33Gt/Gt) have less severe renal dysfunction and reduced tubular histological lesions. Besides, immunologically speaking, endogenous IL-33 was shown to increase myeloid cell trafficking (24hr post-IRI). Ferhat et al. however noted that IL-33 does not affect the early phase of inflammatory cell recruitment post-IRI (1-6hr) but was better described as an amplifier for neutrophils trafficking, which was shown to be mediated by iNKT cell activation and subsequent IFN-γ/IL-17A production [126]. Moreover, IL-33 was shown to exhibit a profibrotic role. For instance, post-IRI induction in mice, Liang et al. recognized an increase in fibrosis indicated by collagen staining which was further potentiated when IRI-mice were treated with exogenous IL-33 (0.5 μg/day) for 2 weeks. IL-33 neutralization by sST2 (100 μg/day for 2 weeks; i.p. injection) mitigated renal fibrosis. Besides the functional and histological improvements, sST2 treatment

resulted in decreased ECM depositions, myofibroblast formation, bone-marrow-derived fibroblasts and inflammatory cells (macrophages and T cells) recruitment, and profibrotic and proinflammatory cytokines and chemokines production [122]. Nevertheless, IL-33 contribution in the resolution of kidney injury has also prevailed specifically by highlighting its effect on group 2 innate lymphoid cells (ILC2) activation and expansion which have emerged as an important axis for IL-33- mediated renoprotection [127]. IL-33 treatment (0.3µg/day) for 5 consecutive days pre or post-IRI, protected kidney structure and function, reduced renal fibrosis, and improved the survival rate [128]. IL-33 administration also was found to induce Th2 cytokines, Tregs, ILC2s, and alternatively activated macrophages (AAMs) while it dampens GR1+ neutrophils infiltration and proinflammatory cytokines production, thereby ameliorating kidney damage [128]. In the search for the key IL-33-induced cell type mediating this renal preservation, Cao et al. reported that the deletion of AAM partially reversed IL-33 protective effect, whereas ILC2 deletion abolished IL-33 mediated renoprotection and thus ILC2 was a predominant major player. Mechanistically, Adoptive transfer of ex-vivo expanded ILC2 was shown to improve renal function and tubular damage in amphiregulin (Areg) dependent manner [128]. Of note, the expression of ILC2s was confirmed in human and mouse kidneys, and ST2+ ILC2 represented a major subtype of ILCs [129]. IL-2 cytokine is known to maintain Tregs homeostasis and was shown to upregulate its ST2 receptor expression [130] therefore; Stremska et al. manipulated IL233 hybrid fusion protein that implements both IL2 and IL-33 activities. This attempt was successful in demonstrating the synergistic effect of IL233 hybrid by enhancing the effects of Tregs and ILC2s in preserving kidney structure and function following IRI [131].

2. IL-33 in Chemical-Induced AKI

In Cisplatin-induced AKI, high-dose exogenous IL-33 (1 µg, twice a day on days 1, 2, and 3 after cisplatin administration, i.p. injection) revealed a detrimental effect on the kidneys reflected by increased apoptosis, acute tubular necrosis, CD4 T cells infiltration, and increased serum creatinine which was validated by its resolution upon the administration of sST2 fusion protein (sST2; 100 µg/day, on days 1, 2, and 3 of cisplatin administration). The observed exacerbated kidney injury in IL-33 treated mice was shown to be mediated by CD4 T-cells/CXCL1 axis [124, 129]. However, in adriamycin-induced glomerulosclerosis model, exogenous IL-33 (0.4 µg/day) for 4 consecutive days resulted in a sustained expansion of ST2+ ILC2s which resulted in renal protection presented by decreased neutrophils and mononuclear phagocyte infiltration to the kidneys, a shift into type 2 immune response, and reduced glomerular and tubular histopathologies. IL-33-mediated renoprotection was attributed to ST2+ ILC2 expansion, which ameliorates renal injury via eosinophils [129]. In the context of drug-induced nephrotoxic injury, IL233 hybrid cytokine protected the kidney integrity by boosting Tregs and ILC2s proliferation and mobilization [131] [132].

3. IL-33 in Obstructive Renal Injury

Unilateral ureteral obstruction (UUO) model is extensively used to study renal fibrosis and tubular cell injury mechanisms, which are two important representatives of kidney damage [133]. Chen et al. demonstrated that IL-33 and IL1RL1 are upregulated in surgically induced obstructive renal injury, and interstitial myofibroblasts were shown to be

major sources of IL-33 [123]. By comparing IL-33 knockout mice to the wild type, Chen revealed the significant contribution of IL-33 to renal fibrosis and tubular cell loss after injury. Therefore, IL-33 was postulated as an important promoter of kidney disease progression and a potential therapeutic target. Similarly, this upregulation of IL-33 and IL1RL1 in UUO model was recognized by Li et al [134]. Exogenous IL-33 increased kidney fibrosis and macrophages infiltration. Li suggested that IL-33-induced renal fibrosis is mediated via macrophages polarization by reporting the concomitant increase in renal AAMs and the reduction in renal fibrosis with macrophages deletion even with IL-33 administration.

Collectively, these results show that IL-33 exerts pleiotropic immunomodulatory effects in kidney damage progression. Endogenous IL-33, upregulated at the early stage, plays a role in the acute inflammatory phase by mediating immune cell recruitment and amplifying the inflammatory response. Whereas, exogenously, short-term IL-33 treatment at low dose seems beneficial potentially through the activation of ILC2s, AAMs, and Tregs. Long-term treatment with high-dose exogenous IL-33, however, may be detrimental by promoting tissue fibrosis. [125]

J. Thesis Rationale and Aim

Myocardial infarction is an immense public health concern, with the developing countries being in the frontline [134]. AKI represents a major unresolved issue in patients with MI that associates with high morbidity and mortality. Based on the nature of IL-33 as a nuclear alarmin with pleiotropic immunomodulatory effects, investigations were directed

to study its effect in inflammatory diseases. In the setting of MI, however, the impact of IL-33 on LV remodeling is still inconclusive with some studies suggesting a protective role [120] while others revealing that IL-33 could worsen adverse left ventricular remodeling [121]. Similarly, IL-33 mediated kidney disease progression and development has been shown to be disease-context and dose dependent [122, 128]. To date, no study has assessed the impact of IL-33 on kidney damage in the setting of CRS1. Therefore, given the well documented effects of IL-33 treatment in multiple models of kidney injury, we aimed to evaluate the effect of IL-33 treatment on kidney damage at 4 and 7 days post-MI in male C57Bl6 mice. .

K. Hypothesis

Our understanding of kidney damage in the presence of MI following IL-33 administration is not well elucidated. To date, the exact mechanisms behind the impact of IL-33 treatment on MI induced-kidney damage through the cardiorenal interrelationship remain poorly understood and this is the focus of our study. Based on our rationale supported by published evidence, we hypothesize that IL-33 administration in the settings of MI-induced kidney damage might be influenced by LV progression into adverse remodeling. This is the first study to assess the impact of IL-33 on kidney damage following MI. The main aim of this study is to investigate the impact of the IL-33/ ST2 axis on kidney damage 4 and 7 days post-MI.

CHAPTER II

MATERILAS AND METHODS

A. Study input

1. Animals

Three months old wild-type (WT) male C57BL/6J mice weighing 20–25 g were maintained in the Animal Care Facility at the American University of Beirut Medical Center under optimal conditions with 12 light/12 dark hours cycle with free access to standard chow and water. All animal experiments were conducted with approval from the Institutional Animal Care and Use Committee (IACUC # 18-2-RN560) in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, 8th edition.

2. Experimental Design

Three months old WT male C57BL6/J mice were allocated into 4 groups: sham ±IL-33, MI± IL-33. Mice received either a daily intraperitoneal injection of mouse recombinant IL-33 (BioLegend, San Diego, CA, U.S.A.) (1 μg suspended in 200 μl PBS) or PBS (200 μl/day) starting from the surgical day continued 4 and 7 days thereafter. Echocardiography was performed at baseline, day one post-MI, and right before sacrifice. Heart and kidneys were harvested for histological and molecular analysis.

B. Surgical Procedures

1. MI Induction

MI was induced by left anterior descending (LAD) coronary artery ligation. First, to induce analgesia, intraperitoneal injection of 0.05mg/kg tramadol was given and mice were placed on a heating pad to maintain body temperature at 37 °C during surgery. Then, they were anesthetized with 2% isoflurane and intubated orotracheally followed by securing the airway with a MiniVent ventilator (HSE Harvard, Germany). Animal's chest was shaved and a skin cut was made over the left thorax between the left third and fourth intercostal space. The heart left ventricle was exposed and the LAD artery was ligated using a 7-0 prolene suture (Ethicon, Norderstedt, Germany), 2 mm below the left atrioventricular border. Successful MI induction was confirmed by the blanching of the tissue downstream of the ligation site and by echocardiography 24 hours after surgery. A 6-0 polypropylene suture (Ethicon, Norderstedt, Germany) was used in a simple continuous pattern to close the ribs and then the muscle layer. The chest was then tightly sealed and isoflurane was decreased. The intubation tube was removed and mice were monitored to ensure a full recovery. The sham operation was performed in a similar manner, except for ligation of the coronary artery.

