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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology
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REGULATION OF NK CELL-MEDIATED TUBULAR EPITHELIAL CELL DEATH AND KIDNEY ISCHEMIA-REPERFUSION INJURY BY THE NKR-P1B RECEPTOR AND CLR-B

(Spine title: Clr-b regulates NK cell-mediated TEC death and kidney IRI)

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by

James Yip

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of the requirements for the degree of
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The School of Graduate and Postdoctoral Studies
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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

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ABSTRACT

Renal ischemia-reperfusion injury (IRI) occurs following reduced renal blood flow and is a major cause of acute injury in both native and transplanted kidneys. We have previously demonstrated that NK cells can mediate tubular cell death and kidney IRI. Natural killer receptor-protein 1B (NKR-P1B) has been shown to interact with C-type lectin-related protein B (Clr-b), resulting in the suppression of NK cell-mediated cytotoxicity.

Clr-b mRNA and protein expression in the kidney were up-regulated after renal IRI. Similar upregulation of Clr-b expression was seen in cytokine-challenged primary-cultured tubular epithelial cells (TEC). Furthermore, NK cytotoxicity assays demonstrated enhanced necrotic death in TEC after Clr-b siRNA knockdown.

Our results indicate that Clr-b expression in TEC and the kidney is upregulated after injury. The blockade of Clr-b enhances NK cell-mediated TEC death and kidney injury. These studies suggest that enhancing the inhibitory Clr-b in transplant patients may protect the kidney from NK cell-mediated cytotoxicity.

KEYWORDS

Tubular epithelial cell, NKR-P1B, Clr-b, cell death, ischemia-reperfusion injury

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIRL	Antibody-induced redirected lysis
ARF	Acute renal failure
bFGF	Basic fibroblast growth factor
CAN	Chronic allograft nephropathy
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
Clr	C-type lectin-related
CK2	Casein kinase 2
CKD	Chronic kidney disease
C-TGF	Connective tissue growth factor
DAP10	DNAX-activation protein of 10 kDa
dsRNA	Double-stranded tibonuvlriv svif
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting

FADD	Fas-associated protein with death domain
FasL	Fas ligand
FcRγ	Fc receptor γ-chain
FLIP	Fas-associated death domain-like interleukin-1β converting enzyme like inhibitory protein
GEF-H1	Guanine nucleotide exchange factor H1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H60	Histocompatibility 60
HGF	Hepatocyte growth factor
HMGB-1	High-mobility group protein 1
IAP	Inhibitor-of-apoptosis protein
ICAM-1	Intracellular cell adhesion molecule-1
IFN-γ	Interferon-γ
IFNGR1	IFN-γ receptor 1
IFNGR2	IFN-γ receptor 2
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRI	Ischemia-reperfusion injury
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KARAP	Killer activating receptor-associated protein
KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAK	Lymphokine-activated killer
Lck	Lymphocyte-specific tyrosine kinase
LLT1	Lectin-like transcript-1
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Mononuclear chemoattractant protein-1
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MULT-1	Murine UL16 binding protein-like transcript 1
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NKC	Natural killer gene complex
NKG2D	Natural-killer group 2 member D
NKR-P1	Natural killer receptor-protein 1

Nkp46	Natural killer cell p46-related protein
NO	Nitric oxide
PCR	Polymerase chain reaction
PDGF-BB	Platelet-derived growth factor-BB
PI3K	Phosphatidylinositol 3-kinase
RAE-1	Retinoic acid early inducible-1
RCTL	Rat cytomegalovirus C-type lectin-like
RhoA	Ras homolog gene family member A
RIPK	Receptor-interacting protein kinase
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
SHP-1	Src homology phosphatase-1
shRNA	Short-hairpin RNA
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TACE	Tumour necrosis factor-α converting enzyme
TEC	Tubular epithelial cells
TFIID	Transcription factor II D
TGF-β	Transforming growth factor-β

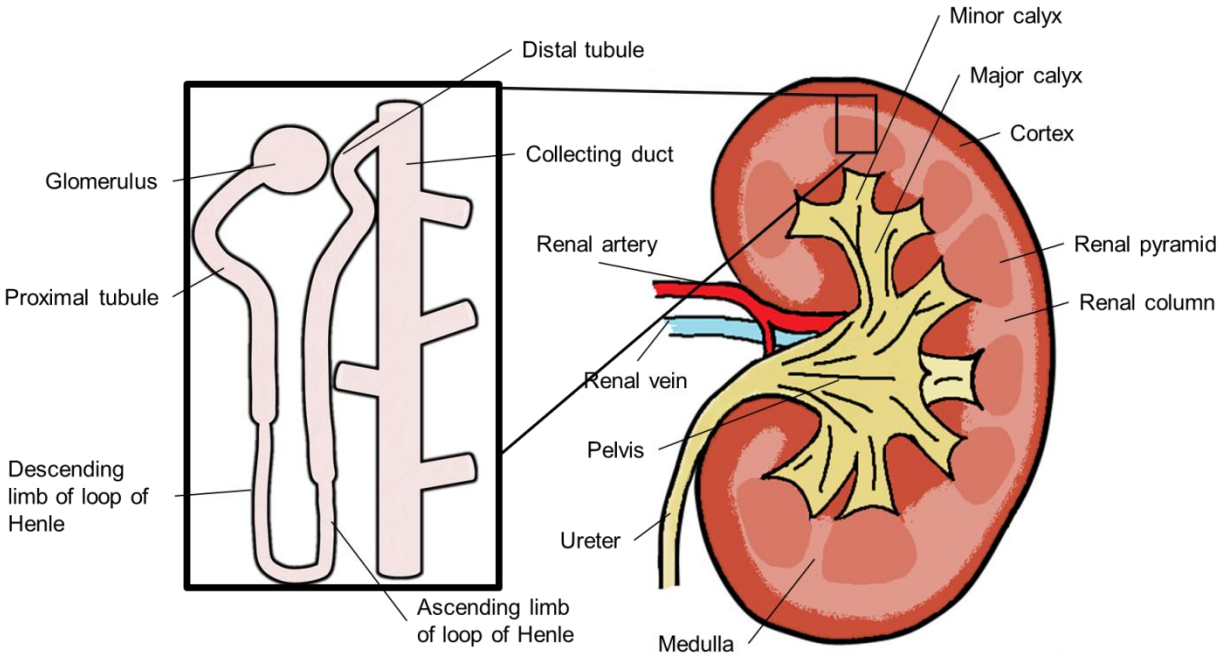
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TNFR1	Tumour necrosis factor receptor type 1
TNFR2	Tumour necrosis factor receptor type 2
TRADD	Tumour necrosis factor receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1

1 Introduction

1.1 The anatomy of the kidneys

The kidney is a bean-shaped structure with a concave and convex surface (Figure 1). The concave surface lies the renal hilum, where the renal artery enters the organ and the renal vein and ureter exit. Surrounding the kidney is the renal capsule, composed of fibrous tissue, which is in turn surrounded by the adipose capsule, renal fascia and pararenal fat. The parenchyma is divided into the superficial renal cortex and the deep renal medulla. These structures consist of cone-shaped renal pyramids and renal columns spanning both the renal cortex and medulla. Within these structures are nephrons acting as the functional units of the kidney (Figure 1). The initial filtering portion of a nephron is the renal corpuscle, located in the cortex, followed by a renal tubule in the medullary pyramids. The medullary ray is a collection of renal tubules and part of the renal cortex that drains into a single collecting duct. Each renal pyramid empties urine into a minor calyx. The minor calyces empty into major calyces, which empty into the renal pelvis.


Distal tubule
Collecting duct
Glomerulus
Proximal tubule
Descending limb of loop of Henle
Ascending limb of loop of Henle
Minor calyx
Major calyx
Cortex
Renal artery
Renal pyramid
Renal column
Renal vein
Pelvis
Ureter
Medulla

.

Figure 1. Structure of the kidney

The kidney is divided into the renal cortex and medulla, consisting of renal pyramids and renal columns. Nephrons are functional units that span the cortex and medulla. Damage and the resulting inflammation in these structures may lead to renal dysfunction and organ loss in severe cases.

1.2 Challenges in kidney transplantation

The most common risk factors associated with chronic kidney disease (CKD) between 2005 – 2010 in the United States are *diabetes mellitus*, hypertension, and cardiovascular disease[223]. In early stages of CKD, proper diet and medication may help the kidney maintain function. When kidney function drops below 10-15%, however, complications may develop and can lead to death[254]. In these cases, patients will require dialysis or a kidney transplant. Kidney diseases may involve the glomerulus such as diabetic nephropathy, bacterial endocarditis, Alport Syndrome, systemic lupus erythematosus and focal segmental glomerulosclerosis[81]. Diseases such as polycystic kidney disease[144] and reflux nephropathy[61] cause damage to the tubulointerstitium, while vascular damages may result from renal artery stenosis[48] and Hemolytic Uremic Syndrome[16]. Kidney stones[46] and prostate diseases[187] may also lead to obstruction of the kidney. These diseases result in renal dysfunction and, if severe, may require patients to undergo kidney transplantation or dialysis.

Kidney transplantation may be necessary for patients with end-stage renal diseases. A typical kidney transplant patient is expected to live 10 to 15 years longer than on dialysis[256]. However, kidney transplantation may involve an array of complications that ultimately result in organ injury and rejection. Factors affecting organ viability includes donor age, prolonged cold ischemic time, delayed graft function and acute tubular necrosis, living or deceased donor, renal mass, and donor brain injury[103]. Side effects of immunosuppressants may lead to sepsis and viral infections, including human cytomegalovirus and Bren nan-Krohn polyomavirus[103]. Recipient factors affecting kidney grafts may include lipid disorders, diabetes, recurrent diseases, compliance, hypertension, and obstruction[103]. At the immunological level, allograft injury may be caused by direct or indirect allorecognition, donor-host mismatch, subclinical inflammation, co-stimulatory signaling, inadequate immunosuppression, or antibody-mediated rejection[103]. The resultant inflammation may lead to kidney failure and graft loss.

1.3 Acute allograft injury in kidney transplantation

Transplantation is invariably associated with organ injury resulting from ischemia-reperfusion injury (IRI)[127], inflammation[218], drug toxicity[45] and rejection[235]. The kidney is vulnerable to diverse forms of injury leading to dysfunction and, in most severe instances, delayed graft function. Similarly, IRI, which occurs during the early stage of kidney transplant surgery, is a major cause of delayed graft function and if severe can lead to early allograft loss[214]. Renal IRI is a major cause of acute renal failure (ARF) and is associated with reduced renal blood flow, contributing to up to 50% mortality rate[225]. Following ischemia, there is oxidative stress causing cytokine and chemokine upregulation and also cell death. The result is the recruitment of inflammatory cells, which further mediates injury through direct cell-cell contact or indirect cytokine and chemokine production inside the graft[164, 232]. Recruitment of leukocytes into the site of injury is mediated by cell adhesion molecules[37, 164].

A broad range of extracellular and intracellular factors contribute to IRI. Studies have demonstrated that Fas-Fas ligand (FasL) interactions between tubular epithelial cells (TEC) lead to self-induced and inflammatory cell-mediated apoptosis[67, 68, 173]. This death receptor pathway of apoptosis involves caspase-8 activity, as Fas stimulation by anti-Fas antibody causes liver destruction and mortality only in mice with functional caspase-8[175]. Lethality in caspase-8-deficient mice has been traced to failure in early vascularization and haematopoietic development[238]. Lack of functional Fas, FasL or caspase-8 also accumulates T lymphocytes and results in progressive severe lymphoaccumulation[175, 215].

Disruption of Fas-FasL interactions or Fas signaling through caspase-8 inhibition *in vivo* has proven to be successful in rodent kidney transplant models by our group[69, 166, 266]. It has been difficult, however, to establish a role for this receptor in allograft injury because Fas expression may vary nonspecifically in the graft[25].

A study on genes involved with host-mediated cellular response in renal biopsies from transplant patients found high expression of perforin, granzyme B and FasL[211]. In acute renal allograft rejection, TEC death commonly occurs *via* both the perforin and granzyme B lytic pathways and Fas-FasL system[25, 84, 253]. Studies examining perforin and granzyme B found that this pathway was responsible for cluster of differentiation (CD)4^+ T lymphocyte-mediated renal tubular cell destruction[129, 177, 253]. The Fas-FasL system, on the other hand, has been shown to reduce immune responses by initiating apoptosis of immune cells[58, 196] and apoptosis of TEC[180, 239]. FasL is expressed not only on macrophages, neutrophils, T, B and natural killer (NK) cells, but also TEC[25, 179], where it induces the elimination of antigen-activated $CD4^+$ T lymphocytes[3]. Fas-FasL interactions have also been associated with chronic renal injury and graft survival, and Fas polymorphisms may affect graft survival[202].

Involvement of endogenous tumour necrosis factor-α (TNF-α) has also been documented in kidney IRI[53, 62, 63]. Resident dendritic cells appear to be the predominant source in the early-stages[62], and TNF-α binding with its receptor induces one of the death receptor pathways of apoptosis[11]. This pathway involves the adaptor molecule Fas-Associated protein with Death Domain (FADD), which binds to the intracellular region of the receptor. FADD activates caspase-8 and initiates apoptosis[23]. Fas-associated death domain-like interleukin-1β converting enzyme-like inhibitory protein (FLIP) is an endogenous inhibitor that resembles caspase-8 but lacks a catalytic site and antagonizes this pathway[227]. However, biochemical and structural studies have indicated that FLIP can heterodimerize with caspase-8 to impart catalytic activity, even in the absence of interdomain cleavage which is typically required for caspase-8 homodimer stabilization and activity[22, 39]. Gene ablation of caspase-8[238], FADD[259] or FLIP[258] leads to embryonic lethality, illustrating the physiological significance of these proteins.

Recent studies have revealed that knockdown of caspase-8 does not simply prevent cell death but also sensitizes cells to necrosis[240]. Necrotic induction by

TNF-α in response to caspase-8 inhibitors involves the kinase activity of receptor-interacting protein kinase (RIPK)-1[57, 94] and -3[44, 89, 263]. NADPH oxidase, mitochondrial reactive oxygen species (ROS)[240], and metabolic enzymes[263] production have been demonstrated as possible downstream effectors of RIPK3. Interestingly, while caspase-8 deletion is lethal during embryonic development, RIPK3 ablation rescues the lethal phenotype of caspase-8-deficient mice[175]. RIPK1 and RIPK3 associate with FADD to induce necrosis only after caspase-8 or FLIP knockdown, as the caspase-8-FLIP heterodimer prevents their stabilization through RIPK1 cleavage[175, 185]. TNF receptor type 1 (TNFR1), Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors are all capable of triggering RIPK-dependent necrosis in the absence of caspase-8[94].

Besides conventional Fas- and TNF-α-induced cell death, TEC apoptosis can also result from oxidative stress and its associated mediators, including ROS, cytokines, chemokines and nitric oxide (NO)[66, 82, 164, 232]. Under physiological conditions, ROS are broken down by superoxides and other oxidases, including superoxide dismutase, catalase, and glutathione peroxidase. During IRI, however, the excessive production of ROS overwhelms the free radical scavenging system. The consequence is lipid peroxidation of cell membranes, DNA fragmentation, and sulfhydryl-mediated protein cross-linkage, all of which leads to cellular injury[232]. Compromise of mitochondrial ATP production can also inhibit the sarcoplasmic reticulum's calcium ATPase and sodium-potassium ATPase activities, leading to cellular calcium accumulation[92]. It has been demonstrated that oxygen radicals result in the chemotaxis of neutrophils, which are sources of further radical generation[195].

NO production or inducible nitric oxide synthase (iNOS) upregulation during inflammation has been reported in infiltrating mononuclear cells[167] and within the kidney, including glomerular, mesangial, smooth muscle and tubular epithelial cells[153, 184, 193]. NO, while at low levels exert cytoprotective effects, are potentially toxic at higher levels[129]. Studies have revealed that the production of cytokines such as IL-1β, TNF-α and interferon-γ (IFN-γ) leads to increased expression of

iNOS[15, 167, 241]. The resulting increase in nitric oxide promotes FasL expression, causing Fas- and caspase-8-mediated apoptosis[71, 86, 241]. Many of the toxic effects of NO are mediated indirectly by its oxidative reaction products, such as peroxynitrite. In the kidney, higher nitric oxide concentrations have been demonstrated to promote cellular injury[153, 184, 193].

Chemokines are also early major mediators of inflammation, promoting pro-inflammatory cytokine expression, upregulating endothelial cell adhesion molecules, leukocyte infiltration and activation. The CC, CXC, and CX3C chemokine families have been implicated in kidney IRI[102, 220]. Renal IRI results in activation of the complement system in TEC and induces cells to produce pro-inflammatory chemokines[4]. TEC have been shown to express toll-like receptor (TLR)-2 and -4 which cause the production of a variety of chemokines, some of which can also be induced by complement activation fragments, including CXCL1, macrophage inflammatory protein 2 (MIP-2), IL-6, and TNF-α[4].

Previous studies have demonstrated that macrophages and neutrophils infiltrate the kidney post-injury, playing an important role in tubular dysfunction[24, 56, 65, 261]. It has also been suggested that many cell types of the adaptive immune system participate in kidney IRI[10, 27, 54, 55, 188, 201, 262]. $CD4^+$ T cells have been demonstrated to infiltrate the organ within the first 4 hours but vanish after a day following ischemia, coined the “hit-and-run” phenomenon[10, 77, 135] and likely through the Fas-FasL pathway[121]. In addition, $CD19^+$ B cells, $CD4^+$ $NK1.1^+$ NKT cells, and $CD3^-$ $NK1.1^+$ NK cells also infiltrate the kidney shortly after injury, suggesting that a complex multi-factorial inflammatory response is triggered by kidney IRI[10]. This is further supported by studies showing that NKT cells promote neutrophil infiltration and IFN-γ production after IRI[142], while NK cells also promote neutrophil infiltration and TEC expression of CD137[115]. As kidney injury results from multiple molecular events in TEC and inflammatory cells, further investigation is necessary for a more thorough understanding of inflammation-mediated kidney injuries.

1.4 Chronic allograft injury in kidney transplantation

While acute rejection rates are very low in the era of modern immunosuppression, chronic renal injury remains a significant clinical issue. There are several characterizations of chronic allograft nephropathy (CAN), including progressive tubular atrophy and interstitial fibrosis, as well as microvascular and glomerular damage[103]. Graft function may decline within months to years after transplantation[103]. The incidence of this disorder can be up to 60% of grafts 10 years post-transplant. As there are currently no specific strategies to prevent CAN, a more direct approach to limit TEC atrophy and fibrosis may produce better long-term outcomes for kidney grafts.

Tubular epithelial cells comprise more than 75% of renal parenchymal cells and their susceptibility to injury directs long-term function of kidney allografts, as tubular injury can be a primary cause for nephron loss[191]. Apoptosis is an innate mechanism required for kidney remodelling and repair. When cell death exceeds the kidney`s regenerative capacity, there may be loss of function and even premature graft failure. In many patients, a gradual loss of renal function occurs after transplantation, causing chronic allograft injury and becoming a critical problem in clinical settings[214]. Although kidney tubular cells are highly capable of regeneration after injury[174], it remains unclear as to how early-stage ischemic injury negatively influences allograft function over long-term survival.

Studies have suggested that IRI may play a role in permitting kidney allograft tolerance[40, 50, 128, 169]. Recent studies have demonstrated that lymphatic neoangiogenesis may contribute to chronic renal graft injury[112, 113]. Tertiary lymphoid tissues have been found in patients with graft loss[114] and are associated with chronic rejection and alloantibody production[226]. Others have suggested that epithelial-to-mesenchymal transition (EMT) may be a common pathway leading to chronic graft function loss[244, 251]. EMT has been described in other chronic kidney diseases with fibrosis, and TEC injury has been associated

with the development of fibroblasts within the interstitium of the kidney[108]. Consistent with this, connective tissue growth factor (C-TGF) and transforming growth factor-β (TGF-β) have been shown to be involved in the fibrotic response in kidney allografts[42, 51, 52, 78]. In kidney transplant models, C-TGF can induce transformation of kidney TEC into fibroblasts[42]. Furthermore, high-mobility group protein 1 (HMGB-1) has been observed as an extracellular secreted protein in serum of patients with sepsis and regulates the production of interleukin (IL)-1 and TNF-α during inflammation[70]. HMGB-1 has been suggested to be a key mediator of immune-mediated EMT in TEC by reducing E-cadherin expression, increasing α-smooth muscle actin expression and enhancing cell migration[151]. Other factors involved in augmenting TGF-β signalling, such as endothelin-1[231], platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF)[247], or counteracting TGF-β signalling, such as bone morphogenic protein-7 and hepatocyte growth factor (HGF), are also potentially important in the response to injury.

Although progress has been made, greater knowledge about the pathophysiology of IRI and chronic kidney graft injury is required to develop rational and effective therapeutics. There are currently no specific treatments available to prevent IRI, as it involves a complex biological process and many contributing factors that have not yet been clearly identified. Understanding the precise mechanisms behind IRI and late kidney graft loss is therefore vital for identifying clinically-effective approaches.

1.5 NK cell biology

NK cells are predominantly derived from the bone marrow and provide a first line of defense against pathogens and cancer. Immature NK cells lie in the bone marrow and lymph nodes, whereas mature NK cells can be primarily found in the spleen, peripheral blood, and lungs[6, 88, 116]. Studies have demonstrated the existence of bipotent NK/T progenitor cells in the thymus[35, 64, 192]. NK cells are

responsible for attacking abnormal or foreign cells by releasing perforin and granzymes to initiate cytotoxicity, or secreting inflammatory cytokines, such as IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF). NK cells are believed to be triggered based on the “missing-self” hypothesis, where target cells with defective major histocompatibility complex (MHC) class I expression are identified and eliminated by NK cells. Viruses such as cytomegaloviruses and human immunodeficiency viruses can evade the NK cell’s immune surveillance system by manipulating the balancing of NK receptor signalling interactions[178].

The natural killer gene complex (NKC) encodes type II integral membrane proteins with C-type lectin-like extracellular domains[260]. Receptors from the same family have been demonstrated to exhibit opposing functions. For example, Ly49A, Ly49C, and Ly49G recognize MHC Class I antigens and act as inhibitory receptors, while Ly49D and Ly49H stimulate NK cell activity[109, 118, 156, 217]. Inhibitory Ly-49 engagement prevents NK cells from attacking “self-MHC” class I-expressing cells, whereas activating receptors such as natural-killer group 2 member D (NKG2D) permit NK cells to attack “non-self” MHC molecules and cells deficient of self-MHC class I molecules. In the presence of normal MHC class I molecules, inhibitory receptors tend to dominate NK cell function[182]. However, activating receptors can overcome the inhibition depending on the target cell’s regulation of its surface ligands. For example, stress-induced expression of ligands for NKG2D can activate NK cell-mediated cytotoxicity even in the presence of MHC class I molecules[17, 59]. As NK cell activity is modulated by multiple receptors, further studies need to be conducted to elucidate their functions.

NK cell receptor expression can also mark different developmental stages. Natural killer receptor-protein 1 (NKR-P1) acquisition is an early developmental event, followed by CD94/NKG2. Final NK cell maturation is reached after Ly49 expression, consisting of a full set of NK cell receptors and effector functions[73, 117]. In mice and humans, fully mature NK cells can be identified by expression of

the integrin CD11b[116]. Interestingly, immature murine NK cells are $CD11b^-$ $CD27^-$ and transit through $CD11b^-$ $CD27^+$ and $CD11b^+$ $CD27^+$ until fully maturing as $CD11b^+$ $CD27^-$ cells[43, 88]. In humans, NK cells can be differentiated based on their surface density of CD56[76, 137, 206]. Fully differentiated NK cells are associated with low CD56 expression and are highly cytotoxic, whereas less mature and cytotoxic NK cells have high expression of CD56[216, 245]. NK cells expressing high levels of CD56 are believed to be the main contributors of IFN-γ release upon cytokine stimulation, while NK cells with low CD56 can produce IFN-γ upon interaction with target cells[72]. Low-expressing CD56 NK cells are also associated with Killer cell immunoglobulin-like receptor (KIR) expression, and high-expressing CD56 NK cells express CD94/NKG2 receptors[73]. In rats, Ly49 expression is found primarily in NK cells with Ly49 stimulatory receptor 3, while NKG2 receptors are mainly expressed in NK cell subsets with NKR-P1B[100, 133]. NK cell development undergoes various stages of maturation through the programmed acquisition of receptors and ligands.

1.6 Role of NK cells on kidney injury

NK cells are important participants in early-stage innate immune responses. In solid organ transplantation, NK cells have emerged as a particular focus of interest because of their potent cytolytic activity and their ability to distinguish between allogeneic MHC antigens. NK cells have been shown to participate in both acute and chronic rejection of solid organ allografts[18] and are influenced by immunosuppressive drugs such as calcineurin inhibitors, steroids, or therapeutic antibodies. After ischemia, NK cells quickly infiltrate kidney grafts and participate in renal IRI[265]. It has been demonstrated in several studies that NK cells can determine transplant survival by rejecting an allograft indirectly by influencing the alloreactivity of T cells or by killing antigen-presenting cells[28]. Recent findings also demonstrated that NK cells can induce allograft tolerance, suggesting that the role of NK cells in graft rejection and tolerance needs to be reconsidered. In

order to understand the role of NK cells in kidney injury, it is important to decipher the mechanisms regulating NK cell function in the kidney.

Unlike T and B cell responses, NK cells are constitutively cytotoxic and can lyse target cells without pre-sensitization. Along with cellular toxicity, IFN-γ and TNF-α production by NK cells form an essential component of their innate immune response[150, 246, 268]. We have recently shown that NK cells can lyse TEC in a perforin-dependent manner and contribute to renal IRI[264, 265]. NK cells infiltrate the kidney as early as 30 minutes after IRI and regress to low levels after 24 hours[265]. Depletion of NK cells in mice inhibited kidney IRI and adoptive transfer of NK cells worsen injury[265]. We also previously found that, while osteopontin is important for tissue repair and can inhibit TEC apoptosis, it is highly expressed in the kidney after IRI and contribute to NK cell activation and mediate TEC death[264]. Furthermore, increases in the NKG2D ligands retinoic acid early inducible-1 (Rae-1), murine ULBP-like transcript 1 (MULT-1) and histocompatibility 60 (H60) were also observed in mouse kidneys after renal IRI[41, 265]. Ectopic expression of Rae-1b or H60 in various tumour cell lines have resulted in NK cell-mediated rejection of the tumour cells by syngeneic mice[60]. As a subset of NK cells remain long-term in kidney grafts, a potential role for NK cells exist in chronic graft dysfunction as well as regulation of NK cell function by the kidney.

1.7 Regulatory roles of NKR-P1 receptors

An early method for NK cell purification involved the immunization of C3H mice with CE thymocytes to produce α-Ly1.2 anti-serum, capable of depleting NK cell activity[80]. Afterwards, it was discovered that anti-NK cell specificity could be isolated serologically by selectively adsorbing the C3H α-CE anti-serum into BALB/c thymocytes or splenocytes[80]. The NK alloantigen, called “NK-1,” was broadly used to define and purify NK cells[124, 126]. This led to the development of an α-NK1.1 monoclonal antibody (mAb), PK136, by Koo and Peppard[125]. PK136

identifies NK cells from CE, B6, NZB, C58, Ma/My, ST, and SJL mice, but not BALB/c, AKR, CBA, C3H, DBA, or 129 mice[80, 126]. It was reported that blocking the NK1.1 antigen did not alter NK cell-mediated cytotoxicity and that the 39 kDa glycoprotein stimulated NK cell function upon cross-linking[110, 125, 210].

The NK1.1 alloantigen is now widely used as a marker for NK cell identification. Previous studies have revealed that NK1.1 is a member of the NKR-P1 family of disulfide-linked homodimeric type II transmembrane C-type lectin-like receptors[34, 79, 183, 200], which includes NKR-P1, killer cell lectin-like receptor subfamily G member 1 (KLRG1), Ly49, CD94-NKG2A/C/E, and NKG2D receptors. It has further been determined that the NK1.1 alloantigen is NKR-P1C in C57BL/6 mice and NKR-P1B in the SJL/Sw strain, while the anti-NK1.1 mAb recognizes neither NKR-P1B nor NKR-P1C in the BALB/c strain[32, 34, 131, 200]. Furthermore, the *Nkr-p1* cluster was found to be located on chromosome 6 in mice and chromosome 12 in humans[200]. Currently, the *Nkr-p1a*, *-b*, *-c*, *-d*, *-e*, *-f* and *-g* genes have been identified, although further analysis suggests that *Nkr-p1e* is nonfunctional[34, 183]. During development, NK cells express NKR-P1C, NKR-P1D and NKR-P1F before the expression of most members of the Ly49 family in C57BL/6 mice[12]. It has also been demonstrated that (C57BL/6 × BALB/c)F_1 NK cells and (C57BL/6 × 129/Sv)F_1 NK cells express proportionally the same C57BL/6 isoform of NKR-P1C and NKR-P1D by immunostaining[12]. Studies have identified that the inhibitory NKR-P1B and NKR-P1D receptors are recognized by C-type lectin-related (Clr)-b[31], and the stimulatory NKR-P1F receptor is recognized by Clr-g[99].

Sequencing and functional studies indicate that the NKR-P1A, NKR-P1C and NKR-P1F receptors exert stimulatory effects on NK cells, whereas NKR-P1B and NKR-P1D are inhibitory[9, 32, 99, 110, 131, 181]. NKR-P1A, NKR-P1C and NKR-P1F contain positively charged amino acid residues in its transmembrane domain, and association of ligands with its Fcγ chain delivers stimulatory signals, transmitted via the FcRγ adaptor molecule, that triggers cytotoxic activity and cytokine secretion[9, 110]. FcRγ is known to transduce signals for FcγRIII and FcεRI in immunoglobulin[9]. It has been suggested that association with FcRγ involves

the charged transmembrane R residue in the NKR-P1 receptors and the corresponding transmembrane D residue in FcRγ[32]. Stimulatory NKR-P1 receptor cross-linking presumably results in phosphorylation of the FcRγ immunoreceptor tyrosine-based activation motif (ITAM) Y residues, leading to downstream recruitment of the spleen tyrosine kinase (Syk), and activation of NK cytotoxicity[9, 32]. ITAMs are also known to associate with other adaptor molecules such as killer activating receptor-associated protein (KARAP) or DNAX-activation protein of 10 kDa (DAP10). Protein tyrosine kinases with Src homology 2 domains or phosphatidylinositol 3-kinase (PI3K) can then bind to phosphorylated tyrosine residues and modulate downstream signalling (Figure 2)[182]. Antibody cross-linkage to rat NKR-P1A in a previous study provoked antibody-induced redirected lysis (AIRL), phosphatidylinositol turnover, and calcium flux[38, 197]. In C57BL/6 murine NK cells, NKR-P1C was also observed to mediate AIRL and IFN-γ production[7, 110]. The murine NKR-P1C receptor has been implicated in allorecognition by studies involving the NK-mediated F1 anti-parent “hybrid resistance” phenomenon[130].

NKR-P1B and NKR-P1D, however, lack charged transmembrane residues and possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain, which interacts with Src homology phosphatase-1 (SHP-1) in a phosphorylation-dependent manner to produce inhibitory signals (Figure 2)[32, 99, 131, 181]. Sequence analysis revealed that mouse NKR-P1B lacks a charged transmembrane R residue due to a single amino acid deletion in its sequence[25]. Mutation of the NKR-P1B cytoplasmic ITIM Y residue was found to eliminate both SHP-1 recruitment and inhibitory responses[147]. NKR-P1B was also demonstrated to produce inhibitory signals in PK136-mediated AIRL assays[32, 131], and cross-linking of both NKR-P1B and NKR-P1C on (B6 x Sw)F1 LAK cells using PK136 mAb revealed a dominance of NKR-P1B inhibitory response over NKR-P1C stimulatory signaling[32]. In rats, NKR-P1B displayed dominant inhibitory function over 2B4-mediated cytotoxicity[141]. Similarly, mouse NKR-P1B overexpression in the human YTSeco NK cell line also showed dominant inhibition over 2B4 function[147].

Furthermore, Inngjerdingen and colleagues have demonstrated in rats that NKR-P1B's expression pattern varies between compartments. Particularly, in comparison with other organs, NKR-P1B is prevalent in the peripheral blood, mesenteric lymph nodes, Peyer's patches and the liver[100]. Relatively low levels of NKR-P1B were detected on the surface of NK cells residing in the bone marrow and cervical/inguinal lymph nodes[100], suggesting developmental-stage specific NKR-P1B expression. Interestingly, a distinct pattern of chemokine receptors was observed between these NK cell populations. In the highly NKR-P1B$^+$ NK cell population there were higher levels of CX_3CR1, CXCR6 and CCR5, and lower levels of CCR7 and CCR9 than the low NKR-P1B$^+$ population[100]. NK cells with high NKR-P1B expression were demonstrated to be more responsive in terms of IFN-γ production[100].

Sequence analysis of mouse and rat NKR-P1 receptors revealed that all isoforms possess a cytoplasmic CxCP motif similar to those found in the CD4 and CD8 receptors[30, 199, 236]. This motif is known to associate with Lymphocyte-specific tyrosine kinase (Lck), a Src-related protein tyrosine kinase[236]. A study demonstrated that association between Lck and rat NKR-P1A was dependent on the CxCP motif, as mutation of both C residues abrogated the interaction[30]. Mutation of a single C residue in the CxCP motifs of mouse NKR-P1B and NKR-P1C either abolished or greatly reduced the capacity of the receptors produce intact signals[147]. NK cells from C57BL/6 *lck*$^{-/-}$ mice displayed significantly less PK136-mediated AIRL of FcR$^+$ P815 mastocytoma targets, demonstrating the necessity of Lck in NKR-P1C function. Lck-deficient bone marrow-derived lymphokine-activated killer (LAK) cells were more severely impaired in NK1.1-mediated AIRL than splenic LAK cells, suggesting other signalling mechanisms may compensate for the Lck deficiency during NK cell maturation[147]. Upon receptor activation, Lck phosphorylates the ITIM tyrosine residue, facilitating recruitment of the SHP-1 tyrosine phosphatase. Thus, both the stimulatory and inhibitory NKR-P1 receptors utilize the CxCP motif to initiate their signalling pathways.

As NKR-P1A, NKR-P1D and NKR-P1F are weakly expressed on NK cells, activation of these receptors has been shown to have no significant effect on NK cell-mediated cytotoxicity[12]. NKR-P1A is expressed at very low levels on NK cells and some activated T cells, while undetectable on NKT cells. IL-2-activated NKT cells were demonstrated to express NKR-P1C, low levels of NKR-P1A, and no NKR-P1D and NKR-P1F[12]. While the endogenous ligand is unknown, rat NKR-P1A was shown to functionally recognize a xenogeneic ligand expressed on several mouse tumour lines[198]. A study has suggested that oligosaccharides may bind to rat NKR-P1A with high affinity, however the results could not be reproduced[20, 21, 122, 123].

NKR-P1D, on the other hand, has been found to be expressed on approximately half of the NK cell population in C57BL/6 mice[12]. NKR-P1D expression increased in a progressive manner during NK cell development, independently of Ly49E and CD94/NKG2[12]. Notably, the study also demonstrated that NKR-P1D$^+$ NK cells express low levels of Ly49 receptors and high levels of CD94/NKG2 receptors relative to NKR-P1D$^-$ NK cells[12]. The activating receptors CD16, NKG2D, natural killer cell p46-related protein (Nkp46), NKR-P1C and NKR-P1F expression were shown to be independent of NKR-P1D expression[12], and antibody cross-linking or Clr-b ligand interaction with NKR-P1D resulted in receptor down-regulation[12]. Interestingly, activated NKR-P1D$^-$ NK cells produce less IFN-γ and were less cytotoxic relative to NKR-P1D$^+$ NK cells. The reduced cytotoxicity may be similar to NK cells lacking inhibitory Ly49 receptors, either because cells lacking inhibitory receptors are subjected to chronic low-level activation that induces self-tolerance[190], or because the ligand-receptor interactions deliver signals that are required for function[117].

Another study demonstrated that CD3ζ/NKR-P1D fusion receptors expressed on BWZ.36 reporter cells can detect ligands on *ex vivo* bone marrow and spleen cells, but not thymocytes[99]. Further analysis of hematopoetic cells identified macrophages and dendritic cells as the stimulators for the BWZ.CD3 ζ/NKR-P1D reporter cells[99]. Clr-b overexpression on tumour cells was found to inhibit

C57BL/6 NK cell activity, which was reversible by an NKR-P1D blocking mAb[99]. However, another study revealed that Clr-b expression on BWZ.4A6^{+} cells was insufficient to inhibit lysis by NKR-P1D-expressing C57BL/6 NK cells, but significantly inhibited NKR-P1B-expressing Sw or CD-1 NK cells[31]. Due to the low affinity of NKR-P1D-Clr-b interaction[99], physiological Clr-b levels expressed on normal lymphocytes appear to be insufficient in delivering signals *via* NKR-P1D receptors on mature NK cells[12].

A study on YB2/0 rat plasmacytoma cells transfected with the NKR-P1F ligands Clr-g and Clr-x demonstrated that this pathway does not affect IFN-γ synthesis by NK cells[12]. Similarly, although anti-NKR-P1C mAb acted as a potent inducer of IFN-γ production, anti-NKR-P1A and anti-NKR-P1F mAbs failed to stimulate IFN-γ synthesis[12]. Interestingly, Clr-x ligand interaction with NKR-P1F resulted in receptor down-regulation, but not Clr-g[12]. It has been reported, however, that NKR-P1F is highly expressed in bone marrow-derived dendritic cells and facilitates T cell activation through interaction with Clr-g expressed on T cells[229, 230].

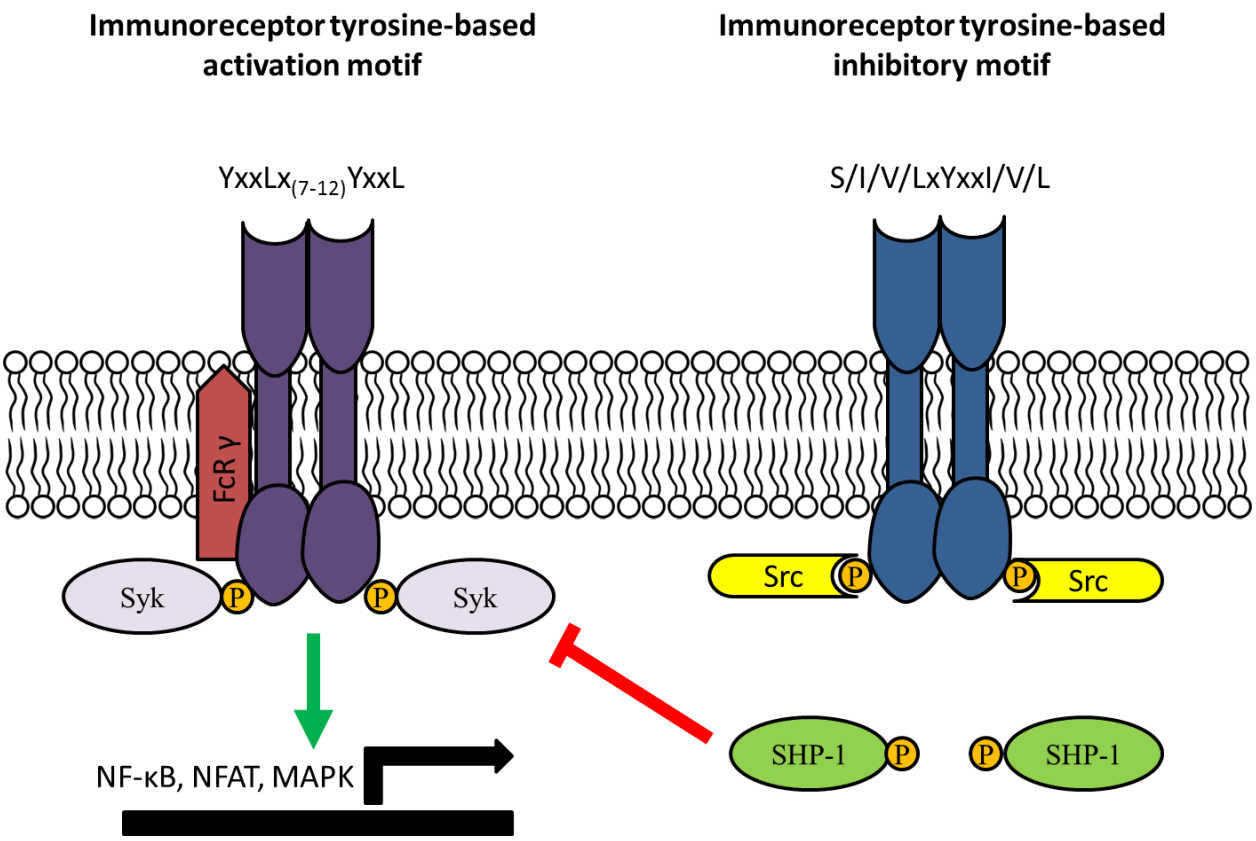

Immunoreceptor tyrosine-based activation motif
Immunoreceptor tyrosine-based inhibitory motif
YxxLx(7-12)YxxL
S/I/V/LxYxxI/V/L
FcR γ
Syk
P
P
Syk
Src
P
P
Src
NF-κB, NFAT, MAPK
SHP-1
P
P
SHP-1

Figure 2. NKR-P1 receptors contain classical ITAM ITIM motifs

An illustration demonstrating the molecular signalling of the classical immunoreceptor tyrosine-based activation (ITAM) and inhibitory motifs (ITIM) upon ligand interaction. After recruitment of the FcRγ adaptor molecule, the phosphorylated Y residue on ITAM initiates tyrosine kinase activity and downstream signalling cascades such as the Nuclear factor κB (NF-κB), Nuclear factor of activated T cells (NFAT) or Mitogen-activated protein kinase (MAPK) pathways. In ITIM, phosphorylation by Src results in the phosphorylation and activation Src homology phosphatase-1 (SHP-1), which inhibits Spleen tyrosine kinase (Syk) to block cascade activation.