2. Echocardiography

The recording of the parasternal 2 Dimensional M-mode and B-mode echocardiograms were obtained along the long axis using a Visual Sonics Echo system

(Vevo 2100, High Resolution Imaging System, VisualSonics, Inc., Toronto, Canada) equipped with a 22–55 MHz (MS550D) linear transducer (VisualSonics). ECGs were measured successively to monitor cardiac functional alterations. A record was taken at baseline, following surgery on day 1 (sham operation or MI-induction) and at the day of sacrifice (day 4 or day 7). Prior to echocardiography, mice were anesthetized using 2% isoflurane in oxygen and then placed on a temperature-controlled platform. After shaving the chest and applying the ultrasonic gel on heart area, the probe was directed at the mid papillary muscle level in order to obtain B-mode and M-mode echocardiography images, in the parasternal long and short axis views. Heart rate and body temperature were maintained constant throughout the procedure. At least three images were taken from consecutive cardiac cycles and analyzed to measure ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) by calculating the average at baseline and right before killed.

3. Necroscopy

Mice were first injected intraperitoneally with 100 µl of Heparin 30 minutes prior to the operation. Anesthesia was then induced by isoflurane vapor (4% in oxygen). Blood was evacuated via cardiac puncture and centrifuged at 2200 rpm for 10 minutes. Plasma was collected, flash-frozen in liquid nitrogen and stored at −80°C. Mice were then subjected to cervical dislocation. To arrest the cardiac cycle at the diastole phase, 100 µl cardioplegic solution was injected directly to the heart. For kidney collection, left kidney was harvested into 4% zinc formalin tubes for histology, whereas the right kidney was harvested and immediately placed into a cryo tube in liquid nitrogen followed by storage at -80 for

molecular work. The LV remote and infarcted areas were snap-frozen in liquid nitrogen for RNA extraction.

C. Immunohistochemistry

1. Hematoxylin and Eosin (H&E) Staining

H&E stain was used to evaluate glomerular retraction. Four µm thick renal sections from each mouse were stained with H and E according to standard laboratory protocol. Briefly, kidney tissues were fixed in 4% formalin, then dehydrated and embedded in paraffin. Dewaxing and rehydration steps of paraffin-embedded kidneys were performed and the tissue was stained with H and E and examined under light microscope with 40x magnification. Glomeruli with visible afferent and efferent arterioles were included in the analysis.

2. Massons' trichrome (MTC) Staining

MTC was used to assess total renal fibrosis. First, after dewaxing and rehydration, tissues were soaked in Bouin's solution for 1 hour at 56 °C to improve staining quality then washed and rinsed with distilled water for 5-10 minutes. The nuclear dye, Weigert's iron hematoxylin solution, was then applied for 10 minutes. After washing, tissues were incubated with Biebrich scarlet-acid fuchsin solution to stain the acidophilic tissue elements (cytoplasm and collagen) with red. To decolorize the collagen fibers for discrimination, this was followed by the application of phosphomolybdic-phosphotungstic acid solution for 10

minutes. Afterward, sections were directly transferred to an aniline blue solution for collagen to be stained in blue. Tissues were observed under a light microscope at 10× magnification and fibrosis was measured using image-j software (https://imagej.nih.gov/ij/).

3. Periodic Acid Schiff (PAS) Staining

PAS was used to detect Bowman's capsule and glomerular capillary area. Following dewaxing and rehydration, 4µm thick kidney sections were cleared with xylene, rinsed in increasing concentration of ethanol then immersed in 0.5% PAS solution for 10 minutes. After rinsing well in distilled water slides were covered with Schiff's reagent for 15 minutes then washed for 5 minutes. Images were obtained under the light microscope (Olympus CX41 microscope) at 40× magnification. Bowman's capsule area and capillary area were measured using Image J software (https://imagej.nih.gov/ij/)

D. Molecular Analysis

1. Dihydroethidium (DHE) Staining

DHE was used to quantify total ROS score in the kidneys. Four µm thick kidney sections were stained with 10 µM DHE (Calbiochem, Darmstadt, Germany) then incubated in a humidified chamber under dark condition for 45 minutes at room temperature. Images were obtained by Laser Scanning Fluorescent Microscope (Zeiss Axio) under 20x magnification and quantified using Image J software (https://imagej.nih.gov/ij/).

2. Protein Extraction and Western Blots

Kidney tissues were crushed in liquid nitrogen then homogenized in radioimmunoprecipitation assay lysis and extraction buffer (RIPA) and left overnight on a rotary mixer at 4°C. The supernatant was then collected and protein concentrations were measured by the Thermo Scientific NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA). Samples were adjusted with RIPA buffer to achieve the same final protein concentration and heated in Laemmli buffer for 10 minutes at 95°C, then stored at -20°C. Protein samples (200µg) were loaded into the wells of 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then run at thirty amperes/gel till the die reached the bottom of the gel then electroblotted into nitrocellulose membranes at 100 volts, at 4ºC for 1 hour. The membranes were then blocked with 5% non-fat dry milk in 0.1% TBST (Tris buffer saline with 0.1% Tween 20) for 1 hour at room temperature then probed overnight at 4°C with primary antibodies against alpha-smooth muscle actin (α-SMA) and Interleukin-13 (IL-13) diluted in 0.1% TBST. Membranes were washed four times with 0.02% TBST and incubated with the peroxidase-conjugated secondary antibody (1/40000) for 1 hour at room temperature. Finally, membranes were washed twice with TBST 0.02% followed by two washes with TBS 1x and bands were visualized with an enhanced chemiluminescence kit (Biorad) using the chemidoc MP imaging system-Biorad machine. The protein expression level was normalized to total protein to confirm equal loading by incubating the membrane with a total protein stain (Ponceau Red). Bands were analyzed using Image J software (https://imagej.nih.gov/ij/).

Primary Antibody	Dilution
Anti-α-SMA (Abcam, catalog# ab5694)	1/200
Anti IL13 (Abcam, catalog#ab106732)	1/500

Table 1: Antibodies used for western blot analysis

3. RNA Extraction and Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from frozen kidney tissues using Trizol according to manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY, USA) and RNA concentrations were determined by the NanoDrop® ND-1000 UV-Vis Spectrophotometer. Purity of extracted RNA was confirmed by the 260:280 absorbance ratio. cDNA was synthesized from 1 µg RNA using Revert Aid 1st Strand cDNA synthesis kit (Thermo, USA) and mRNA expression was then analyzed in a CFX96 real-time PCR system (Bio-Rad, Germany). RT-qPCR was performed in duplicate with a final volume of 10µl using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and gene-specific primers listed in **Table 2** to quantify the expression of the following genes: soluble suppression of tumorigenicity-2 (s ST2), collagen type III (Col3), matrix metalloproteinase-8 (MMP8), BCL (B Cell Lymphoma)-Associated X (BAX), B-cell lymphoma 2 (BCL2), nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide riboside kinase-1 (NMRK1), sirtuin-3 (SIRT-3), and poly [ADP-ribose] polymerase (PARP-1). For each 10µl PCR mixture, 4 µl cDNA was used with 0.9µl DNase free water, 5µl SYBR green, and 0.05µl of the forward and reverse primers. A control (no template) was used to eliminate unspecific

amplifications. Cardiac gene expression between different samples was normalized to hypoxanthine-phosphoribosyl transferase (HPRT), whereas renal genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were reported as $2^{-\Delta\Delta Ct}$ values.

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
HPRT	GTTGGGCTTACCTCACTGCT	TAATCACGACGCTGGGACTG
GAPDH	TGTGTCCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
sST2	ACGCTCGACTTATCCTGTGG	CAGGTCAATTGTTGGACACG
Col3	TCTCTAGACTCATAGGACTGACC	TTCTTCTCACCCTTCTTCATCC
MMP8	CACACTCCGTGGGGAGATTT	GCCTGAAGACCGTTGGGTAG
BAX	ATCCAAGACCAGGGTGGCT	CCTTCCCCCATTCATCCCAG
BCL2	AGTACCTGAACCGGCATCTG	TATGCACCCAGAGTGATGCAG
NAMPT	ACCAGCGGGGAACTTTGTTA	ACATAACAACCCGGCCACAT
NMRK-1	CTTGAAGCTTGCTCTGCGAC	GTGTCGTCTTCCCTCCGTTT
SIRT-3	GATTCGGATGGCGCTTGAC	TCTCCCACCTFTAACACTCCC
PARP-1	ACACCACAAAACCTCAGCCA	ACAAACCACAAACAACCGGC

Table 2: Primers used for RT-PCR analysis

4. Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma IL-33, TNFα and IL1-β levels were measured using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. All samples were analyzed in duplicates.