1.8 NKR-P1B and Clr-b interaction

Recently, Carlyle and colleagues demonstrated that NKR-P1B and NKR-P1D interact with Clr-b[31]. Clr-b expression exhibits a broad spectrum including adult splenocytes, thymocytes and lymph node cells. Its expression pattern is also similar to that of MHC Class I molecules: high levels are observed in hematopoietic cells, low levels in $CD4^+$ $CD8^+$ thymocytes, and negligible during erythropoiesis and in terminally-differentiated erythrocytes[31]. Clr-b expression and function, however, were found to be independent of β_2m and MHC I expression[31]. Clr-b is also frequently downregulated in mouse tumour cell lines, as NKR-P1B tetramers were shown to bind endogenous Clr-b on cell lines, including MNK-1 pre-NK cells and NIH-3T3 fibroblasts, but not several tumour lines[31]. Tumour cell lines that expressed Clr-b exhibited dramatically reduced NK cell-mediated lysis[31]. SJL/Sw NK cells transduced with NKR-P1B were shown to decrease CD107a degranulation and TNF-α expression against Clr-b-expressing target cells[234].

Although it is unknown whether there is bidirectional communication, the Clr-b cytoplasmic tail contains motifs for casein kinase 2 (CK2) phosphorylation (SxxE), TNF receptor-associated factor 2 (TRAF2) binding (SPQE), ubiquitinylation and endocytosis[267]. CK2 overexpression is common in tumour cell lines, and active CK2 is known to regulate the nuclear factor-κB (NF-κB) pathway, caspase activation and Bcl-X levels[1, 145, 208], leading to lymphoproliferation and accelerated lymphomagenesis[207, 208]. Phosphorylation of CK2 in certain receptors also results in endocytosis[49, 176]. TRAF2, on the other hand, is required for TNF-α-mediated activation of the mitogen-activated protein kinase (MAPK)8/c-Jun N-terminal kinase (JNK) and NF-κB[249]. TRAF2 forms a complex with TRAF1 to interact with the inhibitor-of-apoptosis protein (IAP), functioning as a mediator of anti-apoptotic signals. The interaction of this protein with TNF receptor type 1-associated DEATH domain (TRADD), a TNF receptor adaptor protein, ensures the recruitment of IAPs for the direct inhibition of caspase activation. Thus, it is tempting to postulate that NKR-P1B-Clr-b

interaction functions as a cell survival pathway in Clr-b-expressing cells (Figure 3).

The *Clr* genes are closely linked and interspersed among the *Nkr-p1* genes in the NKC, which may ensure the co-inheritance of receptor and ligand genes and provide a mechanism for "missing self-recognition"[34]. Whereas the *Ly49* genes are divergent between the C57BL/6 and BALB/c mice strains, the *Nkrp1* and *Clr* families are conserved[34]. Interestingly, NKR-P1B expression is missing in certain mouse strains, such as C57BL/6[34]. Similarly, sequencing analysis revealed that *Ly49h* and *Ly49d* are absent in the BALB/c strain[5], resulting in uncontrolled mouse cytomegalovirus replication, early host death[8, 26], and decreased NK cell cytotoxic efficiency[98]. It has been suggested that the *Nkr-p1d* gene in these mice represent a divergent allele to the *Nkr-p1b* gene found in BALB/c and other mouse strains[34]. Protein sequence alignment revealed only three amino acid substitutions in the extracellular regions between the $NK1.1^+$ Sw/SJL and $NK1.1^-$ BALB/c mice, and only a single amino acid mutation (position 191) in the BALB/c NKR-P1B receptor was able to confer NK1.1 reactivity[34]. In contrast to the other conserved *Nkr-p1* genes, the C57BL/6 and BALB/c *Nkr-p1c* alleles have diverged significantly[34]. It is possible that the variable prevalence of NKR-P1 receptors may account for the different susceptibilities of mouse strains to kidney injury.

The existence of NKR-P1 and Clr-like genes within the MHC region of chicken and quail genomes suggests that the MHC and NKC regions may share a common ancestral origin[111, 213]. Also, Clr-like genes were found in the rat cytomegalovirus genome, suggesting that some viruses may have evolved strategies to bypass NKR-P1 recognition during infection[243]. In humans, it is interesting to note that only the isoform NKR-P1A exists and interacts with lectin-like transcript-1 (LLT1), a ligand which shares significant homology with the Clr family[2, 194]. Similar to mouse NKR-P1B and Clr-b, NKR-P1A and LLT1 interaction inhibits NK cell function[2]. Notably, LLT1 was observed to stimulate IFN- γ production in human T cells but inhibited cytotoxicity and IFN-γ secretion in NK

cells[2]. Unlike murine NKR-P1 receptors, the human NKR-P1A receptor was found to lack the CxCP Lck-recruitment motif[136, 199].

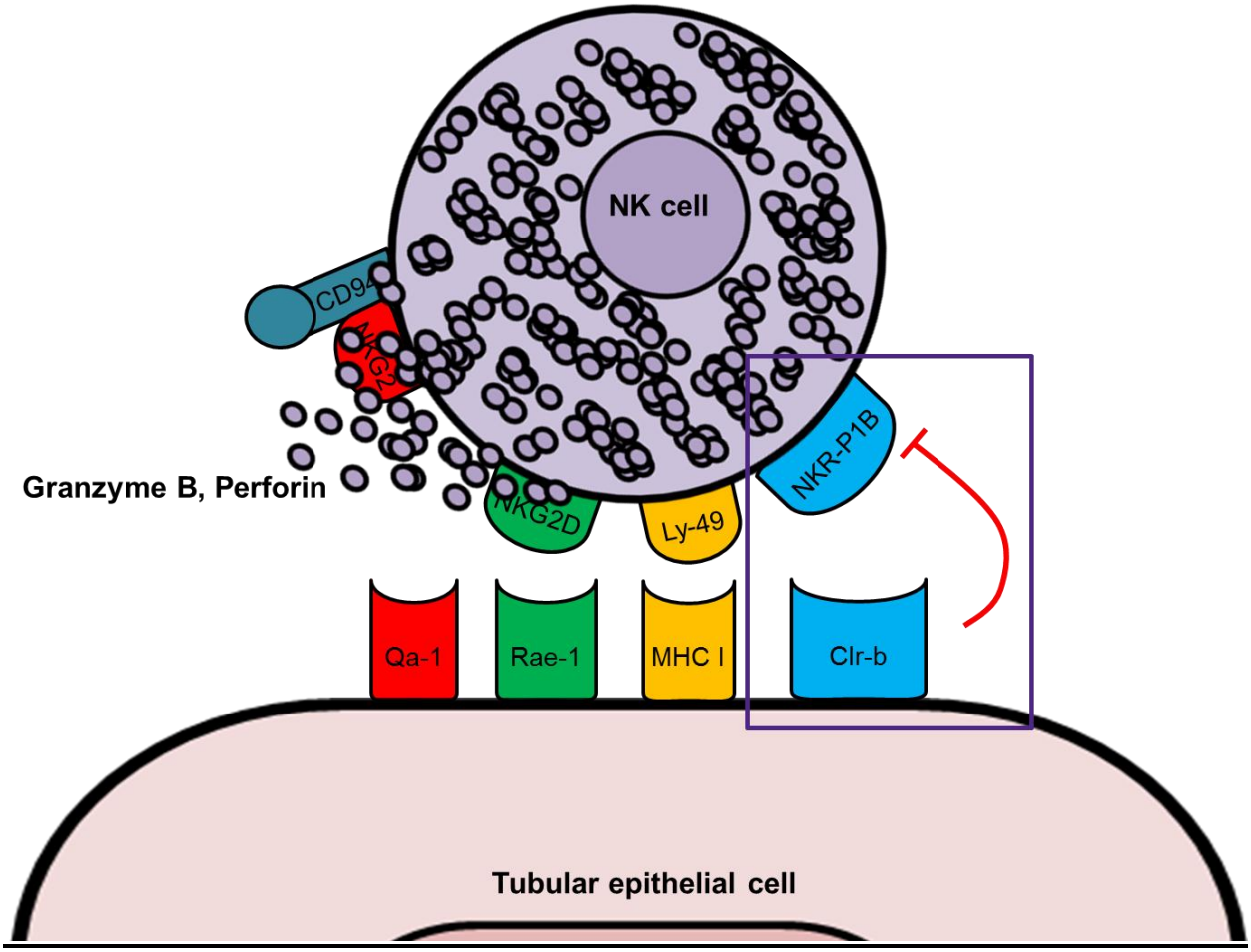

NK cell
CD94
NKG2
Granzyme B, Perforin
NKG2D
Ly-49
NKR-P1B
Qa-1
Rae-1
MHC I
Clr-b
Tubular epithelial cell

Figure 3. NK cells express a plethora of receptors to regulate cytotoxicity

An illustration demonstrating multiple activating and inhibitory receptor interactions with TEC ligands to form an immunological synapse and cause a graded cytotoxic NK cell-mediated response.

1.9 Hypothesis

We hypothesize that Clr-b is up-regulated in TEC during tissue injury and this up-regulation provides protection to kidney grafts against NK cell-mediated cytotoxicity.

1.10 Specific aims

1. To determine if Clr-b expression is up-regulated in TEC after cytokine treatment, hypoxia, ischemia and renal injury
2. To investigate the effect of NKR-P1B and Clr-b interaction on NK cell-mediated TEC death
3. To investigate the effect of stimulated/deficient NKR-P1B and Clr-b interaction on ischemia and renal injury in mice

Chapter 2

2 Materials and Methods

2.1 Animals

C57BL/6 (H-2^b), BALB/c (H-2^d), C3H (H-2^k) and *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in the animal facility at Western University using approved protocols and procedures. Animal experiments were conducted in accordance with the Canadian Council of Animal Care guidelines with protocols approved by the Animal Use Subcommittee of Western University.

2.2 Antibodies and reagents

Cells were characterized with the following fluorescent-conjugated antibodies: FITC anti-mouse CD26 (eBioscience, San Diego, CA), PE Conjugated Streptavidin (eBioscience), and a 4A6 monoclonal antibody that specifically recognizes Clr-b (kindly from JR Carlyle, University of Toronto, Toronto, ON). Mouse cytokines TNF-α, and IFN-γ were purchased from PeproTech (Rocky Hill, NJ). Cell viability assays identifying necrotic and apoptotic cells employed propidium iodide (PI) and annexin V, respectively (eBioscience). Data were acquired and analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter, Mississauga, ON).

Primary TEC were obtained from C57BL/6 or BALB/c mouse kidneys after digestion with 1 mg/mL of collagenase V (Sigma-Aldrich, St. Louis, MO) for 15 min and then sequentially sieved. All TEC were grown in complete K1 culture medium (DMEM:HamsF12, 50:50; Invitrogen-Life Technologies, Carlsbad, CA), supplemented with 5% bovine calf serum, hormone mix (5 µg/mL insulin, 1.25 ng/mL PGE1, 34 pg/mL triodothyronine, 5 µg/mL transferrin, 1.73 ng/mL sodium selenite, and 18 ng/mL hydrocortisone), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), and 25 ng/mL epidermal growth factor. Cells

were trypsinized before each passage. Proximal tubular phenotype of TEC was confirmed by staining with anti-CD26 antibody (eBioscience).

2.3 Primary kidney tubular epithelial cell isolation and culture

Primary kidney cell cultures were obtained from the cortex region of either C57BL/6 or BALB/c mouse kidneys after digestion with collagenase V at 1 mg/mL (Sigma) for 15 min in complete K1 media in a humidified incubator at 37 °C. The digested cells were then passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA) and washed with 20 mL of PBS. The single cell suspension was then grown in complete K1 media in a 75 cm^2 vented flask (BD Biosciences) in a humidified incubator at 37 °C. Trypsin-EDTA at 0.125% was used for each passage cycle with prior washing using PBS without magnesium and calcium (Invitrogen).

2.4 Isolation of NK cells

NK cells were purified from C57BL/6 or BALB/c mouse spleens by gently passing the spleen through a 40 µm cell strainer (BD Biosciences), washed with 10 mL of PBS and spun down at 500 × g for 5 min. The pellet was subsequently resuspended in 1 mL of ACK lysis buffer (Invitrogen) for 30 sec and washed with 10 mL of PBS. Subsequent isolation was performed as described in MACS cell separation kit (Miltenyi Biotec, Auburn, CA). Briefly, a single cell suspension was made in 490 µL of MACS buffer (PBS, 1% FBS, 0.5% EDTA) and incubated with 10 µL of magnetic beads conjugated with anti-CD3 (Miltenyi) for 15 min at 4 °C. Cells were then washed with 9.5 mL of MACS buffer and centrifuged for 5 min again. Washed cells were then resuspended in 500 µL of MACS buffer and transferred into a MACS midsize column (Miltenyi) that was subjected to a magnetic field. The column was washed three times with 500 µL of MACS buffer and the flow through was collected and spun down. Cells were subjected to a

second round of purification using anti-CD49b (DX5; Miltenyi). Purity of NK cells was confirmed by flow cytometry analysis and was found to be > 94% $CD3^-$ $CD49b^+$.

2.5 Cytokine induction

TEC were challenged with 25 ng/mL of IFN-γ (PeproTech) and 25 ng/mL of TNF-α (PeproTech) in K1 culture media without glucose for 0, 24, 48, 72 and 96 h. Cell death was confirmed with annexin V and propidium iodide staining and analyzed by flow cytometry, according to the manufacturer's protocol (BD Biosciences).

2.6 Hypoxia induction

Hypoxic culture conditions were produced by placing the cells in a mini-incubator perfused with 95% N_2 saturated with water. Temperature was maintained on a heating pad at 32 °C. The cells were exposed to hypoxia for 20 min. Cells were cultured in deoxygenated K1 culture media without glucose for 0, 24, 48, 72 and 96 h. Cell death was confirmed with annexin V and propidium iodide staining.

2.7 siRNA transfection

TEC were transfected with 2 μg/mL of Clr-b siRNA oligonucleotides using 6 μg/mL of Lipofectamine (Invitrogen), and then cultured for an additional 96 h before use in NK:TEC co-cultures or analyzed by flow cytometry. Reduction in expression of the appropriate marker was then calculated using flow cytometry or RT-PCR. Clr-b siRNA oligonucleotides were purchased as a pool of 3 different duplexes from Santa Cruz Biotechnology (Santa Cruz, CA). siRNAs included: 5′-CAG UAC CAG GAU CUA UUC Att -3′ and 5′- UGA AUA GAU CCU GGU ACU Gtt -3′; 5′- GCU ACA CUG UAC UUU AUA Ctt -3′ and 5′- GUA UAA AGU ACA

GUG UAG Ctt -3′; 5′- GGA CGA AAU AAC AGG CUA Att -3′; 5′- UUA GCC UGU UAU UUC GUC Ctt -3′.

2.8 NK cell killing assay

NK cells were purified by CD49b$^+$ selection on a MACS beads column (Miltenyi), then activated with IL-2 (1000 U/mL) in RPMI 1640, supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 mg/mL), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and b-mercaptoethanol (0.5 mM). Activated or naïve NK cells were used as effector cells in a killing assay. Cell death was confirmed with propidium iodide staining, according to the manufacturer's protocol (BD Biosciences). TEC were transfected with Clr-b siRNA (Santa Cruz) 4 days prior to the killing assay. TEC were labeled with 0.5 mM Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for 8 min and washed three times with medium. The CFSE-labeled TEC were mixed with NK cells or cultured alone for 5 h. PI staining and flow cytometry were used to determine the percentage of TEC death by gating on CFSE$^+$ cells.

2.9 Quantitative real-time polymerase chain reaction

Total RNA were extracted from kidneys with a spin column (Qiagen, Mississauga, ON). cDNA pools were synthesized with the First Strand Synthesis System (Stratagene, La Jolla, CA). Primers were designed using Oligo Perfect Designer, (Invitrogen-Life Technologies). Clr-b: 5'- CTC GGT TTG ACA ACC AGG AT -3' and 5'- GAT CCC GTT GTT GTT CAG GT -3'; NKR-P1A: 5'- CCT GCT CAC CAG TTC AGT GA -3' and 5'- CTT CTT GGT CTT GAA TGA GCA T -3'; NKR-P1B: 5'- CTC CTG ACT GTG AAT CCC ATC CCC A -3' and 5'- GCC CCT CAG CTA CCA GGG CTT -3'; NKR-P1C: 5'- TCC CTT CTC ACC ACC AGT TA -3' and 5'- CAG TCT TGT GGG CAC TCT AAA -3'; NKR-P1D: 5'- GTT TGT CCC TGT TCA CGA GG -3' and 5'- TCT TGA ACA TCC GCG CAG AT -3';

NKR-P1E: 5’- GGA AAG GAG CCA CTT TGC TGC TCA -3’ and 5’- TCC TTT TGG CAG ATC CAA CGT CC -3’; NKR-P1F: 5’- TGT CAC TGT TCA CAG TTC CC -3’ and 5’- ATC TCG GGC ACT CTA GTA TG -3’; NKR-P1G: 5’- AGC TGT GCC CTC ATC TCA CAA ACA -3’ and 5’- CAG GAG TCA GGA CAC AGG GTT TCA -3’; and β-actin: 5’- CCA GCC TTC CTT CCT GGG TA -3’ and 5’- CTA GAA GCA TTT GCG GTG CA -3’. Real-time quantitative PCR were performed on standardized quantities of cDNA using the Brilliant SYBR Green QPCR Master Mix kit, and amplified DNA products were generated and detected using the Mx4000 system (Stratagene). Each PCR amplification condition was set up in triplicate. β-actin amplification was used as the endogenous control. The normalized δ threshold cycle value and relative expression levels ($2^{\Delta\Delta Ct}$) were calculated.

2.10 Histological analyses

Formalin-fixed kidney sections were stained with H&E, and injury were scored in a double-blinded fashion by a single pathologist at London Health Sciences Centre, using an arbitrary scoring system (0: no change; 1+: < 25% area change; 2+: 25–50% area change; 3+: 50–75% area change; 4+: > 75% area change). Criteria for kidney injury included tubular necrosis, immune cell infiltration, tubular casts, and glomerular necrosis. Kidney sections were embedded in OCT compound and snapped frozen in liquid nitrogen.

2.11 Kidney ischemia-reperfusion injury

Ischemia and reperfusion injuries were performed by a single microsurgeon at the Matthew Mailing Centre for Translational Transplant Studies. Ischemia was induced by clamping the left kidney for 45 min at 32 °C on a thermo-regulated pad in C57BL/6 or C3H mice, and 60 min at 33 °C in BALB/c mice. To assess function of the ischemic kidney, the clamps were released and the right kidney

was subsequently removed. Kidneys were sampled at different time points after being flushed with cold PBS. Sham controls were treated with the same operative procedure as in the injury group but kidneys were not clamped. Serum was collected for creatinine detection by a Jaffe reaction method with an automated CX5 clinic analyzer (Beckman, Fullerton, CA).