E. Total NAD Extraction and Quantification

NAD was extracted and purified from 20 milligrams of kidney tissues using 75% ethanol and 25% HEPES (10 mM pH 7.1) then diluted with water 1:20 to ensure the concentration is within the standard curve. In a 96-well microtiter-plate, 25 microliters of NAD samples were loaded followed by 100 microliters of the reaction buffer [600 mM ethanol, 0.5 mM 3-(4.5dimethylthiazol-2-yl)-2.5- diphenyltetrazolium bromide (MTT), 2 mM phenazine ethosulfate (PES), 120 mM Bicine (pH7.8), yeast alcohol dehydrogenase (SIGMA A3263 > 300 μ/mg)]. Using LB 942 Multimode Reader the kinetics of the reaction (OD at 550nm, every 30 seconds for 20 minutes) was tracked. NAD was calculated using the linear regression equation.

F. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7. Data are expressed as fold change or mean ± standard error of mean (SEM). Statistical comparisons were performed using nonparametric two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A P value of < 0.05, < 0.001, and < 0.0001 were considered statistically significant.

CHAPTER III

RESULTS

A. IL-33 Treatment Decreased EF and FS 7 Days Post-MI

At day 1, **Figure 1** shows a significant decrease in EF, FS, and CO in MI mice in the presence and absence of IL-33 treatment when compared to baseline. At day 4, **Figure 1A, 1B,** and **1C** reveals no significant decrease in EF, FS, and CO in neither of the MI groups when compared with their relative groups at day 1. At day 7, however, only EF significantly decreased in the MI+V group when compared with their relative group at day 1 while each of EF, FS, and CO markedly decreased in the MI mice treated with IL-33 when compared with their relative group at day 1. Of note, a remarkable decrease in EF and FS in the MI+IL-33 group when compared with the MI+V mice was also observed at day 7. **(Figure 1D, 1E and 1F)**

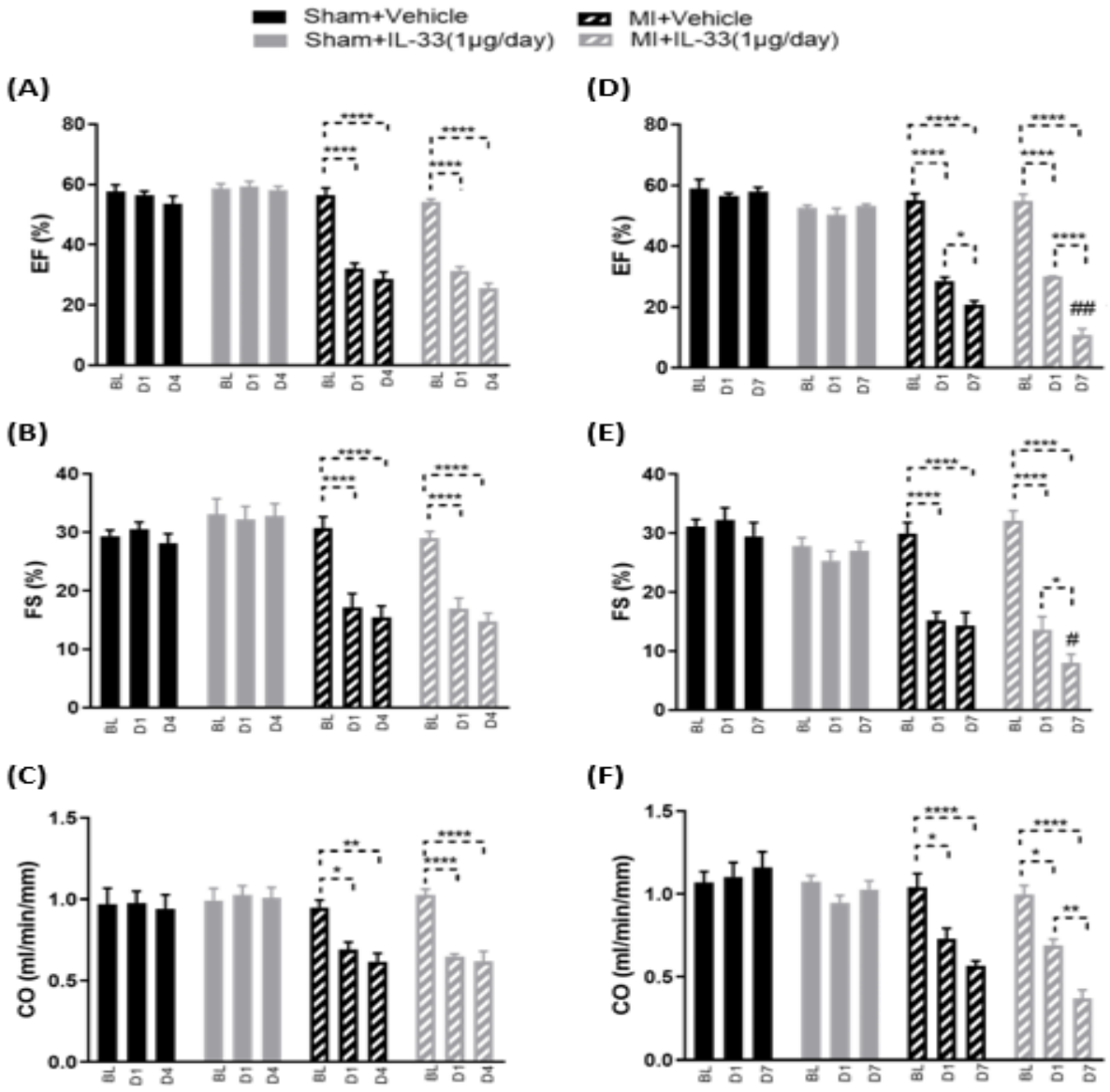

Figure 3: Effect of IL-33 treatment on EF, FS, and CO

EF and FS significantly decreased 7 days post-MI in IL-33 treated group when compared with vehicle treated mice (D and E). No significant change in CO 7 days post-MI in the presence and absence of IL-33 treatment was observed (F). MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33; BL: baseline echo; D1: echo day 1 post-MI; D7: echo day 7 post-MI. Data were analyzed for significance using Two-way ANOVA (n=5-10). All bars represent mean ± SEM. #: significance between the MI+V group and MI+IL-33 group (*P<0.05, **P<0.01, ****P<0.0001); ##P<0.01 (MI+ IL-33 vs MI+ vehicle)

B. IL-33 Treatment Upregulated Cardiac sST2 mRNA Levels at Day 7

At day 4, **Figure 2A** shows low mRNA sST2 levels in all groups with no significant change. At day 7, however, IL-33 treatment significantly increased mRNA sST2 levels in the presence and absence of MI when compared with the vehicle-treated groups. **(Figure 2B)**

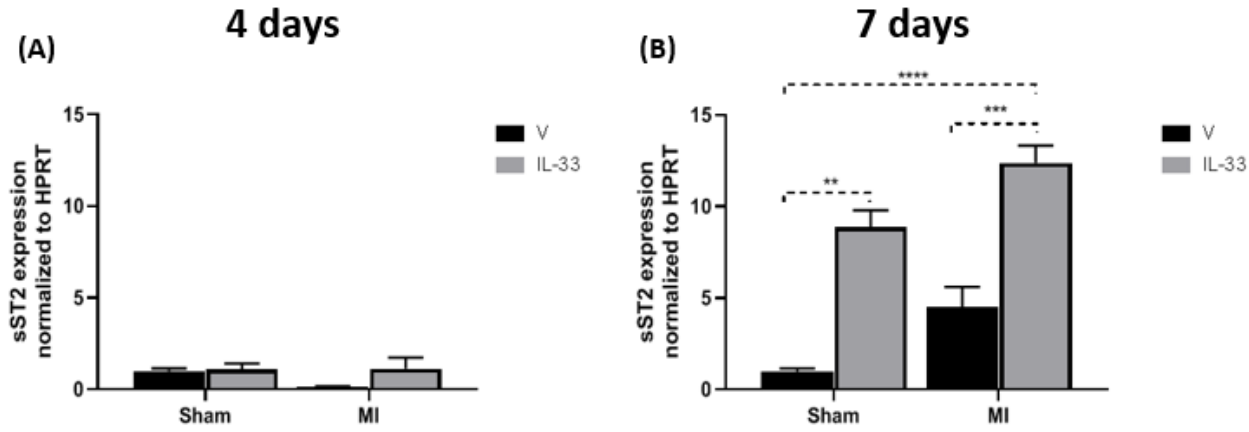

Figure 4: Effect of IL-33 treatment on cardiac sST2 mRNA levels

(B) sST2 significantly upregulated 7 days post-MI in in the sham and MI group treated with IL-33 compared with vehicle-treated groups. MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA .All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

C. IL-33 Treatment Increased Plasma IL-33 Levels in MI Mice at Day 4

At day 4, **Figure 3** shows a significant increase in plasma IL-33 levels in IL-33 treated mice in the presence and absence of MI when compared with the sham-operated group. Importantly, a marked increase in IL-33 plasma levels in the MI group treated with IL-33 when compared with the MI+V group was observed. At day 7, a significant increase in plasma IL-33 levels in sham operated mice treated with IL-33 when compared with their relative sham group was observed. Of note, plasma IL-33 levels substantially decreased in sham and MI mice treated with IL-33 at day 7 when compared with their corresponding groups at day 4.

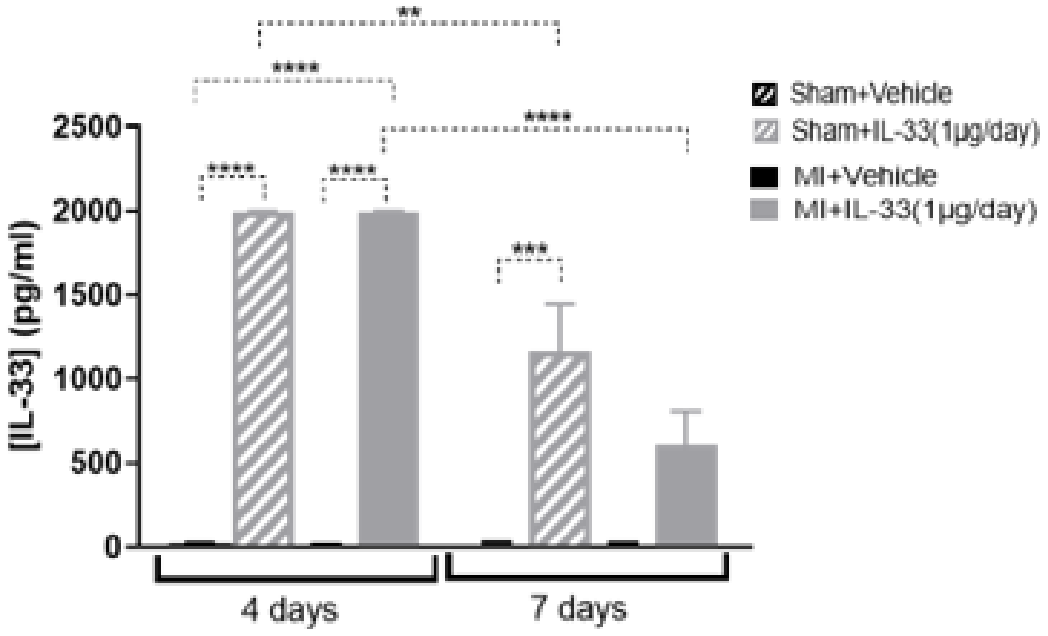

Figure 5: Effect of IL-33 treatment on its plasma levels

On day 7, a notable reduction in these levels was detected while still sustaining higher levels of IL-33 relative to the corresponding sham and MI groups treated with vehicle. MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA. All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

D. IL-33 Treatment Decreased Plasma TNF-α Levels in MI Mice at Day 4 but Increased Plasma IL-1β Levels in MI Mice at Day 7

At day 4, **Figure 4A** shows a significant increase in plasma TNF-α levels in MI mice when compared with their relative sham-operated group. A marked decrease in plasma TNF-α levels in MI+IL-33 mice when compared with the MI+V group was also seen. At day 7, no significant change in plasma TNF-α levels, however, was observed. Of note, plasma TNF-α levels significantly decreased at day 7 in the MI+V mice when compared with their relative group at day 4.

As for plasma IL-1β levels, at day 4, no significant change in plasma IL-1β levels in all mice groups in the presence and absence of MI and IL-33 treatment was observed. At day 7, however, IL-33 treatment markedly increased plasma IL-1β levels in the presence and absence of MI when compared with the vehicle-treated groups. Of note, plasma IL-1β levels significantly increased at day 7 in IL-33 treated mice in the presence or absence of MI when compared with their corresponding groups at day 4. **(Figure 4B)**

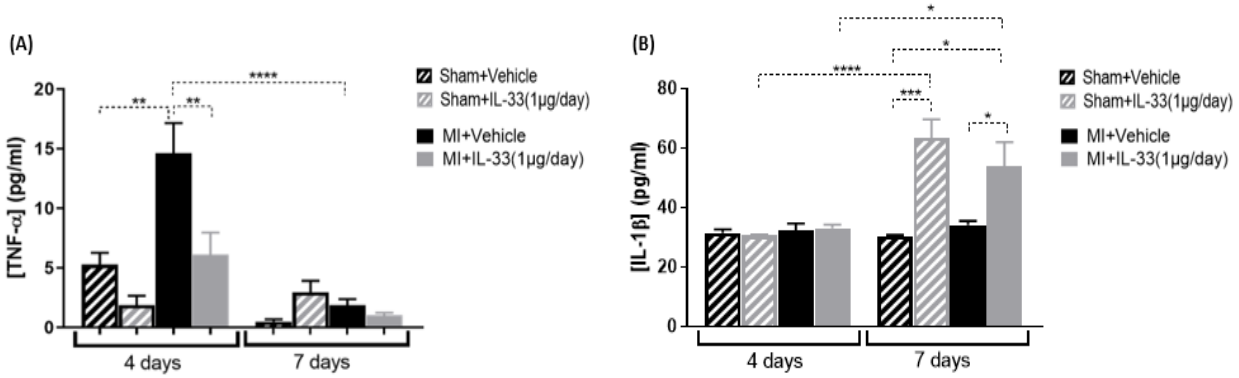

Figure 6: Effect of IL-33 treatment on plasma TNF-α and IL-1β levels

TNF-α systemic expression significantly decreased in the MI group treated with IL-33 for 4 days (A). IL1-β systemic expression significantly increased in the MI and sham groups treated with IL33 at day 7 (B). This recognized elevation in IL- 1β levels in the IL-33 treated mice overpassed significantly the expression levels detected in their counterpart groups at day 4 (A,B). MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-5). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

E. IL-33 Treatment Decreased Bowman's Capsule Area and Glomerular Retraction but Increased Glomerular Capillary Area 4 Days Post-MI

The Bowman's capsule area and glomerular capillary area were assessed using PAS, whereas glomerular retraction was assessed using H&E staining. At day 4, **Figure 5A** and **5C** shows a significant increase in the Bowman's capsule area and glomerular retraction in MI+V mice when compared with their relative shame-operated group. A marked increase in glomerular retraction in MI+V mice when compared with the sham group treated with IL-33 was also observed. Of note, the Bowman's capsule area and glomerular retraction substantially decreased in MI mice treated with IL-33 when compared with the MI +V group. Conversely, **Figure 5B** reveals a significant decrease in glomerular capillary area in MI+V mice when compared with their relative sham-operated group. Glomerular capillary markedly

increased in MI mice treated with IL-33 when compared with MI+V group. At day 7, the Bowman's capsule area and glomerular retraction significantly increased in MI+V mice when compared with sham-operated mice in the presence and absence of IL-33 treatment **(Figure 5D** and **5F)**. Additionally, glomerular retraction markedly increased in MI mice treated with IL-33 when compared with the sham-operated group. Conversely, glomerular capillary area substantially decreased in MI+V group when compared with sham-operated mice in the presence and absence of IL-33 treatment **(Figure 5E)**