2.12 Kidney transplantation

Kidney transplants were performed by a single microsurgeon at the Matthew Mailing Centre for Translational Transplant Studies. Ten week old male BALB/c mice were bilaterally nephrectomized and transplanted with a single kidney from a male C57BL/6 mouse of similar weight. Both donor and recipient mice were anaesthetized with isoflurane. Donor kidney, ureter, bladder, renal artery and vein were recovered together. Vascular vessels of the donor kidney were anastomosed to the recipient's respective abdominal aorta and inferior vena cava below the native renal vessels of the recipient. Renal ischemia time was limited to 30-45 min. Donor and recipient bladders were attached dome-to-dome. Kidney graft survival was assessed daily based on the overall health of the animal. If the recipient mouse was found to be suffering, the mouse was sacrificed for blood and kidney graft retrieval and analysis. Mice were followed up to 60 days and were subsequently sacrificed.

2.13 Statistical analyses

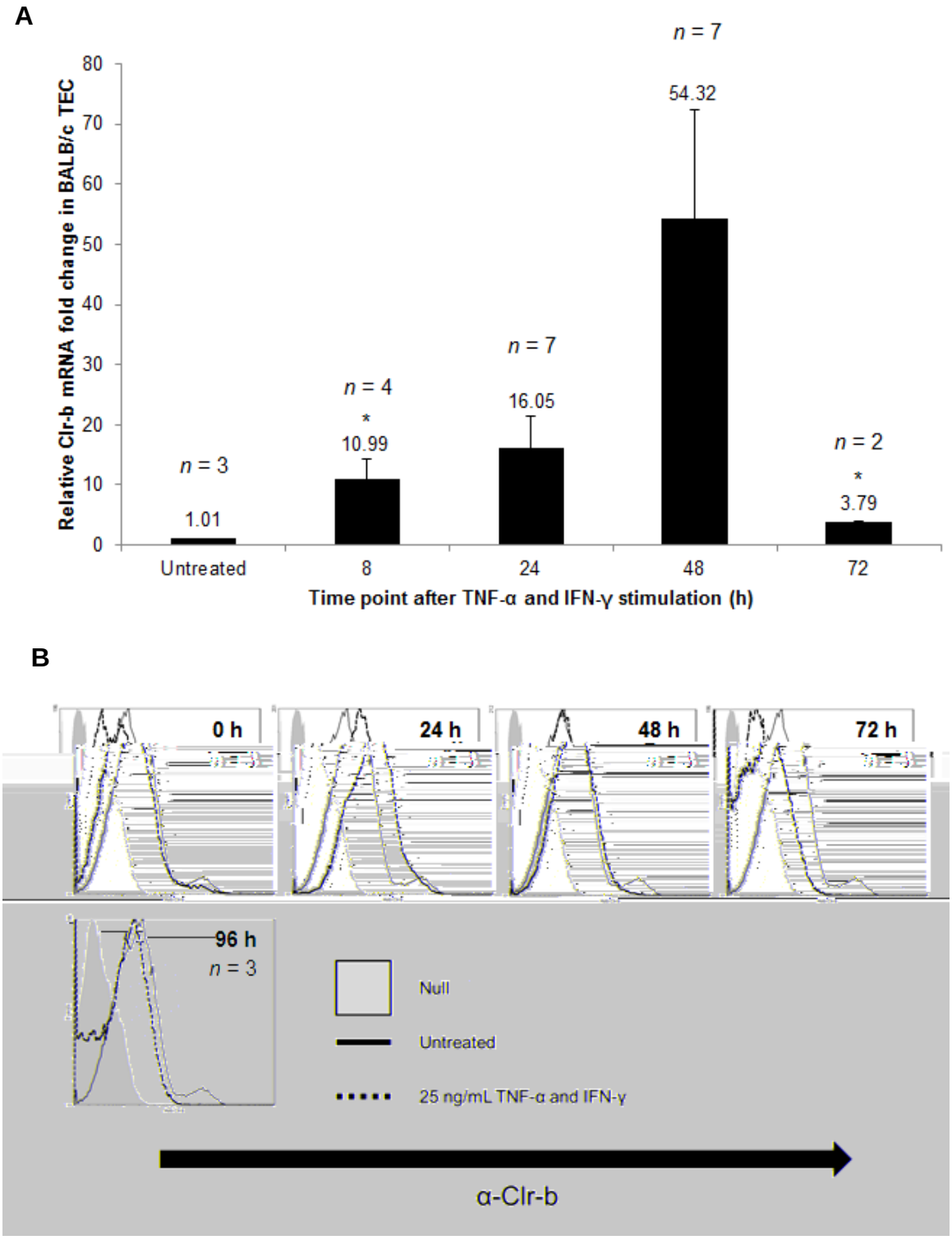
Experimental values were expressed as mean ± SEM. Data were compared using Student's T-Test (Statview, Nesbit, MS) for unpaired values and one-way ANOVA for multigroup differences. The p values < 0.05 were considered significant.

Chapter 3

3 Results

3.1 Clr-b expression in BALB/c TEC increases upon pro-inflammatory cytokines TNF-α and IFN-γ treatment

Our previous work identified NK cells as important participants in renal IRI[265] and that TEC apoptosis can be induced by TNF-α and IFN-γ, pro-inflammatory cytokines which can be secreted by NK cells[68]. As NK cells are regulated by multiple signalling interactions with target cells, we investigated the effect of tubular Clr-b expression upon stimulation by TNF-α and IFN-γ. Real-time quantitative PCR data analysis of Clr-b in primary-cultured TEC from BALB/c (H-2^d) kidneys indicated that a combination of the cytokines TNF-α and IFN-γ effectively upregulated Clr-b expression. At a dose of 25 ng/mL each, TNF-α and IFN-γ significantly increased Clr-b mRNA transcription just 8 h after treatment. This upregulation continued to progress and peaked at 48 h. A decrease in Clr-b mRNA expression level was observed by 72 h (Figure 4A). At the surface protein level, flow cytometry analysis revealed a transient upregulation of Clr-b 24 h after induction by the cytokines (Figure 4B). The effect of these cytokines on tubular cell viability was confirmed by annexin V and propidium iodide (PI) staining, indicators of apoptosis and necrosis, respectively (Figure 4C). Put together, our data demonstrates that the pro-inflammatory cytokines TNF-α and IFN-γ induces cell death and Clr-b upregulation in BALB/c TEC.


A
n = 7
54.32
n = 7
16.05
n = 4
*
10.99
n = 3
1.01
n = 2
*
3.79
Relative Clr-b mRNA fold change in BALB/c TEC
0
10
20
30
40
50
60
70
80
Untreated
8
24
48
72
Time point after TNF-α and IFN-γ stimulation (h)
B
0 h
24 h
48 h
72 h
96 h
n = 3
Null
Untreated
25 ng/mL TNF-α and IFN-γ
α-Clr-b

C

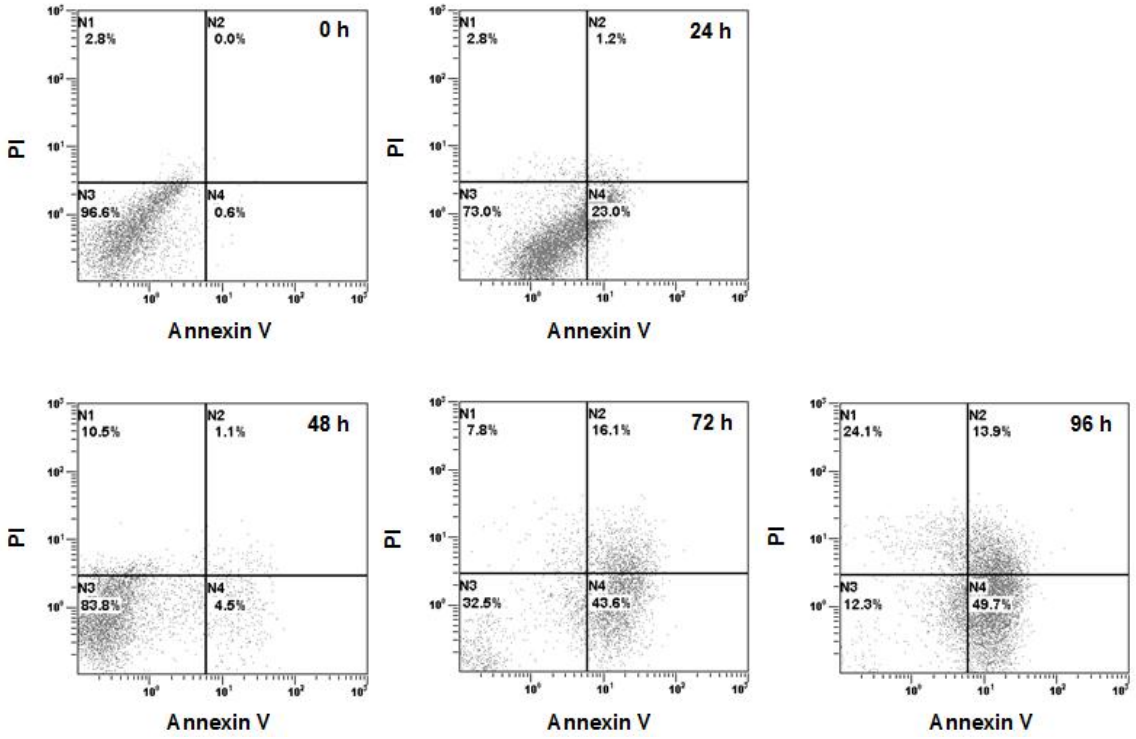

0 h
N1 2.8%
N2 0.0%
N3 96.6%
N4 0.6%
24 h
N1 2.8%
N2 1.2%
N3 73.0%
N4 23.0%
48 h
N1 10.5%
N2 1.1%
N3 83.8%
N4 4.5%
72 h
N1 7.8%
N2 16.1%
N3 32.5%
N4 43.6%
96 h
N1 24.1%
N2 13.9%
N3 12.3%
N4 49.7%
PI
Annexin V

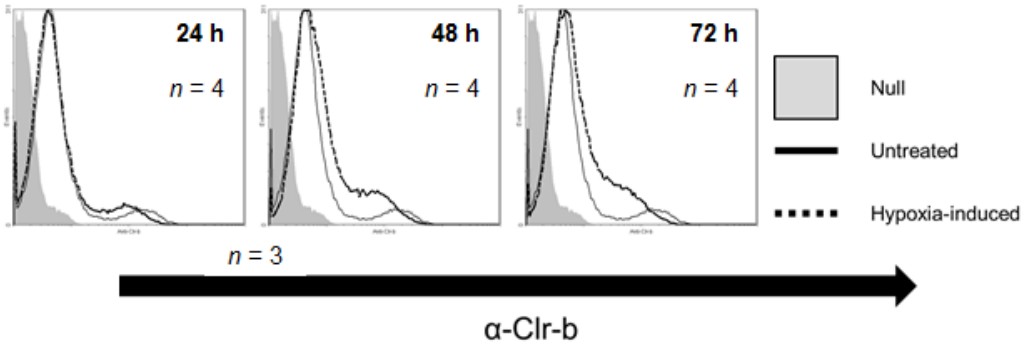
Figure 4. Clr-b ligand in primary-cultured BALB/c kidney TEC increases upon pro-inflammatory cytokines TNF-α and IFN-γ treatment in a time-dependent manner

A) Quantitative RT-PCR of BALB/c TEC Clr-b RNA expression after cytokine induction; $n = 2 – 7$; Statistical analysis was performed with ANOVA. * $p < 0.05$ compared with untreated media control. **B)** Surface ligand Clr-b expression 0 h, 24 h, 48 h, 72 h and 96 h after cytokine induction. BALB/c TEC were cultured and treated as described in *Materials and Methods.* Time points are compared to null (gray) and untreated (solid line); $n = 3$. **C)** Cell death in BALB/c TEC, as measured by annexin V and PI staining 0 h, 24 h, 48 h, 72 h and 96 h after cytokine induction to detect apoptotic and necrotic death, respectively; $n = 1$.

3.2 Clr-b expression in BALB/c TEC is not affected by hypoxia treatment

During IRI, cells are subjected to multiple factors that result in cellular and organ injury. One of the factors includes hypoxia, where cells are subjected to oxygen deprivation until the blood supply can re-nourish the organ. We therefore decided to test whether Clr-b regulation is affected under hypoxic conditions. Albeit not as effective as cytokine stimulation, we found that hypoxic stress also increased Clr-b mRNA expression in BALB/c TEC at the 24 h mark (data not shown). At the cell surface, however, we were unable to convincingly detect a change in Clr-b expression by flow cytometry (Figure 5A). Furthermore, cell viability of BALB/c TEC was not affected by hypoxic stress (Figure 5B). Based on our *in vitro* data, oxygen deprivation is not a factor associated with Clr-b regulation in BALB/c TEC.

A


24 h
n = 4
48 h
n = 4
72 h
n = 4
Null
Untreated
Hypoxia-induced
n = 3
α-Clr-b

B

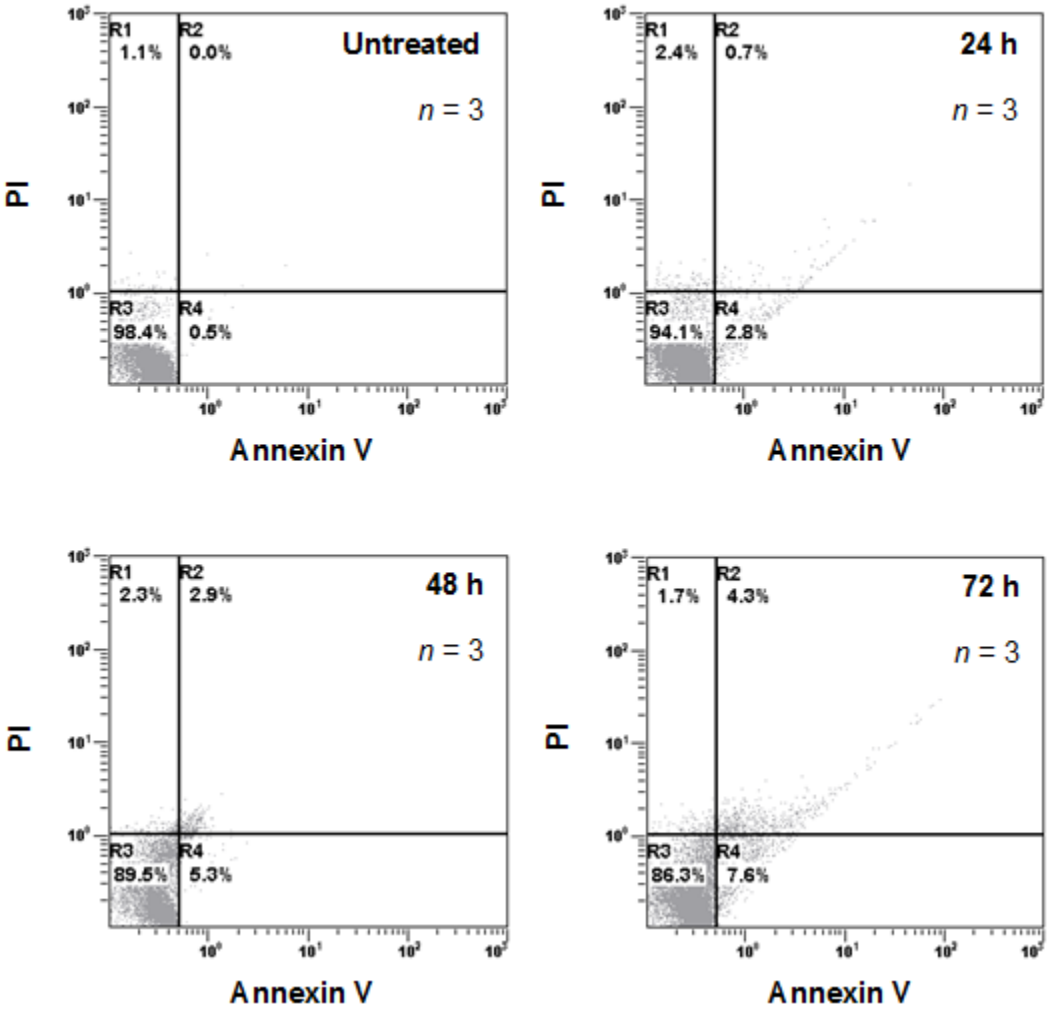

Untreated
n = 3
R1 1.1%
R2 0.0%
R3 98.4%
R4 0.5%
24 h
n = 3
R1 2.4%
R2 0.7%
R3 94.1%
R4 2.8%
48 h
n = 3
R1 2.3%
R2 2.9%
R3 89.5%
R4 5.3%
72 h
n = 3
R1 1.7%
R2 4.3%
R3 86.3%
R4 7.6%
PI
Annexin V

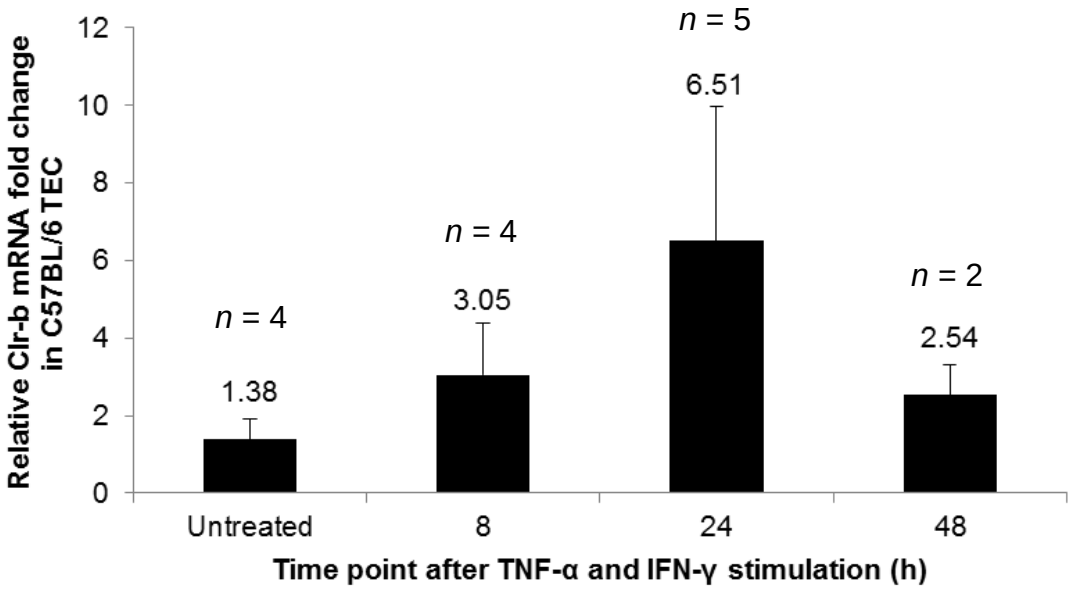
Figure 5. Clr-b ligand in primary-cultured BALB/c kidney TEC is not affected by hypoxia treatment

A) Surface ligand Clr-b expression 24 h, 48 h, and 72 h after being induced by hypoxia for 20 min. BALB/c TEC were cultured and treated as described in *Materials and Methods*. Time points are compared to null (gray) and untreated (solid line); $n = 4$. **B)** Cell death in BALB/c TEC, as measured by annexin V and PI staining 24 h, 48 h and 72 h after hypoxia to detect apoptotic and necrotic death, respectively; $n = 3$.