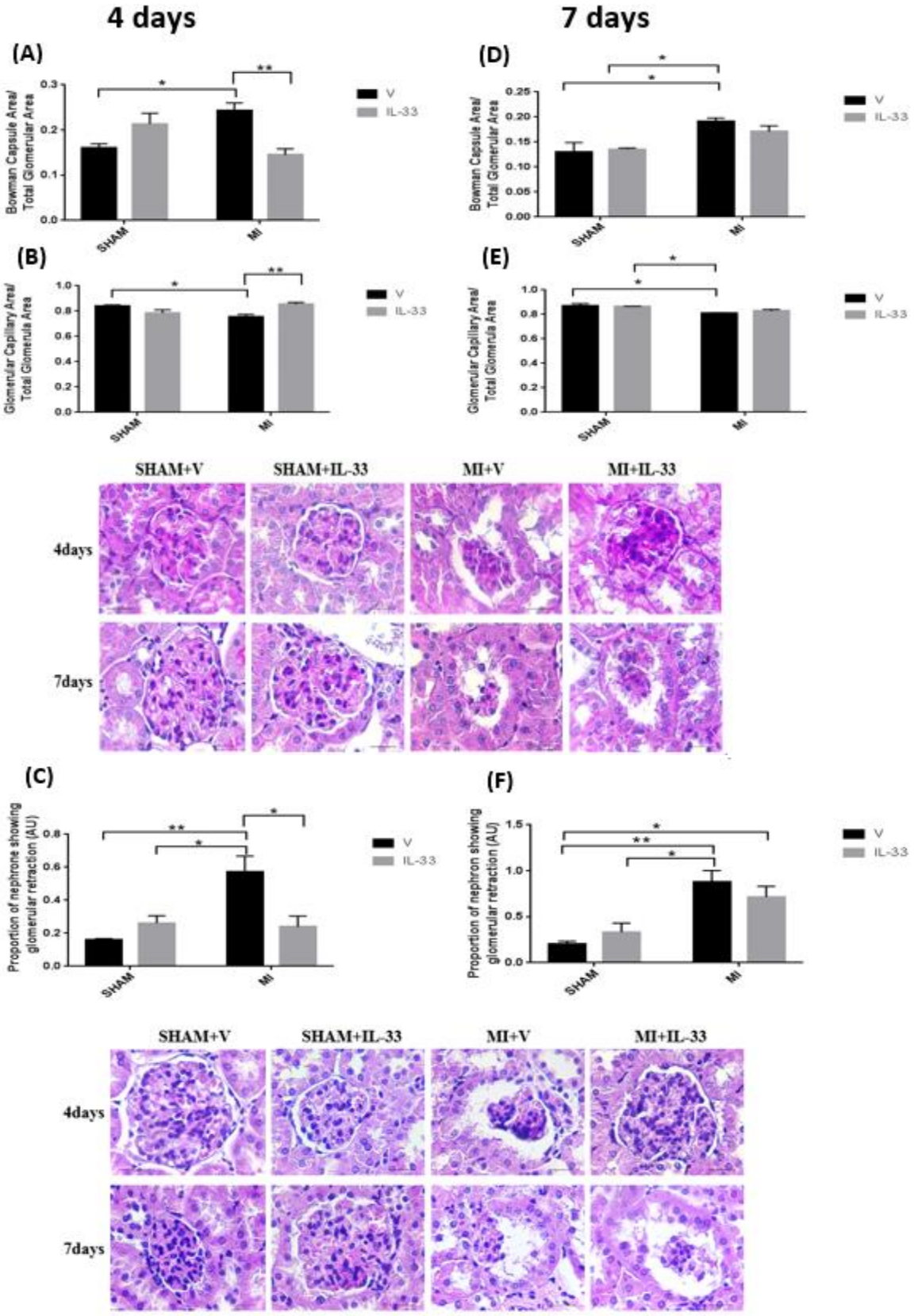
4 days
7 days
(A)
(D)
Bowman Capsule Area/ Total Glomerular Area
V
IL-33
SHAM
MI
(B)
(E)
Glomerular Capillary Area/ Total Glomerula Area
SHAM+V
SHAM+IL-33
MI+V
MI+IL-33
4days
7days
(C)
(F)
Proportion of nephrone showing glomerular retraction (AU)
Proportion of nephron showing glomerular retraction (AU)

Figure 7: Effect of IL-33 treatment on Bowman's capsule area, glomerular retraction and glomerular capillary area

IL-33 treatment decreased significantly the Bowman's capsule area (A) and glomerular retraction (C) 4 days post-MI while increased significantly the glomerular capillary area (B). 7 days post-MI, IL-33 administration showed no considerable effect on Bowman's capsule area (D), glomerular retraction (F) and glomerular capillary area (E). Scale bar 20μm. C: control; MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-8). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

F. IL-33 Treatment Altered Renal Fibrosis and Pro-Fibrotic Markers 4 and 7 Days Post-MI

Total renal fibrosis was assessed using MTC staining. At day 4, **Figure 7A** shows a significant increase in total renal fibrosis in MI mice in the presence and absence of IL-33 treatment when compared with the sham-operated group. A marked increase in renal fibrosis in MI+V group when compared with sham group treated with IL-33 was also observed. Of note, total renal fibrosis remarkably decreased in the MI mice treated with IL-33 when compared with the MI+V group. **Figure 7B** reveals a remarkable decrease in renal α-SMA protein levels in the MI+IL-33 group when compared with the MI+V group. A marked increase in α-SMA protein in MI+V mice when compared to the sham-operated group in the presence or absent of IL-33 was also observed. **Figure 7C** shows significant decrease in Collagen3 renal mRNA expression levels in the MI mice treated with IL-33 when compared with the MI+V mice. MMP8 renal mRNA expression levels significantly increased in the MI group treated with IL-33 when compared with the MI+V and sham-operated mice **(Figure 7D).** At day 7, **Figure 7E** shows a significant increase in total renal fibrosis in MI mice treated with IL-33 when compared with the MI+V and sham-operated mice in the presence and absence of IL-33 treatment. A marked increase in total renal

fibrosis was also seen in MI+V mice when compared with their relative sham-operated group. **Figure 7F** reveals a remarkable increase in renal α-SMA protein levels in the MI+IL-33 group when compared with the MI+V and the sham-operated mice in the presence or absence of IL-33 treatment. **Figure 7G** reveals a significant increase in Collagen3 renal mRNA expression levels in the MI+IL-33 group when compared with the MI+V group. A marked decrease in Collagen3 mRNA expression levels was also seen in the MI+ V group when compared with sham-operated mice treated with IL-33. MMP8 renal mRNA expression levels remarkably increased in MI+V mice when compared with their relative sham-operated group **(Figure 7H)**

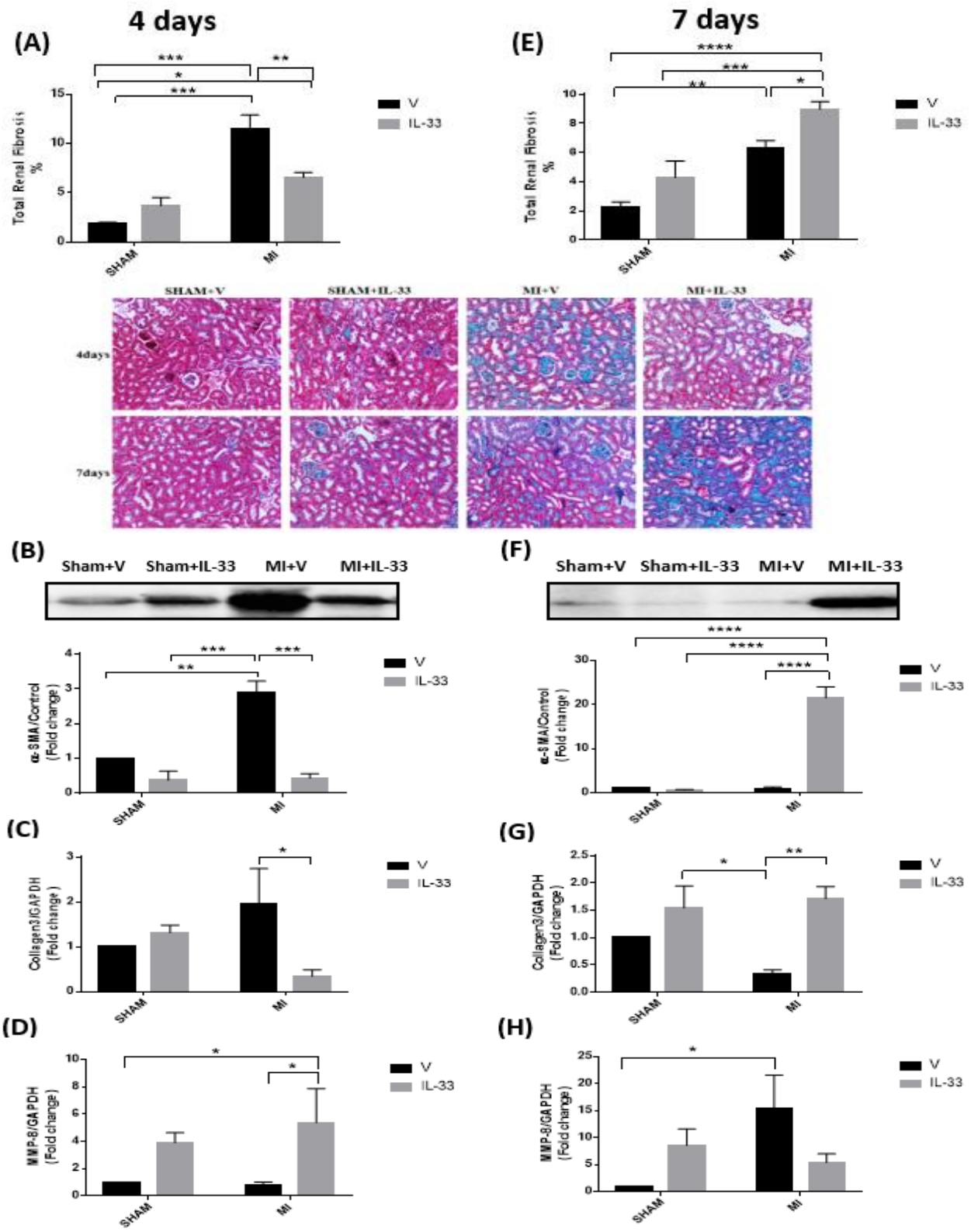
4 days
7 days
(A)
(E)
Total Renal Fibrosis %
SHAM
MI
V
IL-33
SHAM+V
SHAM+IL-33
MI+V
MI+IL-33
4days
7days
(B)
(F)
Sham+V
Sham+IL-33
MI+V
MI+IL-33
α-SMA/Control (Fold change)
(C)
(G)
Collagen3/GAPDH (Fold change)
(D)
(H)
MMP-8/GAPDH (Fold change)