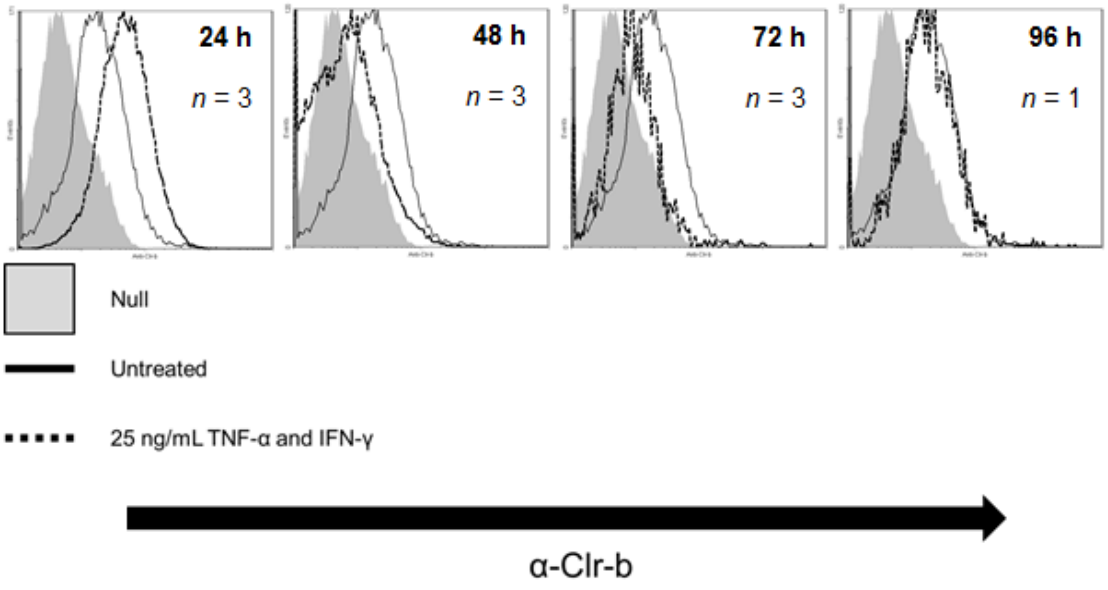
3.3 Clr-b expression in C57BL/6 TEC increases upon pro-inflammatory cytokines TNF-α and IFN-γ treatment

A recent study by Carlyle and colleagues has revealed that NKR-P1B expression is missing in the C57BL/6 mouse strain[34]. From our studies, we know that C57BL/6 mice are more susceptible to renal IRI than BALB/c mice; therefore we opted to compare these two mouse strains. Similar to BALB/c TEC, real-time PCR quantification of C57BL/6 (H-2^b) TEC after TNF-α and IFN-γ also appeared to increase Clr-b expression (Figure 6A). Whereas BALB/c TEC Clr-b mRNA levels peaked at 48 h, C57BL/6 TEC peaked at 24 h. In C57BL/6 TEC, a regression in Clr-b mRNA level was observed by 48 h. However, this increase was not significant upon further statistical analysis. The relative increase of Clr-b in C57BL/6 was also much lower than BALB/c TEC. Although data analysis suggested that this increase in C57BL/6 TEC was not statistically significant at the mRNA level, FACS analysis demonstrated a Clr-b expression pattern similar to BALB/c TEC. In C57BL/6 TEC, Clr-b surface protein upregulation was transiently observed 24 h after cytokine induction (Figure 6B). The effect of these cytokines on tubular cell viability was confirmed by annexin V and PI staining (Figure 6C). In addition, various TNF-α and IFN-γ doses were tested to determine whether Clr-b is regulated in a dose-dependent manner. At doses between 15 - 40 ng/mL each, we could not detect any noticeable differences in Clr-b levels (Figure 6D). Thus, similarly with BALB/c TEC, our data demonstrates that Clr-b is upregulated upon stimulation by the cytokines TNF-α and IFN-γ in C57BL/6 TEC at the surface protein level.

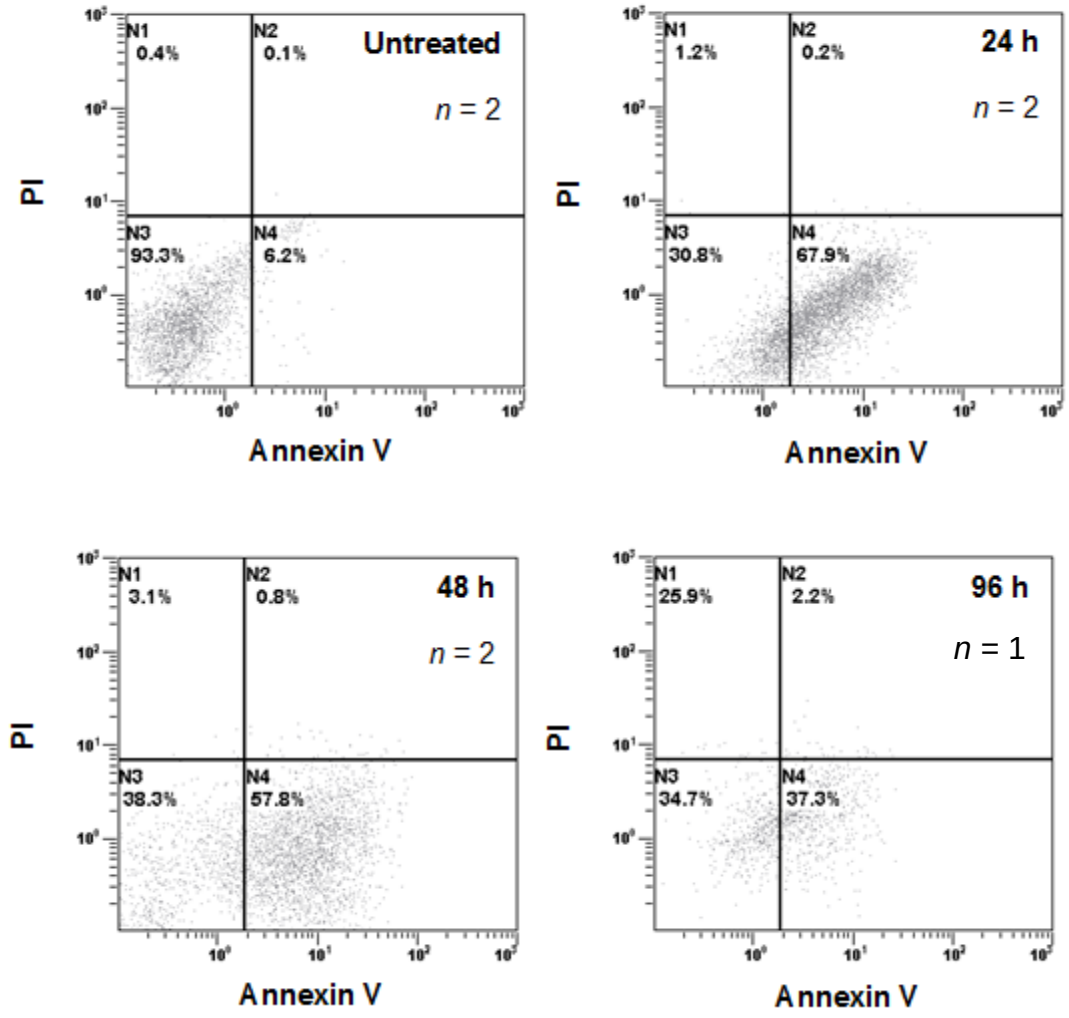
A


Relative Clr-b mRNA fold change in C57BL/6 TEC
12
10
8
6
4
2
0
n = 4
1.38
n = 4
3.05
n = 5
6.51
n = 2
2.54
Untreated
8
24
48
Time point after TNF-α and IFN-γ stimulation (h)

B


24 h
n = 3
48 h
n = 3
72 h
n = 3
96 h
n = 1
Null
Untreated
25 ng/mL TNF-α and IFN-γ
α-Clr-b

C


Untreated
n = 2
N1 0.4%
N2 0.1%
N3 93.3%
N4 6.2%
24 h
n = 2
N1 1.2%
N2 0.2%
N3 30.8%
N4 67.9%
48 h
n = 2
N1 3.1%
N2 0.8%
N3 38.3%
N4 57.8%
96 h
n = 1
N1 25.9%
N2 2.2%
N3 34.7%
N4 37.3%
PI
Annexin V

D

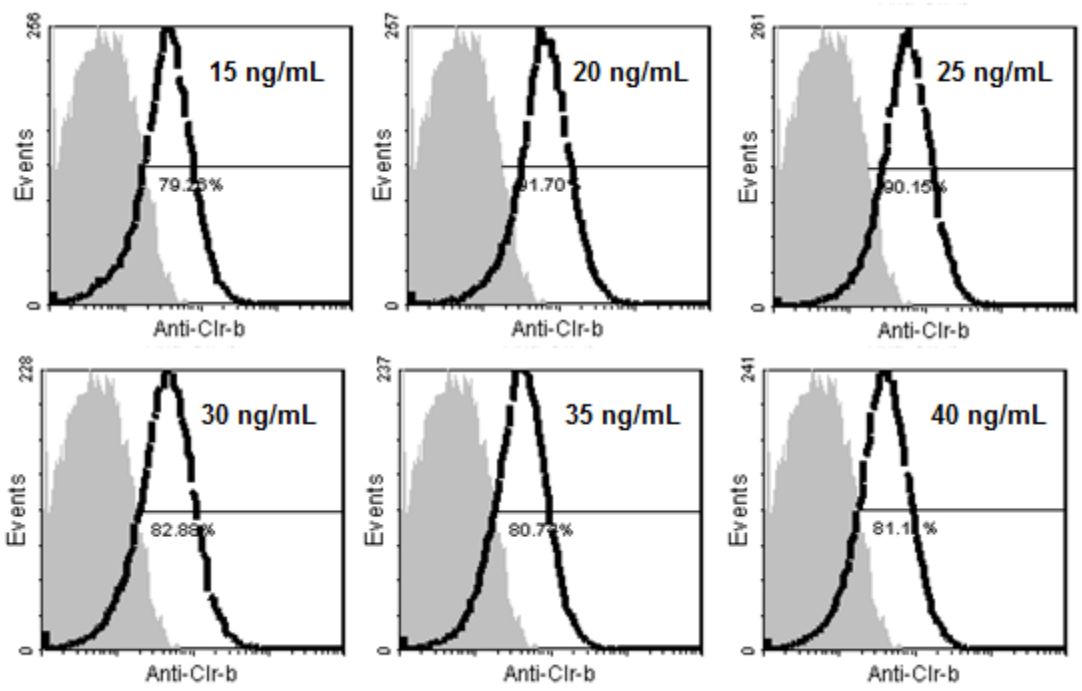

15 ng/mL
79.23%
20 ng/mL
91.70%
25 ng/mL
90.15%
30 ng/mL
82.88%
35 ng/mL
80.72%
40 ng/mL
81.1 %
Events
Anti-Clr-b

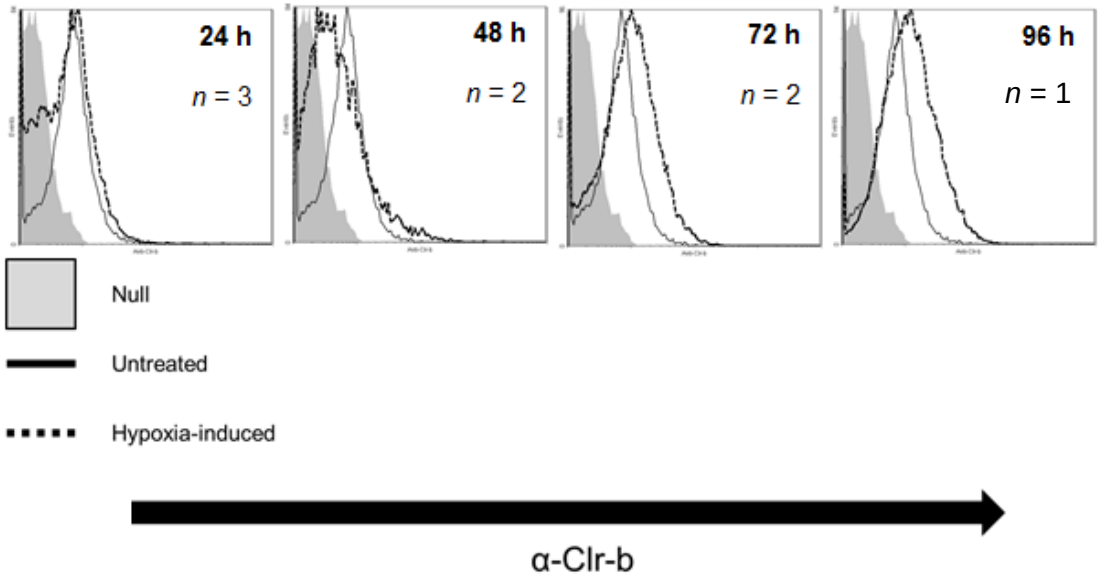
Figure 6. Primary-cultured C57BL/6 kidney TEC Clr-b level increases upon pro-inflammatory cytokines TNF-α and IFN-γ treatment in a time-dependent manner

A) Quantitative RT-PCR of C57BL/6 TEC Clr-b RNA expression after cytokine induction; *n* = 2 - 5. Statistical analysis was performed with ANOVA. **B)** Surface ligand Clr-b expression 24 h, 48 h, 72 h and 96 h after TNF-α (25 ng/mL) and IFN-γ (25 ng/mL) treatment. Time points are compared to null (gray) and untreated (solid line); *n* = 1 - 3. **C)** Cell death in C57BL/6 TEC, as measured by annexin V and PI staining 24 h, 48 h and 96 h after cytokine treatment to detect apoptotic and necrotic death, respectively; *n* = 1 - 3. **D)** Surface ligand Clr-b expression 24 h after TNF-α and IFN-γ treatment, each at doses of 15, 20, 25, 30, 35, and 40 ng/mL. C57BL/6 TEC were cultured and treated as described in *Materials and Methods*. Dosages are compared to null (gray); *n* = 1.

3.4 Clr-b expression in C57BL/6 TEC increases with hypoxic stress

Similarly, we also tested the effect of hypoxia on C57BL/6 primary-cultured TEC. FACS analysis of these tubular cells demonstrated a slight increase in Clr-b surface protein levels 72 and 96 hours after *in vitro* hypoxic stress (Figure 7A). Tubular cell viability was measured by annexin V and PI staining, demonstrating cell death upon oxygen deprivation (Figure 7B). It is possible that the treatments had different capabilities and effectiveness to induce cell injury in different strains of mice, as hypoxic stress was insufficient in BALB/c TEC following the same conditions (Figure 5B).

A


24 h
n = 3
48 h
n = 2
72 h
n = 2
96 h
n = 1
Null
Untreated
Hypoxia-induced
α-Clr-b

B

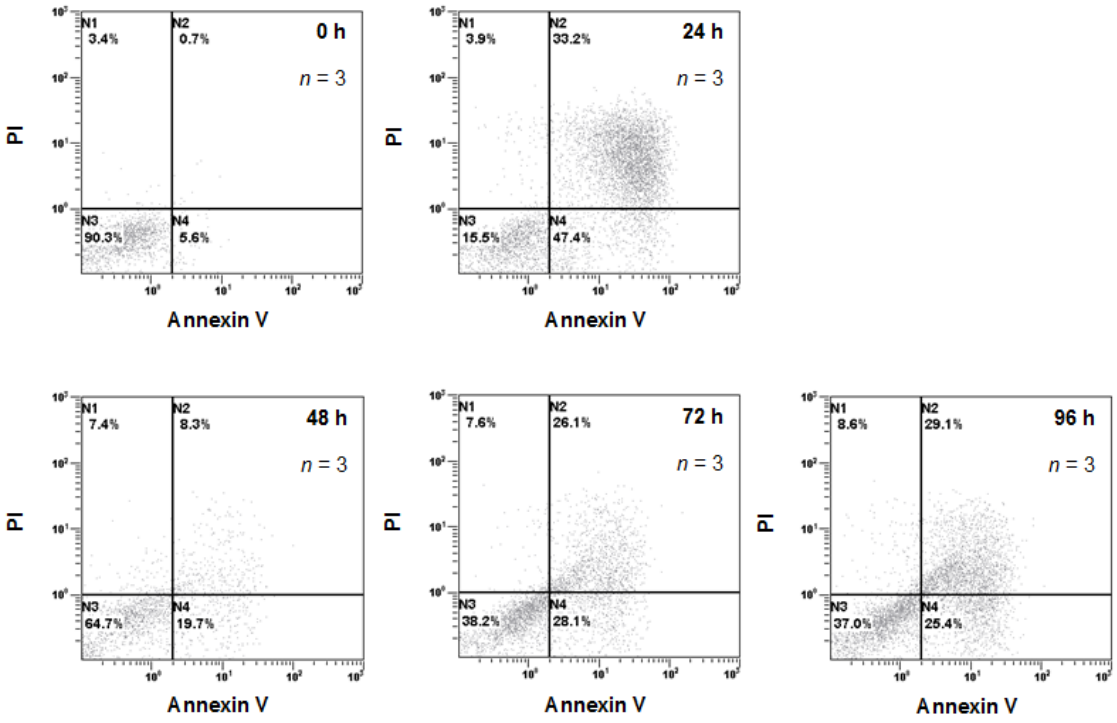
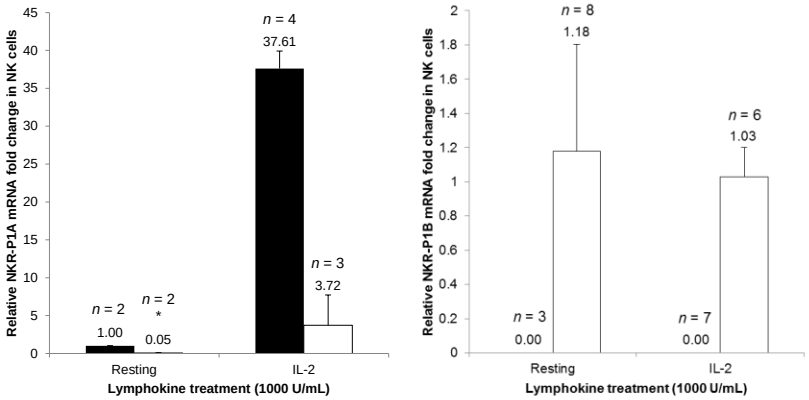

0 h
n = 3
N1 3.4%
N2 0.7%
N3 90.3%
N4 5.6%
24 h
n = 3
N1 3.9%
N2 33.2%
N3 15.5%
N4 47.4%
48 h
n = 3
N1 7.4%
N2 8.3%
N3 64.7%
N4 19.7%
72 h
n = 3
N1 7.6%
N2 26.1%
N3 38.2%
N4 28.1%
96 h
n = 3
N1 8.6%
N2 29.1%
N3 37.0%
N4 25.4%
PI
Annexin V

Figure 7. Primary-cultured C57BL/6 kidney TEC Clr-b level increases with hypoxic stress in a time-dependent manner

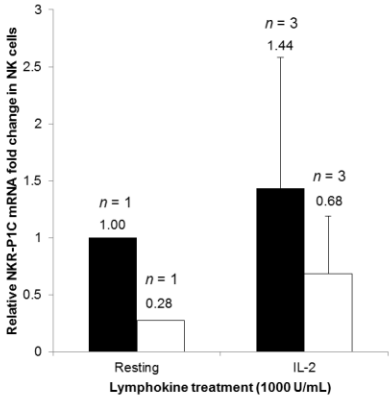
A) Surface ligand Clr-b expression 24 h, 48 h, 72 h and 96 h after being induced by hypoxia for 20 min. C57BL/6 TEC were cultured and treated as described in *Materials and Methods*. Time points are compared to null (gray) and 0 h after hypoxia treatment (solid line); *n* = 1 - 3. **B)** Cell death in C57BL/6 TEC, as measured by annexin V and PI staining 24 h, 48 h, 72 h and 96 h after hypoxia to detect apoptotic and necrotic death, respectively; *n* = 3.

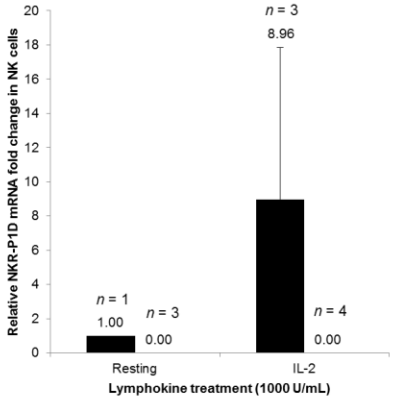
3.5 NKR-P1 expression vary between mouse strains

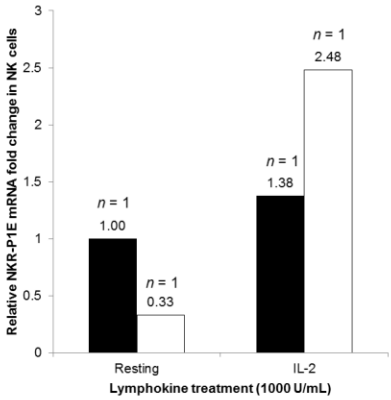
The Ly49 receptors and their ligand MHC class I molecules are important for the prevention of NK cell-mediated self-destruction, yet these molecules are constitutively expressed and contain a high degree of polymorphism[33]. The intricate signalling system of NK cells also involve various NKR-P1 receptor subtypes, acting as either stimulatory or inhibitory mediators. NKR-P1A, NKR-P1C and NKR-P1F receptors have been postulated to exert stimulatory effects on NK cells, whereas NKR-P1B and NKR-P1D are inhibitory[9, 32, 99, 110, 131, 181]. While BALB/c mice express both the stimulatory receptors and the inhibitory NKR-P1B, C57BL/6 mice lack NKR-P1B signalling[34]. Here, we tested splenic naive NK cells and IL-2-activated NK cells from C57BL/6 and BALB/c for NKR-P1 expression. Real-time PCR analysis demonstrated that the stimulatory NKR-P1A expression is significantly higher in C57BL/6 than BALB/c NK cells (Figure 8). As the C57BL/6 genome does not contain the NKR-P1B subtype[34], we did not detect it in C57BL/6 NK cells. In BALB/c NK cells, NKR-P1B expression did not change despite IL-2 activation (Figure 8). Although more experiments need to be repeated for statistical significance, there appears to be variability in the expression of these NKR-P1 receptors between mouse strains. Furthermore, we quantified every known *Clr* ligand in C57BL/6 and BALB/c TEC, as they are interspersed between the *Nkr-p1* genes and may serve to stimulate the NKR-P1 receptors[34]. Based on the mRNA profile, C57BL/6 primary-cultured TEC appear to express higher levels of the Clr subtypes than BALB/c (Figure 9). As BALB/c NKR-P1B did not change upon IL-2 activation, the Clr-b ligand on the TEC side may be more important in regulating this pathway.

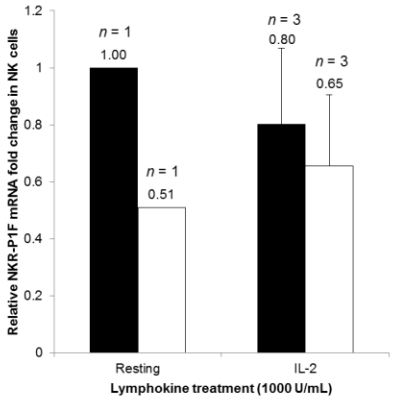

Relative NKR-P1A mRNA fold change in NK cells
n = 2
1.00
n = 2
*
0.05
n = 4
37.61
n = 3
3.72
Resting
IL-2
Lymphokine treatment (1000 U/mL)

Relative NKR-P1B mRNA fold change in NK cells
n = 3
0.00
n = 8
1.18
n = 7
0.00
n = 6
1.03
Resting
IL-2
Lymphokine treatment (1000 U/mL)


Relative NKR-P1C mRNA fold change in NK cells
n = 1
1.00
n = 1
0.28
n = 3
1.44
n = 3
0.68
Resting
IL-2
Lymphokine treatment (1000 U/mL)


Relative NKR-P1D mRNA fold change in NK cells
n = 1
1.00
n = 3
0.00
n = 3
8.96
n = 4
0.00
Resting
IL-2
Lymphokine treatment (1000 U/mL)


Relative NKR-P1E mRNA fold change in NK cells
n = 1
1.00
n = 1
0.33
n = 1
1.38
n = 1
2.48
Resting
IL-2
Lymphokine treatment (1000 U/mL)


Relative NKR-P1F mRNA fold change in NK cells
n = 1
1.00
n = 1
0.51
n = 3
0.80
n = 3
0.65
Resting
IL-2
Lymphokine treatment (1000 U/mL)

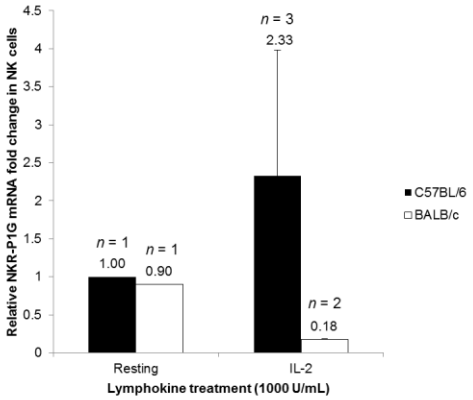
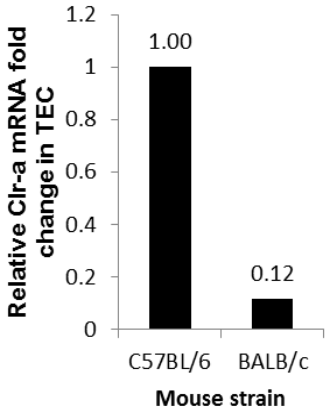
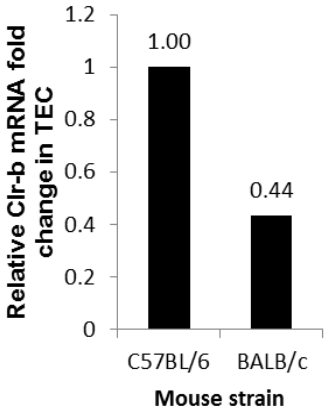
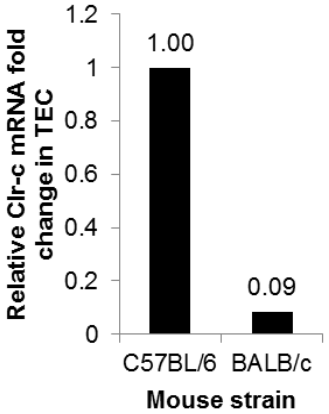

Relative NKR-P1G mRNA fold change in NK cells
n = 1
1.00
n = 1
0.90
n = 3
2.33
n = 2
0.18
Resting
IL-2
Lymphokine treatment (1000 U/mL)
C57BL/6
BALB/c

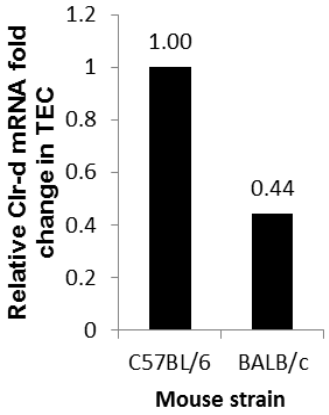
Figure 8. NKR-P1 receptors expression profile in C57BL/6 and BALB/c mice.

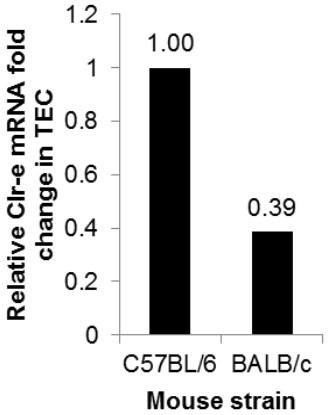
Quantitative RT-PCR of *Nkr-p1* receptor subtypes in C57BL/6 and BALB/c NK cells; n = 1 - 8. NK cells were isolated and cultured as described in *Materials and Methods*. Statistical analysis was performed with ANOVA. * $p < 0.05$ compared with C57BL/6 naïve NK cells.

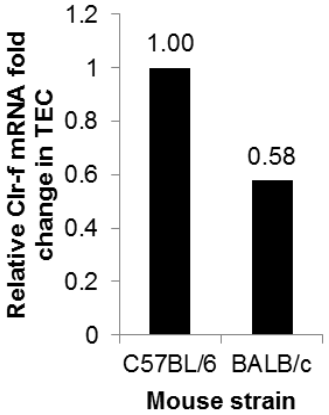

Relative Clr-a mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.12
C57BL/6
BALB/c
Mouse strain


Relative Clr-b mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.44
C57BL/6
BALB/c
Mouse strain


Relative Clr-c mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.09
C57BL/6
BALB/c
Mouse strain


Relative Clr-d mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.44
C57BL/6
BALB/c
Mouse strain


Relative Clr-e mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.39
C57BL/6
BALB/c
Mouse strain


Relative Clr-f mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.58
C57BL/6
BALB/c
Mouse strain

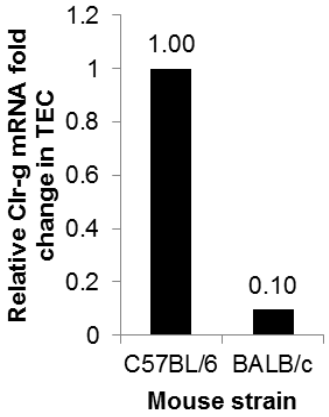

Relative Clr-g mRNA fold change in TEC
1.2
1
0.8
0.6
0.4
0.2
0
1.00
0.10
C57BL/6
BALB/c
Mouse strain

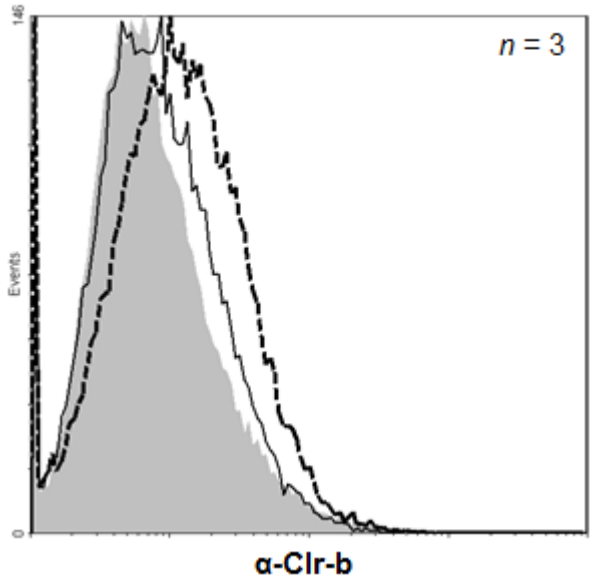
Figure 9. Clr ligands mRNA expression is higher in primary-cultured TEC from C57BL/6 mice than BALB/c mice

Quantitative RT-PCR *Clr* ligand subtypes in BALB/c and C57BL/6 TEC. Total RNA was extracted (Qiagen) and cDNA pools were synthesized (Stratagene). Real-time quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kits and the Mx3005P system (Stratagene). β-actin amplification was used as the endogenous control. The normalized δ threshold cycle value was calculated according to the manufacturer's protocol; $n = 1$.

3.6 TNF-α and IFN-γ did not affect TEC sensitivity to NK cell-mediated cytotoxicity

In this study, we demonstrated that the pro-inflammatory cytokines TNF-α and IFN-γ can upregulate Clr-b in tubular cells (Figures 4 and 6). We decided to test whether cytokine-induced Clr-b expression can overcome other signalling mechanisms and inhibit NK cell activity. While there was less TEC death in the cytokine-treated group, as measured by PI staining, it was not statistically significant (Figure 10). Cytokine-dependent Clr-b up-regulation, therefore, was not sufficient to overcome other stimulatory mechanisms activated upon TNF-α and IFN-γ stimulation at a 10:1 NK cell-TEC ratio.

A


n = 3
146
Events
0
α-Clr-b

B

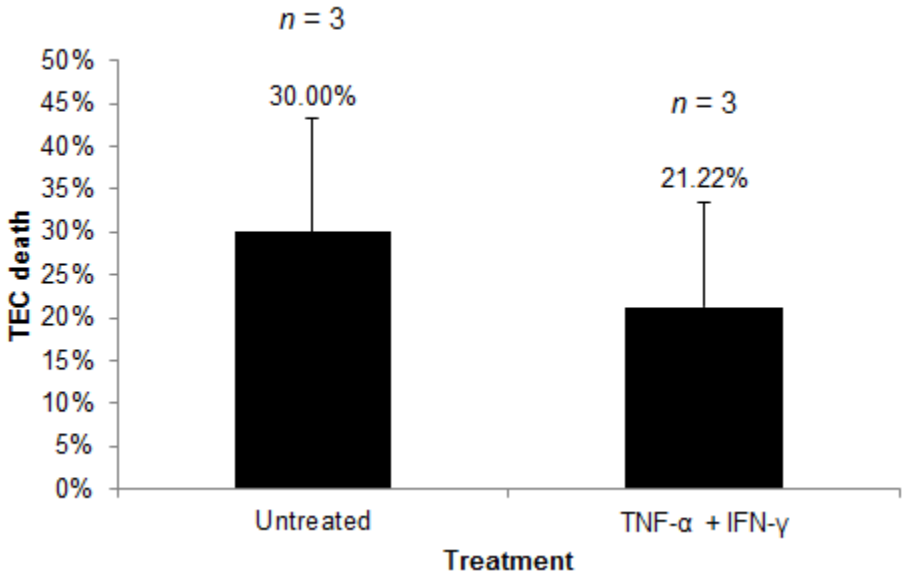

n = 3
30.00%
n = 3
21.22%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
TEC death
Untreated
TNF-α + IFN-γ
Treatment

Figure 10. Clr-b up-regulation by the cytokines TNF-α and IFN-γ did not affect NK cell-mediated cytotoxicity in BALB/c kidney TEC

A) Surface ligand Clr-b expression 24 h after 25 ng/mL of TNF-α and IFN-γ cytokine induction. BALB/c TEC were cultured and treated as described in *Materials and Methods*. Treatment is compared to null (gray) and untreated (solid line); *n* = 3. **B)** Percentage of TEC death from NK cell-mediated cytotoxicity at 10:1 effector-target ratio, with or without cytokine induction. CFSE-stained cells were mixed with IL-2-activated NK cells for 5 h. PI staining and FACs analysis were used to determine the percentage of TEC death by gating on $CFSE^+$ cells. Percentages represent the difference between TEC death before and after incubation with NK cells; *n* = 3. Statistical analysis was performed with Student's T-Test.

3.7 Antibody Clr-b blockade did not affect NK cell-mediated cytotoxicity in TEC

Furthermore, as it has been previously demonstrated that the 4A6 monoclonal anti-Clr-b antibody has ligand blocking capabilities[31], we tested whether the antibody can block the interaction between NKR-P1 and Clr-b, or Clr-b function in TEC. Anti-Clr-b blockade by the antibody at 2.5 μg/mL did not have a significant effect on TEC death at a 10:1 effector – target ratio, as measured by PI staining (Figure 11).

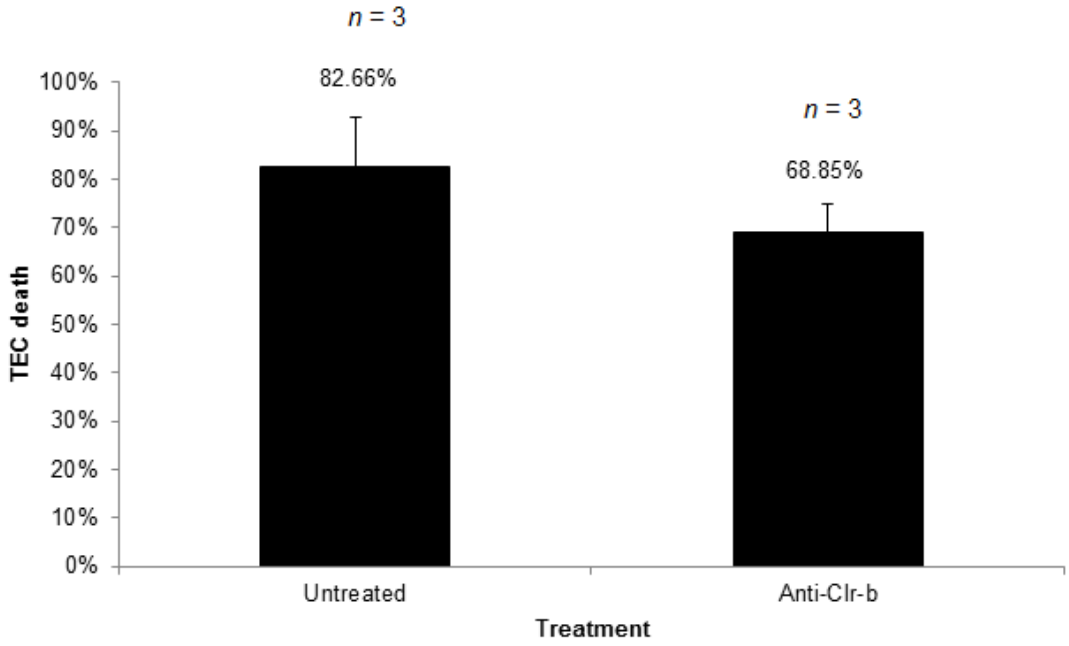
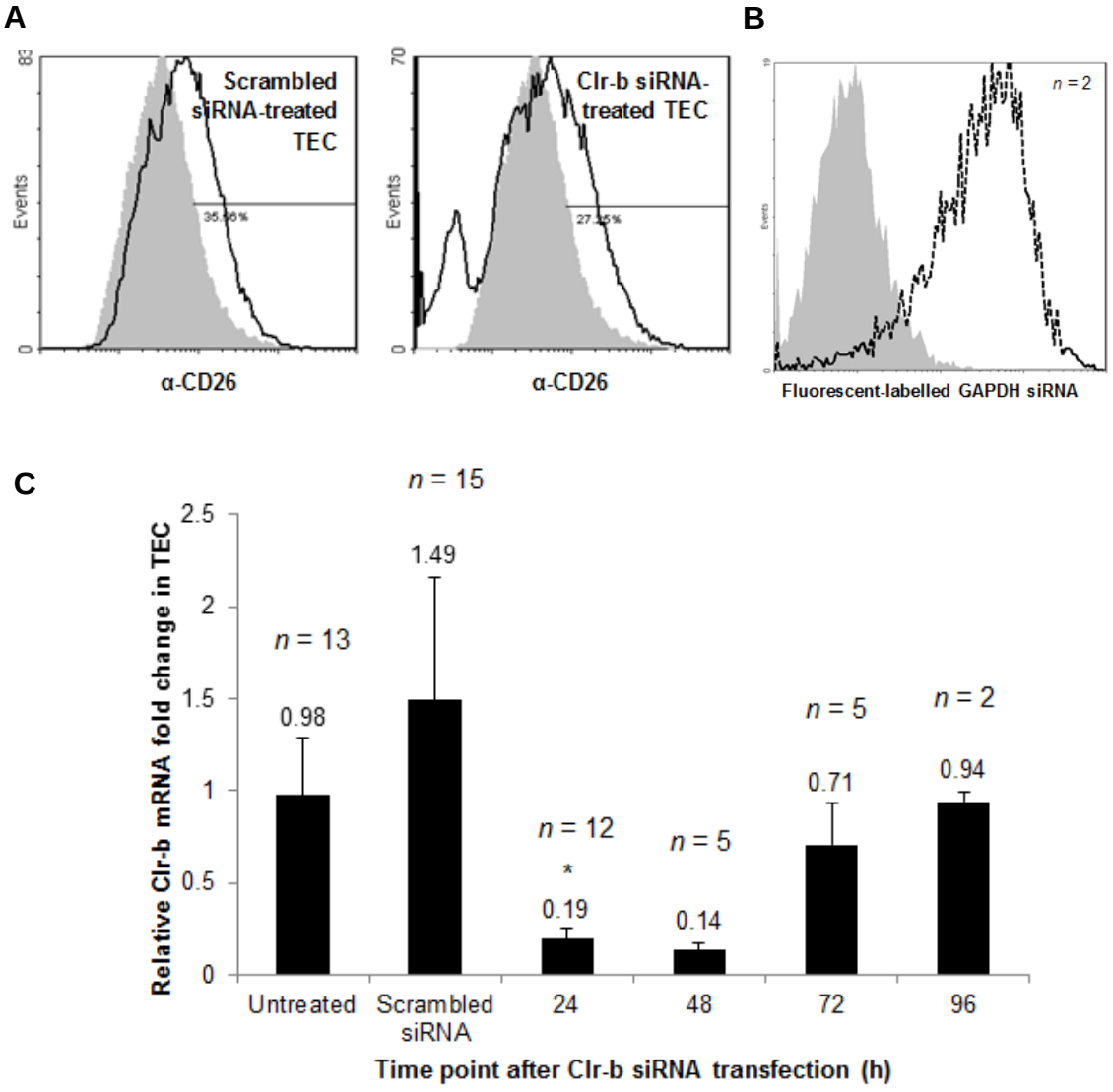

n = 3
82.66%
n = 3
68.85%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
TEC death
Untreated
Anti-Clr-b
Treatment