Figure 8: Effect of IL-33 treatment on renal total interstitial fibrosis and fibrotic markers

IL-33 administration significantly decreased renal fibrosis (A), α-SMA protein (B), and Collagen3 mRNA expression levels (C) 4 days post-MI, however, markedly increased the expression levels of MMP8 (D). 7 days post-MI, IL-33 treatment significantly increased renal fibrosis (E), α-SMA protein (F), and Collagen3 m RNA expression levels (G). Scale bar 20μm. C: control; MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-7). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

G. IL-33 Treatment Increased Total ROS Score 4 Days Post-MI

Total ROS score was assessed using DHE staining. At day 4, **Figure 8A** shows a marked increase in total ROS score in MI mice treated with IL-33 when compared with MI+V and sham operated mice in the presence and absence of IL-33 treatment. At day 7, total ROS score markedly increased in MI mice treated with IL-33 when compared with the sham-operated group. A substantial increase in total ROS score in MI+V and sham-operated mice treated with IL-33 when compared with the sham-operated mice was also seen **(Figure 8B)**

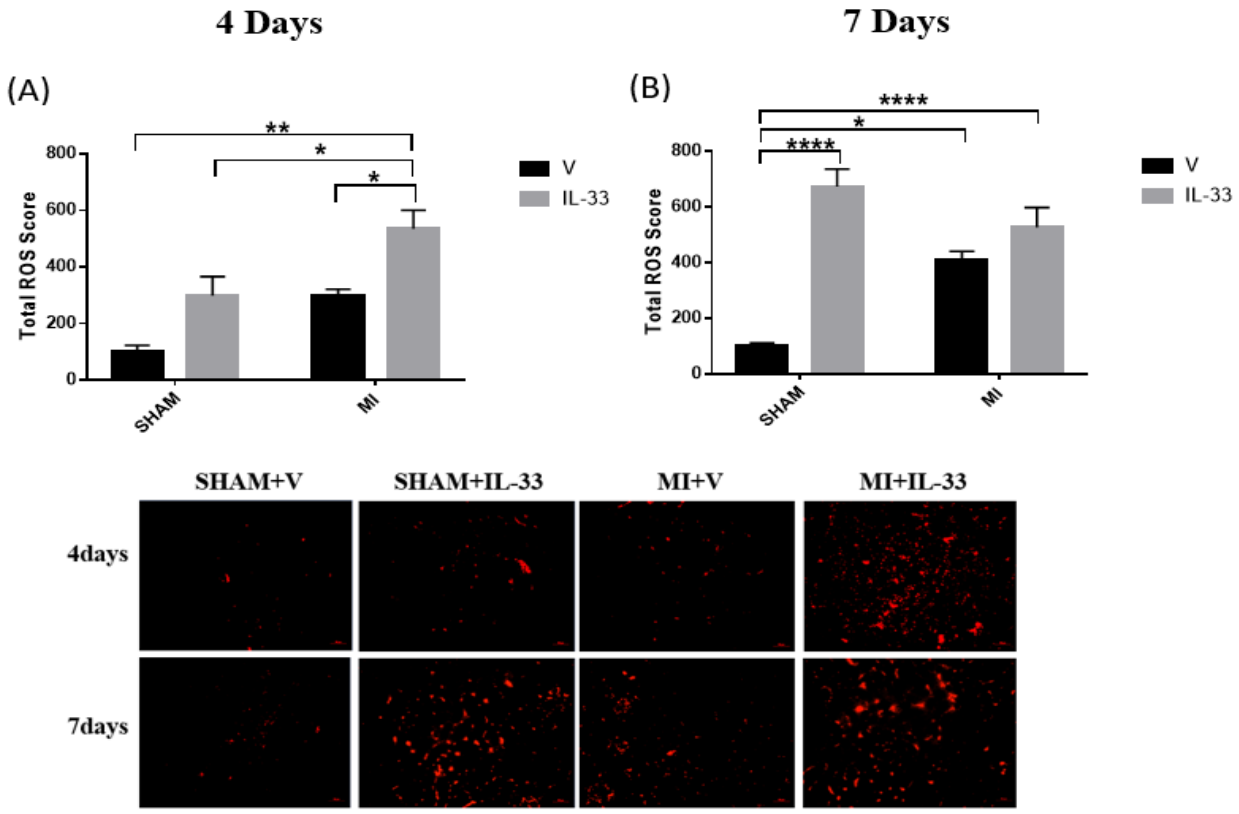

Figure 9: Effect of IL-33 treatment on total renal ROS levels

IL-33 treatment remarkably increased ROS generation in the MI group at day 4 (A). C: control; MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-6). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

H. IL-33 Treatment Decreased the Ratio of Renal BAX/BCL2 mRNA Expression levels at Day 4 and IL-13 Protein Levels at Day 7 Post-MI

At day 4, **Figure 9A** shows a significant decrease in BAX/BCL2 ratio in MI mice treated with IL-33 when compared with the MI+V group and their relative sham operated-mice treated with IL33. A marked increase in renal IL-13 protein levels in MI mice in the presence and absence of IL-33 and sham-operated mice treated with IL-33 when compared

with the sham-operated group was observed **(Figure 9B).** At day 7, BAX/Bcl2 ratio significantly increased in MI+V mice when compared with their relative sham operated-groups in the presence and absence of IL-33 **(Figure 9C). Figure 9D** reveals a remarkable decrease in renal IL-13 protein levels in the MI mice treated with IL-33 when compared with MI+V and sham group in the presence and absence of IL-33 treatment. Of note, a marked decrease in renal IL-13 protein levels was also seen in the MI+V mice when compared with their relative sham-operated group.

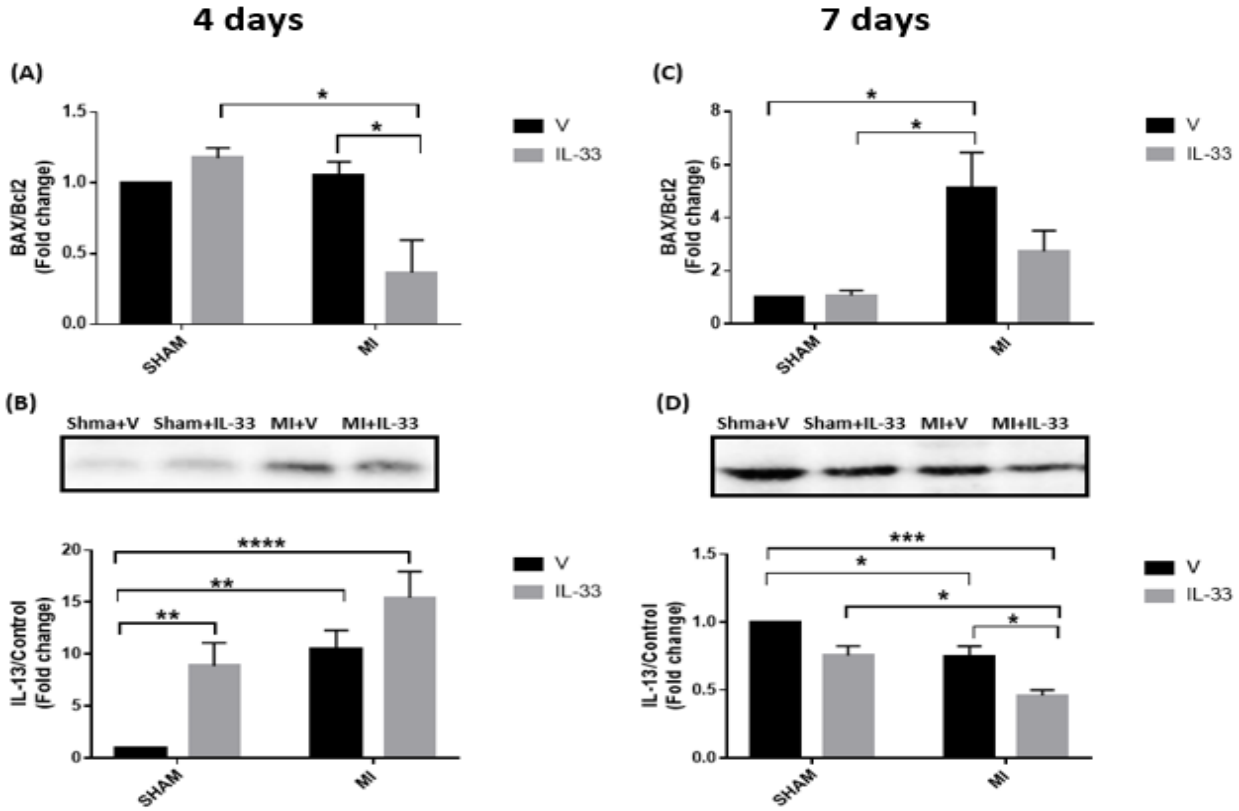

Figure 10: Effect of IL-33 treatment on BAX/BCL2 mRNA expression ratio and IL-13 protein expression levels

BAX/BCL2 ratio decreased significantly with IL-33 treatment 4 days post-MI (A). IL-13 cytokine protein expression in the kidneys increased in the MI+ IL-33 group relative to the all other groups at day 4 (C). IL-13 protein expression levels decreased remarkably following IL-33 treatment post-MI for 7 days (D). MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-7). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

I. IL-33 Treatment Altered NAD Related Enzymes 4 and 7 Days Post-MI

At day 4, **Figure 10A** shows a significant increase in NAMPT renal mRNA expression levels in sham-operated mice treated with IL-33 when compared with their relative vehicle-treated group. A marked decrease in NAMPT renal mRNA expression levels was observed in MI mice treated with IL-33 when compared with their relative sham-operated group treated with IL-33. **Figure 10B** reveals a marked increase in NMRK-1 renal mRNA expression levels in MI mice treated with IL-33 when compared with the sham operated group. **Figure 10C** shows a substantial increase in SIRT-3 renal mRNA expression levels in MI mice treated with IL-33 when compared with MI+V mice and sham operated groups in the presence and absence of IL-33 treatment. PARP-1 renal mRNA expression levels substantially increased in MI+V mice when compared with their relative sham-operated group **(Figure 10D)**. **Figure 10E** shows a significant increase in total renal NAD levels in the MI mice treated with IL-33 when compared with the MI+V group. At day 7, **Figure 10F** shows a significant increase in NAMPT renal mRNA expression levels in IL-33 treated mice in the presence and absence of MI when compared with the sham-operated group. **Figure 10G** reveals a marked increase in NMRK-1 renal mRNA expression levels in MI mice treated with IL-33 when compared with the sham-operated group. **Figure 10H** shows a significant increase in SIRT-3 renal mRNA expression levels in MI mice in the presence and absence of IL-33 treatment when compared with the sham-operated group. PARP-1 renal mRNA expression levels markedly increased in MI mice treated with IL-33 treatment when compared with the MI+V group and sham operated mice. A significant increase in PARP-1 renal mRNA expression levels was also seen in

sham operated mice treated with IL-33 treatment when compared with their relative sham-operated group **(Figure 10I)**. A marked decrease in total renal NAD levels in MI+V mice when compared with the sham-operated group in the presence and absence of IL-33 was seen **(Figure 10J)**

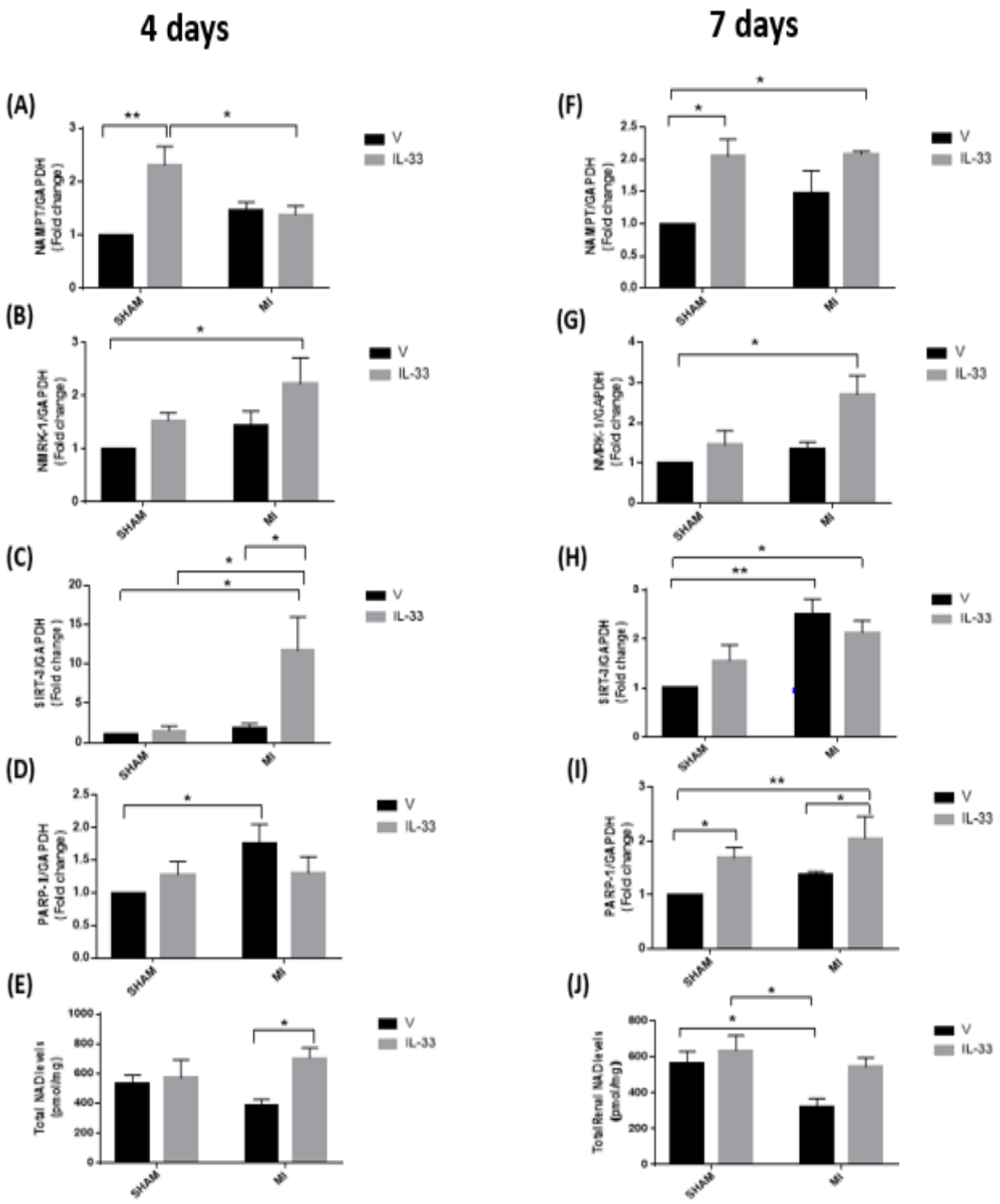

Figure 11: Effect of IL-33 on renal NAD-related enzymes and total NAD levels

IL-33 treatment exerted no significant change in NAMPT (A) and NMRK-1 (B) expression levels 4 days post-MI but increased significantly the m RNA expression levels of SIRT3 (C) and total NAD levels (E) in the kidneys. 7 days post-MI, IL-33 treatment exerted no significant change on NAMPT (F) NMRK-1 (G), SIRT3 (H), and total NAD levels in the kidneys (J) but increased significantly the m RNA expression levels of PARP1 (I). MI: myocardial infarction; V: vehicle; IL-33: Interleukin-33. Data were analyzed for significance using Two-way ANOVA (n=3-8). All bars represent mean ± SEM (*P<0.05, **P<0.01, ****P<0.0001)

CHAPTER IV

DISCUSSION

Mounting evidence reported a strong association between cardiovascular diseases (CVDs) and kidney damage progression and development [47] [135] [136] [137]. IL-33 belongs to the IL-1 family, yet its role in CVDs and kidney diseases remains unclear. Therefore, it is critical to investigate the role that IL-33 plays in the pathological alterations of the kidneys following MI. The main objective of the present study is to assess the impact of exogenous IL-33 administration on kidney damage post-MI. MI was induced by ligating the LAD coronary artery in male mice, followed by the administration of exogenous IL-33 (1µg/day)/PBS for 4 and 7 consecutive days.

Our hemodynamic analysis revealed that IL-33 treatment worsened cardiac dysfunction by depressing left ventricular EF and FS at 7 but not 4 days post-MI. However, no significant change in CO between treated and untreated MI groups was observed. The maintenance of the CO in the presence of a further reduction in EF could be linked to increased activation of the compensatory mechanisms following IL-33 treatment to prevent severe renal hypoperfusion [138]. Of note, previous investigations performed by our lab on the heart revealed a significant increase in infarct size, left ventricular dilatation and hypertrophy, pulmonary congestion, and cardiac rupture 7 days post-MI with IL-33 treatment [121]. These changes might be indicative of a failed compensation in response to the deterioration in cardiac function mediated by IL-33 [139]. A more reliable measurement

of CO could be conclusive. In the kidneys, however, at this early stage of cardiac dysfunction, autoregulatory mechanisms might have kicked in to maintain renal blood flow independently of cardiac function [140]. Renal hemodynamic measurements in this case are important and conclusive.