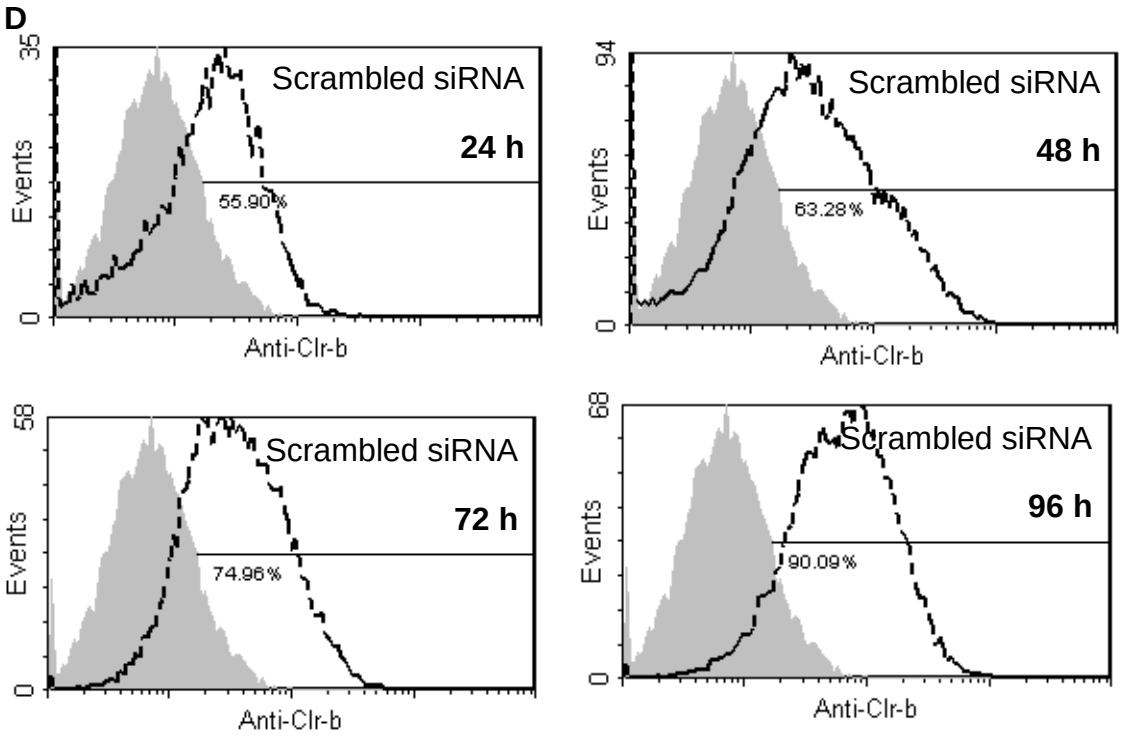
Figure 11. Clr-b blockade by monoclonal antibody did not affect NK cell cytotoxicity in BALB/c kidney TEC

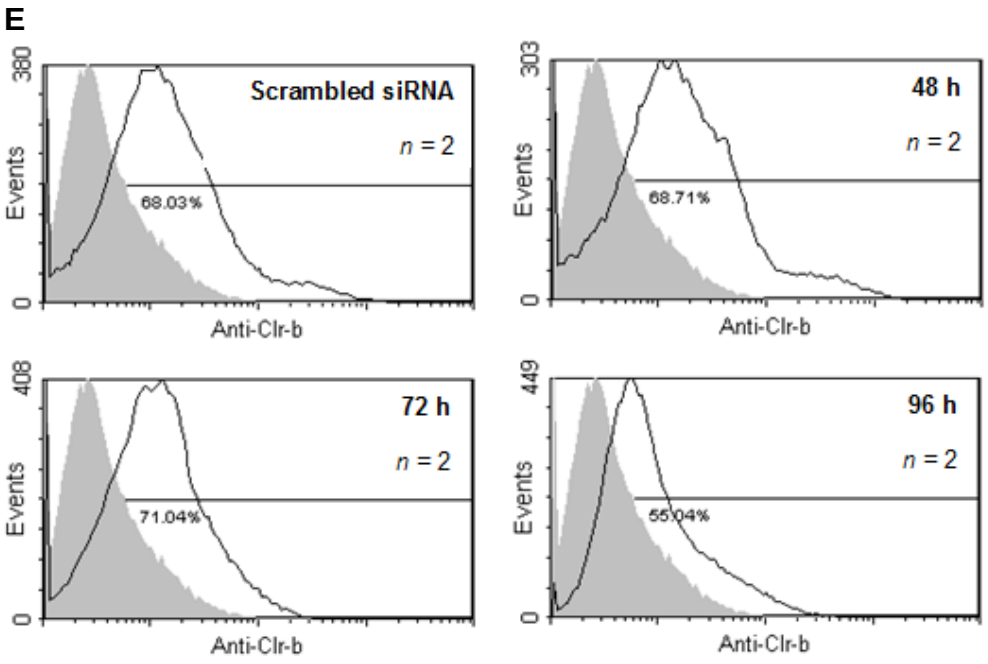
Percentage of TEC death from NK cytotoxicity at 10:1 effector-target ratio. CFSE-stained cells were incubated with or without anti-Clr-b mAb (2.5 μg/mL) for 45 min and mixed with IL-2-activated NK cells for 5 h. PI staining and FACS analysis were used to determine the percentage of necrotic TEC by gating on $CFSE^{+}$ cells. Percentages represent the difference between TEC death before and after incubation with NK cells; n = 3. Statistical analysis was performed with Student's T-Test.

3.8 Clr-b knockdown by siRNA in TEC increased NK cell-mediated death

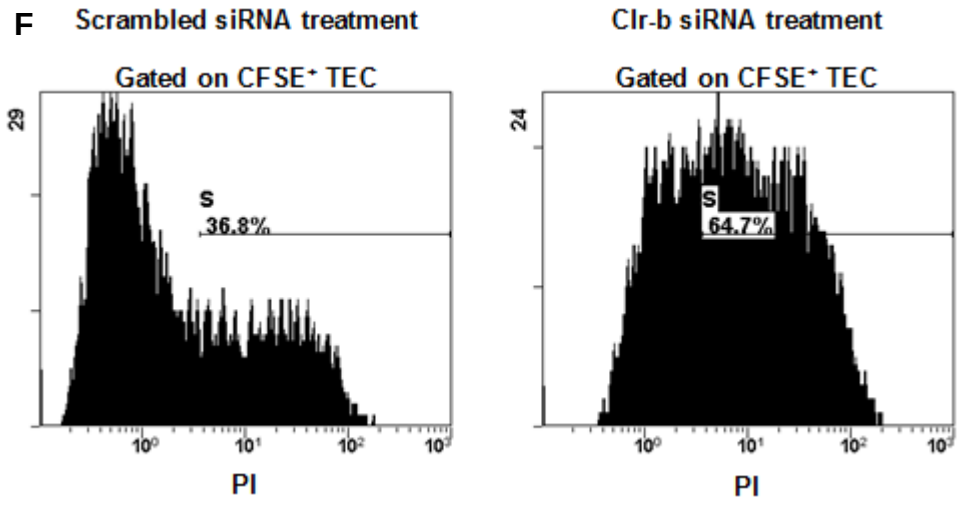
As we had previously used RNAi successfully as a form of therapy to ameliorate renal IRI[69], we decided to knock down *Clr-b* using siRNA technology to decipher its functional significance in tubular cells. TEC were extracted and cultured from BALB/c mice and treated with Clr-b siRNA to prevent Clr-b and NKR-P1B interaction, mimicking C57BL/6's lack of inhibition by this pathway and higher susceptibility to renal IRI. Characterization with α-CD26 confirmed that TEC phenotype was retained after treatment with either scrambled or Clr-b siRNA (Figure 12A). Transfection efficiency in BALB/c TEC was also determined with fluorescent-labelled GAPDH siRNA (Figure 12B). Upon treatment of siRNA, Clr-b mRNA was knocked down most efficiently at 24 and 48 h after transfection (Figure 12C), and surface protein down-regulation was observed 96 h after treatment (Figure 12E). Upon co-culture with IL-2-activated NK cells, BALB/c TEC treated with Clr-b siRNA displayed an increase in tubular death relative to scrambled siRNA, as indicated by PI staining (Figure 12F). Killing assays at various LAK:TEC ratios suggest that this inhibitory mechanism can suppress only up to a certain point, as at 10:1 ratio there were no significant differences between the scrambled and Clr-b siRNA groups (Figure 12G). In conclusion, these data support the notion that Clr-b acts as an inhibitory mechanism for NK cells in kidney tubular cells, since knocking Clr-b down dampened the ligand-receptor interaction and increased NK cell-mediated tubular cell death.


A
Scrambled siRNA-treated TEC
Clr-b siRNA-treated TEC
Events
α-CD26
B
n = 2
Fluorescent-labelled GAPDH siRNA
C
n = 15
n = 13
n = 12
n = 5
n = 5
n = 2
1.49
0.98
0.19
0.14
0.71
0.94
*
Relative Clr-b mRNA fold change in TEC
Untreated
Scrambled siRNA
24
48
72
96
Time point after Clr-b siRNA transfection (h)


D
Scrambled siRNA
24 h
55.90%
Events
Anti-Clr-b
Scrambled siRNA
48 h
63.28%
Scrambled siRNA
72 h
74.96%
Scrambled siRNA
96 h
90.09%


E
Scrambled siRNA
n = 2
68.03%
Events
Anti-Clr-b
48 h
n = 2
68.71%
72 h
n = 2
71.04%
96 h
n = 2
55.04%

F


Scrambled siRNA treatment
Gated on CFSE+ TEC
29
S
36.8%
PI
Clr-b siRNA treatment
Gated on CFSE+ TEC
24
S
64.7%
PI

G

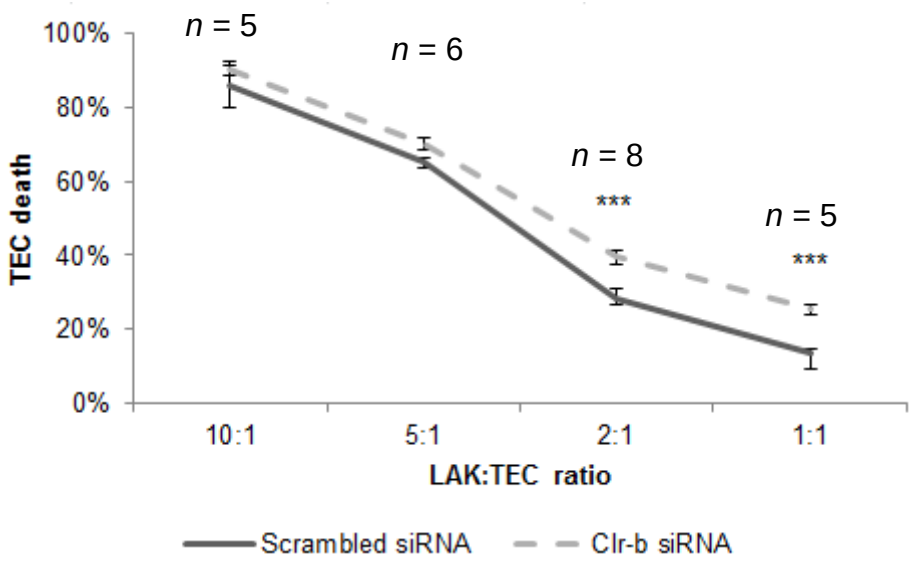

n = 5
n = 6
n = 8
n = 5
***
***
100%
80%
60%
40%
20%
0%
TEC death
10:1
5:1
2:1
1:1
LAK:TEC ratio
Scrambled siRNA
Clr-b siRNA

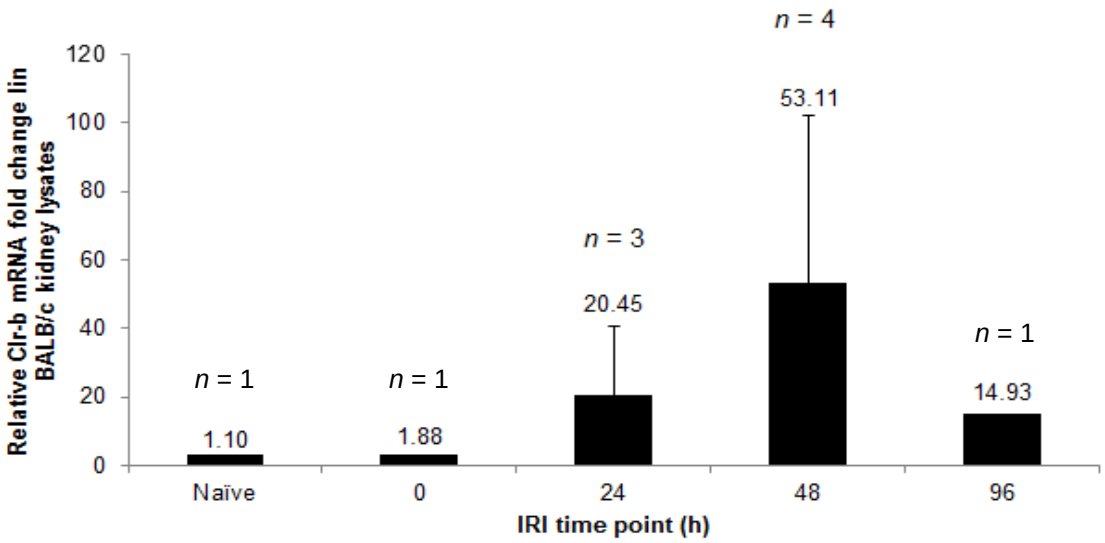
Figure 12. Clr-b knockdown by siRNA transfection in BALB/c primary-cultured TEC increases NK cell cytotoxicity

A) Cell characterization by CD26, a marker for TEC, after treatment with either scrambled or Clr-b siRNA. BALB/c kidney TEC were extracted and treated as described in *Materials and Methods*; n = 1. **B)** Transfection efficiency in BALB/c TEC by fluorescent-labelled GADPH siRNA; n = 2. **C)** Quantitative RT-PCR of BALB/c TEC Clr-b RNA expression after Clr-b siRNA transfection; n = 2 - 14. **D)** Clr-b surface protein detection after scrambled siRNA transfection. Time points are compared to null (shaded); n = 1. **E)** Surface protein knockdown was observed 96 hours after Clr-b siRNA transfection. Time points are compared to null (shaded); n = 2. **F)** TEC viability after the addition of IL-2-activated NK cells. Cytotoxicity assays were conducted as described in *Materials and Methods*. CFSE-stained cells were mixed with IL-2-activated NK cells for 5 h. The cells were used for PI staining to detect necrotic death; n = 11. **G)** Percentage of TEC death from NK activity at various effector:target ratios. CFSE-stained cells were mixed with IL-2-activated NK cells for 5 h. PI staining and FACS analysis were used to determine the percentage of TEC death by gating on CFSE-labelled tubular cells; n = 5 – 8. Statistical analysis was performed with ANOVA. * $p < 0.05$ and *** $p < 0.01$ compared with its scrambled siRNA-treated counterpart.

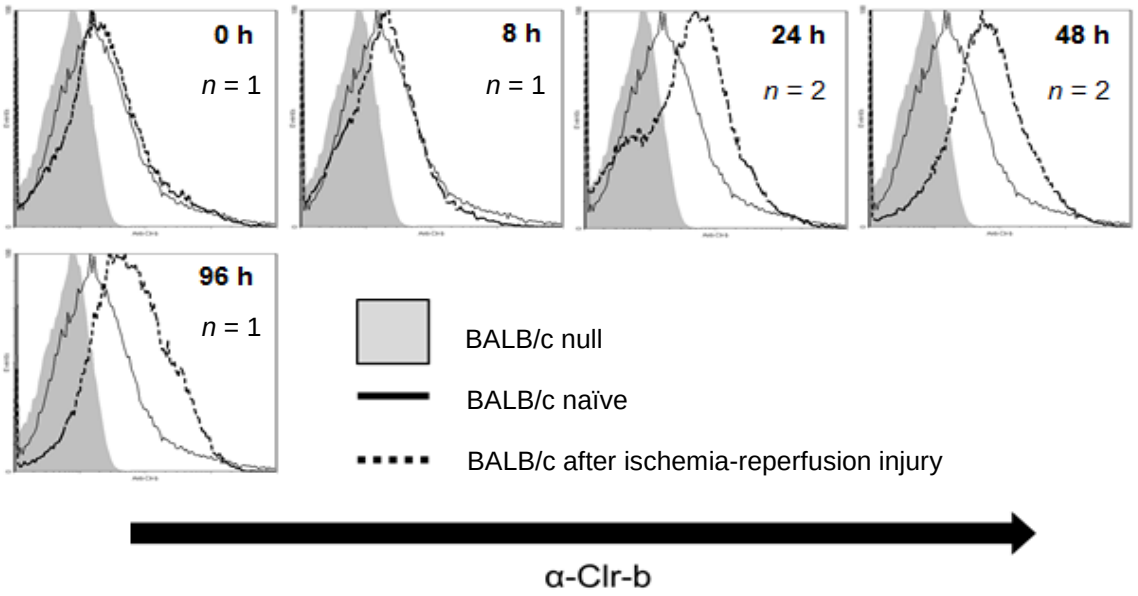
3.9 Clr-b in BALB/c kidneys increases in renal IRI

Ischemia-reperfusion injury in transplantation is an unavoidable phenomenon. Prior to transplantation, the graft is subjected to oxygen deprivation. After transplantation, there is a rush of blood supply, including infiltrating cells such as B cells, T cells, NK cells, macrophages and neutrophils, leading to renal failure. We examined whether Clr-b expression is affected by renal IRI in our animal model. As, under similar conditions, BALB/c mice are particularly resistant against IRI than C57BL/6 mice, we subjected these mice to a more severe condition (60 min at 33°C) than the IRI protocol for C57BL/6 (45 min at 32°C). Surface protein analysis demonstrated that Clr-b surface expression in BALB/c kidneys progressively increased, peaked at 48 hours, and persisted after IRI (Figure 13B). Among the kidney cells stained positive for CD26, a TEC marker, 51.3% were also positive for Clr-b in naïve mice. This percentage increased to 55.2% and 63.0% 24 h and 48 h after IRI, respectively (Figure 13C). In summary, our data concludes that BALB/c kidney Clr-b expression increases as a response to renal injury.