Our findings also revealed a significant increase in cardiac sST2 mRNA expression levels, an IL-33 decoy receptor, 7 days post-MI in the presence of IL-33 treatment. Although clinically a positive correlation between increased serum sST2 levels and exacerbated adverse cardiac remodeling was reported [141], whether the sequestration of IL-33 that might accompany the rise in cardiac sST2 is unfavorable to the heart remains poorly understood. Concomitantly, the availability of plasma IL-33 levels in the IL-33 treated MI group decreased at day 7 compared with day 4 while still preserving higher levels relative to the MI group. Shortening of IL-33 half-life due to increased plasma sST2 levels or the presence of increased post-translational modifications could mediate this reduction in its plasma levels [98].

Dysregulated inflammatory response is a fundamental player in the pathogenesis and progression of cardiovascular and kidney diseases [60] [142]. Evidence suggests that IL-33 modulates innate and adaptive immunity [86]. Our findings revealed that IL-33 dampens systemic inflammation 4 days post-MI by decreasing plasma TNF-α levels. Conversely, 7 days post-MI, IL-33 treatment increased plasma IL-1β suggesting enhanced systemic inflammation. Thus, systemic inflammation represents a vital consequence of IL-33 administration that might serve as an inducer of cardio-renal damage 7 days post-MI as opposed to day 4.

The discrepancy in IL-33 effects between day 4 and day 7 post-MI on cardiac function and systemic inflammation was also recognized at the level of the kidneys. Histologically, the remarkable decrease in Bowman's capsule area and glomerular retraction with IL-33 treatment observed at day 4 post-MI was lost at day 7. Additionally, IL-33 treatment decreased renal fibrosis, α-SMA, and Collagen type III mRNA expression levels at day 4 post-MI while it enhanced fibrosis and the expression of pro-fibrotic markers at day 7 indicating excessive interstitial myofibroblast activation and extracellular matrix (ECM) deposition [143] [144]. The association between enhanced renal fibrosis post-MI and aggravated kidney damage is well-established [69] [145]. However, based on published evidence in AKI models, the impact of IL-33 administration on renal fibrosis is controversial [122] [128]. Studies have pointed out a highly probable association between long duration (high dose) IL-33 treatments and promoted tissue fibrosis, however, further comprehensive analysis is needed [122]. Coherently, IL-33 administration increased MMP-8 levels 4 days post-MI. This neutrophil collagenase is implicated in ECM degradation and turnover by breaking down Collagen type I and type III. Moreover, MMP8 was reported to have a role in renal repair and recovery post-IRI demonstrated using MMP-8 knockout mice resulting consequently in exacerbated morphological alteration and elevated serum creatinine [146] [147].

CVDs induced kidney damage is tightly correlated with ROS overproduction [148] [149]. Our data showed a significant increase in total renal ROS score in MI mice treated with IL-33 at day 4. ROS is a well-known mediator of increased inflammation, tubular and glomerular damage, and cellular death in the kidneys [81] [149]. Recently, Zheng et al reported that IL-33-induced ROS is crucial for the activation and expansion of ILC2 cells

and ILC2-mediated eosinophilia in the lungs and liver [150]. In the kidneys, multiple preclinical studies have reported expansion in resident ILC2s population after IL-33 treatment such as in the setting of adriamycin-induced glomerulosclerosis and renal IRI [128, 129]. IL-33 induced ILC2s was shown to exert renoprotective effects in AKI models by reducing inflammation and promoting reparative responses [128] [129]. In agreement, our recent study revealed the presence of increased ILC2s and eosinophils in the bone marrow, spleen, heart, and blood following IL-33 administration post-MI, which might also be the case in the kidneys [121]. Additionally, the BAX/BCL2 ratio decreased in MI mice treated with IL-33 at day 4, while remained unchanged at day 7. The BAX/ BCL2 ratio controls the mitochondrial outer membrane permeability and thus is categorized as an apoptotic regulator [151]. This finding suggests a potential anti-apoptotic effect of IL-33 4 days post-MI. Further experiments, however, may be needed to validate the anti-apoptotic effect of IL-33 in the context of MI-induced kidney damage. In agreement, the anti-inflammatory cytokine IL-13 protein levels significantly increased in MI mice treated with IL-33 when compared with the sham-operated group. It has been documented that IL-13 is released from $ST2^+$, Th2, and ILC2s, favoring therefore M2 polarization of renal macrophages [152]. This anti-inflammatory response, however, was suppressed at day 7 as presented with a significant decrease in IL-13 protein levels in MI mice treated with IL-33 when compared with the MI+V group.

Metabolically, NAD homeostasis is strictly critical for mitochondrial function and energy production [153]. Depletion of NAD^+ is well-featured in renal diseases [154]. The augmentation of NAD^+ levels was shown to modulate kidney damage and restore renal function revealing a promising therapeutic potential [155]. NAD^+ is synthesized through

the amidated and deamidated pathways [153]. The salvage amidated pathway, however, accounts for 99.9% of NAD generated in the kidney [153]. Therefore, total renal NAD levels and the mRNA expression levels of NAMPT and NMRK1, the main NAD^+ producing enzymes in the kidneys, were assessed. NMRK-1 substantially increased in MI mice treated with IL-33 when compared with the sham-operated group at days 4 and 7. However, NAMPT significantly increased in MI mice treated with IL-33 when compared with the sham-operated group at day 7 only. Renal NAD measurements further fortified our observation by showing a significant decrease in NAD levels following MI relative to the vehicle-treated sham group at day 7 only. With IL-33 treatment post-MI, total renal NAD levels markedly increased at day 4, whereas no significant change was observed at day 7. To the best of our knowledge, this is the first study to report enhanced total renal NAD levels with IL-33 treatment in the context of MI. A study done by Morigi et al. showed decreased NADH/NAD^+ ratio in the IR heart in mice with diabetes mellitus following IL-33 treatment, enhancing therefore ATP production [156]. Further experiments to evaluate the impact of IL-33 on renal NAD^+ and ATP in the setting of MI are warranted.

A growing body of evidence considered NAD^+ as the main regulator of multiple enzymes implicated in mitochondria biogenesis including SIRT3 as well as enzymes involved in DNA repair such as PARP-1 [157] [158]. Our results showed a significant increase in SIRT-3 mRNA expression levels in MI mice treated with IL-33 when compared with the MI+V group at day 4, while remained unchanged at day 7. SIRT3 has been recognized as a key mediator of NAD beneficial effects and has been demonstrated to exert a renoprotective role through dampening fibrosis and apoptosis. For instance, in a mouse model of diabetic kidney disease, SIRT3 deficiency has been associated with induced

profibrotic TGF-β/smad pathway, collagen deposition, and epithelial to mesenchymal transformation [158]. Moreover, SIRT3 overexpression showed protection against ROS overproduction and apoptosis in high glucose-incubated HK-2 cells through enhancing the expression of the antioxidant gene superoxide dismutase 2 enzyme (SOD2) and reducing BAX/BCL2 and cleaved caspase3 levels [159]. These findings are consistent with our results highlighting increased SIRT3 levels along with decreased renal fibrosis and BAX/BCL2 ratio in the IL-33 treated MI group at day 4. We also observed an increase in PARP-1 mRNA expression levels at day 7, only. Although PARP-1 is activated as a reparative mechanism in response to increased ROS-mediated DNA damage [160] [161], multiple investigations reported a detrimental impact of increased PARP-1 levels on the kidneys through depleting NAD levels, subsequently aggravating kidney damage [161] [162].

Collectively, IL-33 treatment seems to improve renal homeostasis 4 days following MI, however, this protection disappears at day 7 coinciding with worsened cardiac dysfunction and increased systemic inflammation. In the murine infarcted heart, IL-33 treatment has been demonstrated to increase eosinophils infiltration at day 7. Eosinophils are generally believed to participate in disease pathogenesis [163]. Although the mechanisms of eosinophils-mediated tissue damage are not fully elucidated, their critical role in promoting thrombosis, oxidative stress, and fibrosis are well-recognized. IL-5, GM-CSF and IL-13 biomarkers that potently contribute to eosinophil's sustainability by promoting its development, migration, and survival have also been overexpressed following IL-33 treatment in either the plasma or cardiac tissue [163]. Therefore, eosinophils

infiltration is proposed as a possible mechanism implemented in IL-33 pathogenesis in the infarcted heart as observed at day 7. This draws interest in uncovering whether eosinophils are also major kidney invaders in the presence of IL-33 treatment and tackling their mediated-renal effects. Further investigations are also required to configure whether the observed shift in IL-33 effect on the kidneys is direct, or indirect through aggravating left ventricular remodeling and systemic inflammation, or the coupling of both. The assessment of local sST2 and IL-33 levels in the kidneys along with cell infiltration and renal function is thus crucial for future analysis.

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