A


Relative Clr-b mRNA fold change lin
BALB/c kidney lysates
120
100
80
60
40
20
0
n = 1
1.10
n = 1
1.88
n = 3
20.45
n = 4
53.11
n = 1
14.93
Naïve
0
24
48
96
IRI time point (h)

B


0 h
n = 1
8 h
n = 1
24 h
n = 2
48 h
n = 2
96 h
n = 1
BALB/c null
BALB/c naïve
BALB/c after ischemia-reperfusion injury
α-Clr-b

C

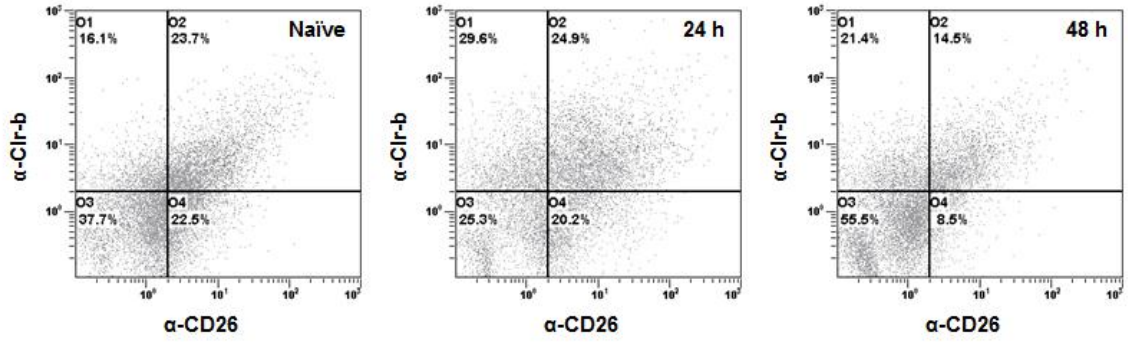

Naïve
O1 16.1%
O2 23.7%
O3 37.7%
O4 22.5%
24 h
O1 29.6%
O2 24.9%
O3 25.3%
O4 20.2%
48 h
O1 21.4%
O2 14.5%
O3 55.5%
O4 8.5%
α-Clr-b
α-CD26

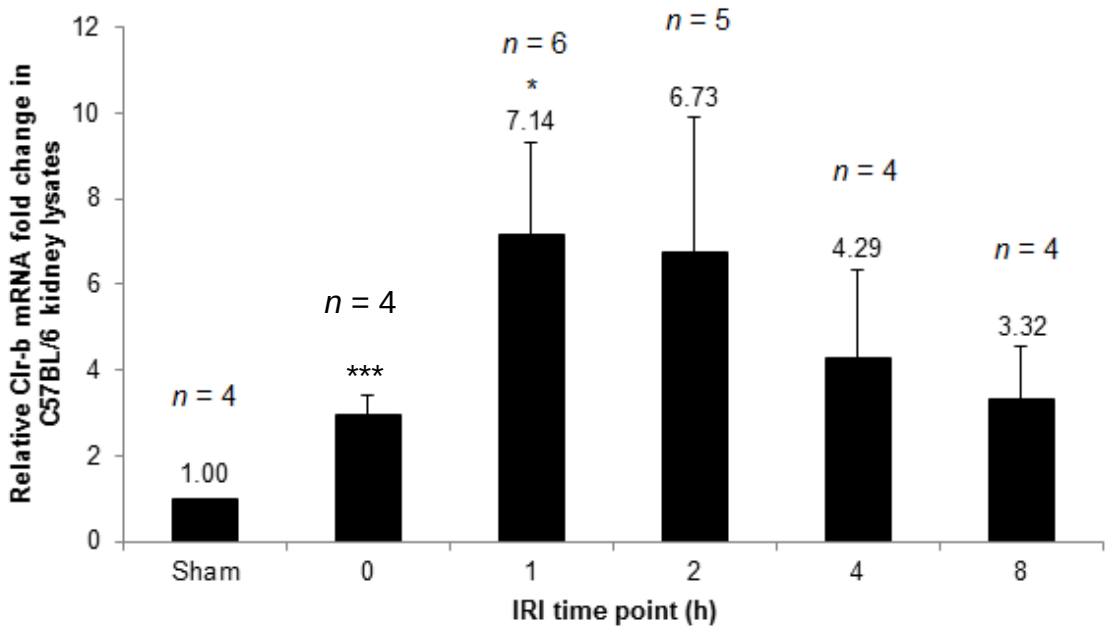
Figure 13. BALB/c kidney Clr-b expression increases with renal IRI in a time-dependent manner

A) Quantitative RT-PCR of BALB/c mice kidney Clr-b RNA expression 0 h, 24 h, 48 h and 96 h after renal IRI. Total RNA was extracted (Qiagen) and cDNA pools were synthesized (Stratagene). Real-time quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kits and the Mx3005P system (Stratagene). β-actin amplification was used as the endogenous control. The normalized δ threshold cycle value was calculated according to the manufacturer's protocol; n = 1 – 4. Statistical analysis was performed with ANOVA. * $p < 0.05$ compared with naïve control. **B)** Surface ligand Clr-b expression 0 h, 8 h, 24 h, 48 h, and 96 h after renal IRI. BALB/c kidney cells were extracted as described in *Materials and Methods* and collected for FACS analysis after α-Clr-b staining. Time points are compared to null (gray) and sham (solid line); n = 1 – 2. **C)** Clr-b expression in $CD26^+$ TEC. BALB/c kidney cells were collected from naïve, 24 h and 48 h after IRI and stained with α-Clr-b and α-CD26 for FACS analysis. R2 quadrant represents cells positive for both Clr-b and CD26; n = 1.

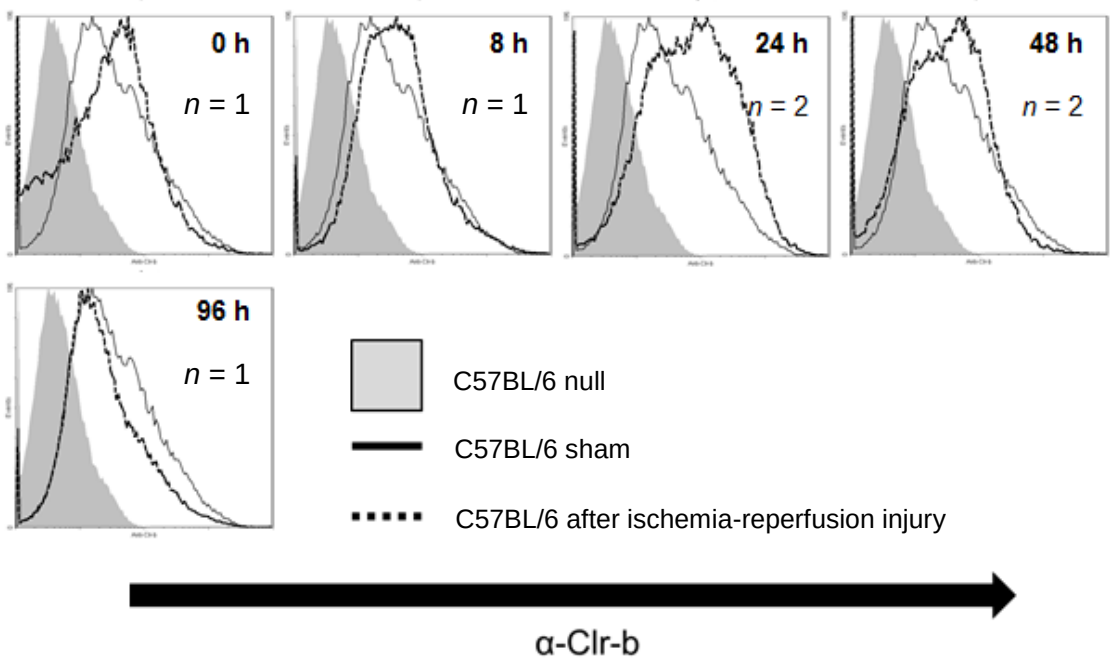
3.10 C57BL/6 and C3H kidney Clr-b increases in renal IRI

Similarly, we tested whether Clr-b expression is affected by renal IRI in other mouse strains. In our IRI injury model, real-time PCR analysis demonstrated that Clr-b mRNA expression in C57BL/6 mice increased within the first few hours after IRI, but began to return to basal levels afterwards (Figure 14A). FACS data also demonstrated that Clr-b surface expression increased 24 hours after IRI, but regressed to physiological levels by day 4 (Figure 14B). Among the kidney cells stained positive for CD26, 61.4% were also positive for Clr-b in naïve mice. This percentage increased to 78.0% and 82.2% 0 h and 8 h after IRI, respectively. Clr-b expression in $CD26^{+}$ TEC began to regress afterwards, as 62.3% were positive at 24 h, 71.3% at 48 h, and 49.4% at 96 h after IRI (Figure 15C). FACS analysis of C3H ($H\text{-}2^{k}$) mice also indicated an increase in kidney Clr-b surface expression within 24 hours post-IRI (Figure 14D). Serum creatinine levels were analyzed to measure C3H renal dysfunction (Figure 14E). In summary, our data shows that Clr-b expression in C57BL/6 and C3H kidneys and, specifically, TEC increases after renal injury.

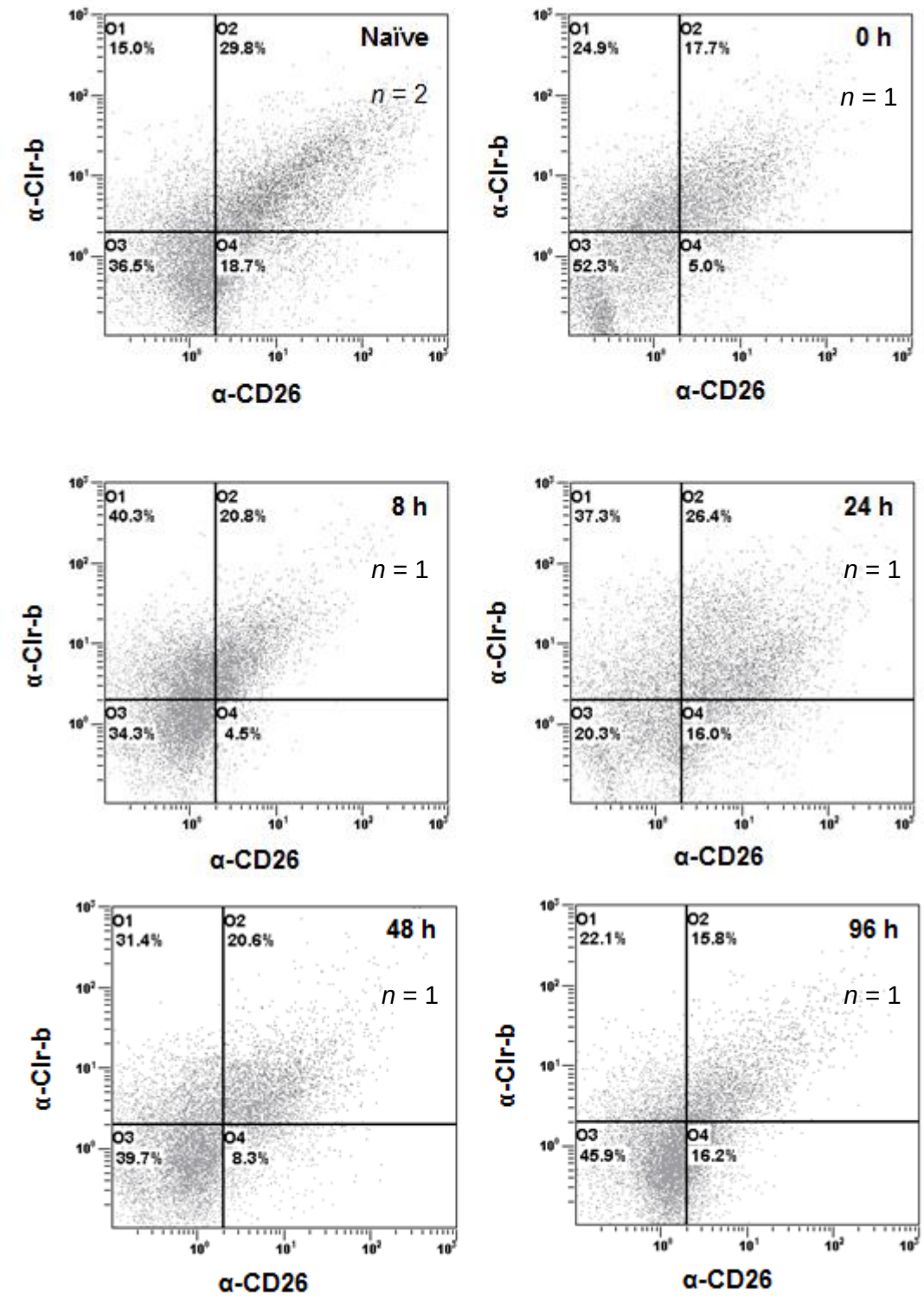
A


Relative Clr-b mRNA fold change in C57BL/6 kidney lysates
12
10
8
6
4
2
0
n = 4
1.00
n = 4
***
n = 6
*
7.14
n = 5
6.73
n = 4
4.29
n = 4
3.32
Sham
0
1
2
4
8
IRI time point (h)

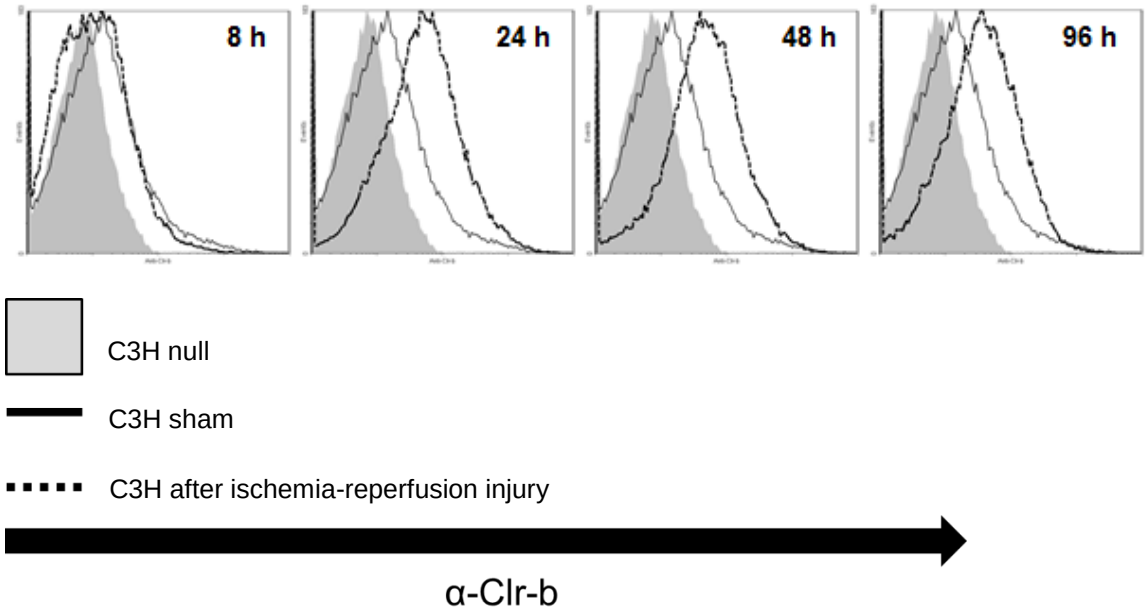
B


0 h
n = 1
8 h
n = 1
24 h
n = 2
48 h
n = 2
96 h
n = 1
C57BL/6 null
C57BL/6 sham
C57BL/6 after ischemia-reperfusion injury
α-Clr-b

C


Naïve
n = 2
O1 15.0%
O2 29.8%
O3 36.5%
O4 18.7%
0 h
n = 1
O1 24.9%
O2 17.7%
O3 52.3%
O4 5.0%
8 h
n = 1
O1 40.3%
O2 20.8%
O3 34.3%
O4 4.5%
24 h
n = 1
O1 37.3%
O2 26.4%
O3 20.3%
O4 16.0%
48 h
n = 1
O1 31.4%
O2 20.6%
O3 39.7%
O4 8.3%
96 h
n = 1
O1 22.1%
O2 15.8%
O3 45.9%
O4 16.2%
α-Clr-b
α-CD26

D


8 h
24 h
48 h
96 h
C3H null
C3H sham
C3H after ischemia-reperfusion injury
α-Clr-b

E

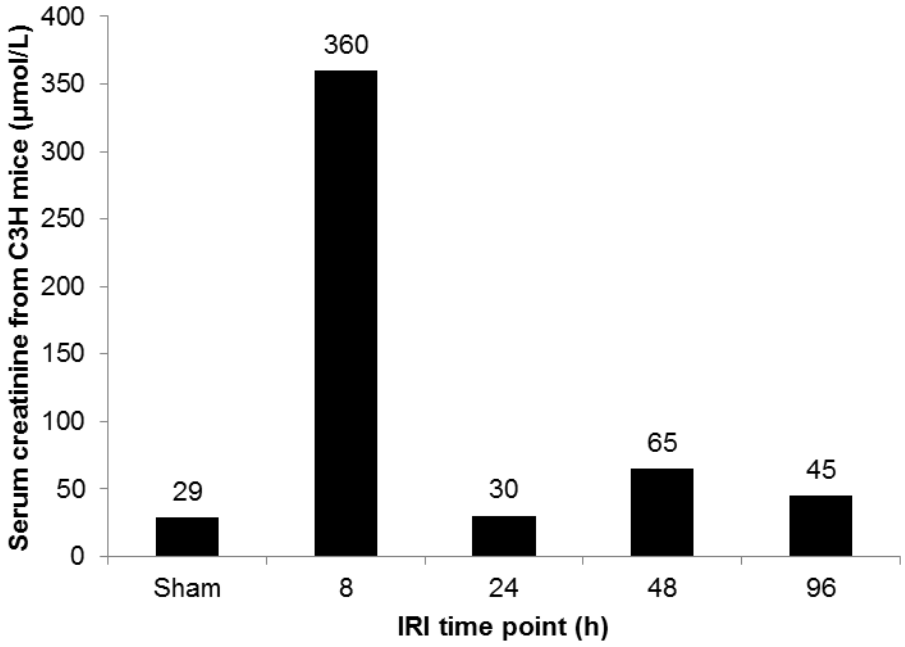

Serum creatinine from C3H mice (µmol/L)
400
350
300
250
200
150
100
50
0
360
29
30
65
45
Sham
8
24
48
96
IRI time point (h)

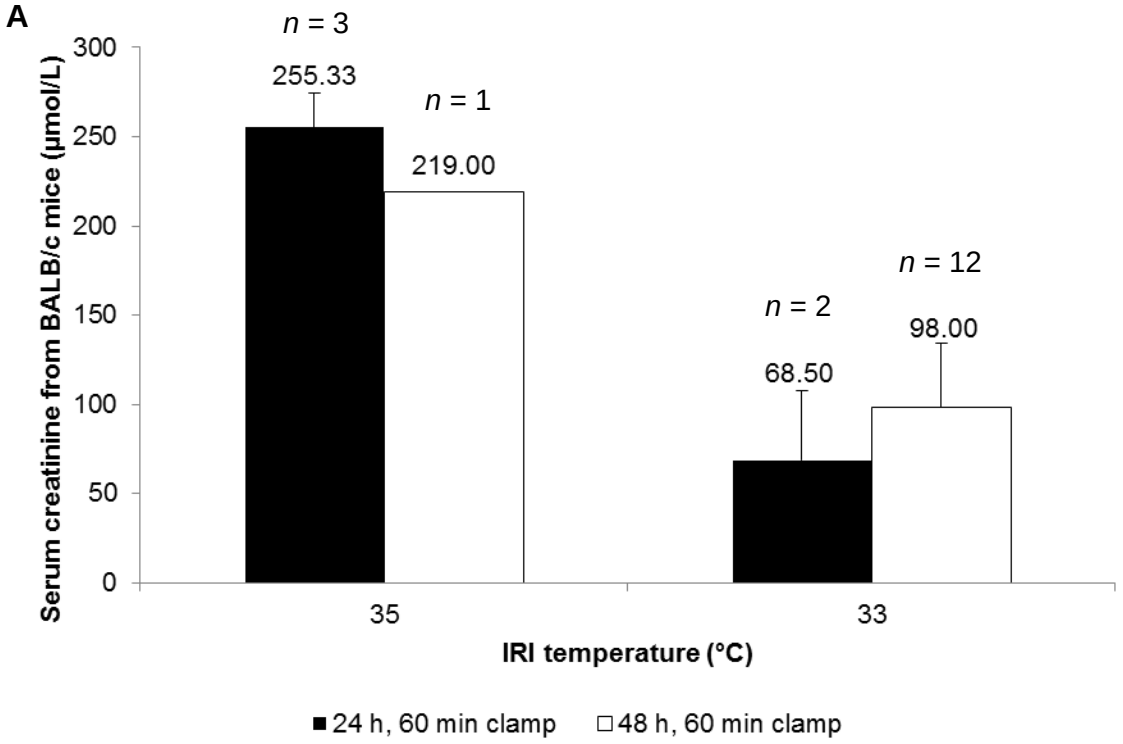
Figure 14. C57BL/6 and C3H kidney Clr-b expression increases with renal IRI in a time-dependent manner

A) Quantitative RT-PCR of C57BL/6 mice kidney Clr-b RNA expression in sham, 0 h, 1 h, 2 h, 4 h, and 8 h after renal IRI; $n = 4 – 6$. Statistical analysis was performed with ANOVA. *** $p < 0.01$ and * $p < 0.05$ compared with sham. **B)** Surface ligand Clr-b expression 0 h, 8 h, 24 h, 48 h, and 96 h after IRI. TEC were purified from C57BL/6 mice as described in *Materials and Methods* and collected for FACS analysis with α-Clr-b staining. Time points are compared to null (gray) and naïve (solid line); $n = 1 – 2$. **C)** Clr-b expression in $CD26^+$ TEC. C57BL/6 kidney cells were collected from naïve, 0 h, 8 h, 24 h, 48 h and 96 h after IRI and stained with α-Clr-b and α-CD26 for FACS analysis. O2 quadrant represents cells positive for both Clr-b and CD26; $n = 1 – 2$. **D)** Surface ligand Clr-b expression 8 h, 24 h, 48 h, and 96 h after IRI. TEC were purified from C3H mice as described in *Materials and Methods* and collected for FACS analysis with Clr-b staining. Time points are compared to null (gray) and sham (solid line); $n = 1$. **E)** Serum creatinine levels of sham, 8 h, 24 h, 48 h, and 96 h after IRI. C3H kidney TEC were extracted as described in *Materials and Methods*. The cells were used for annexin V and PI staining to detect apoptotic and necrotic death, respectively; $n = 1$.

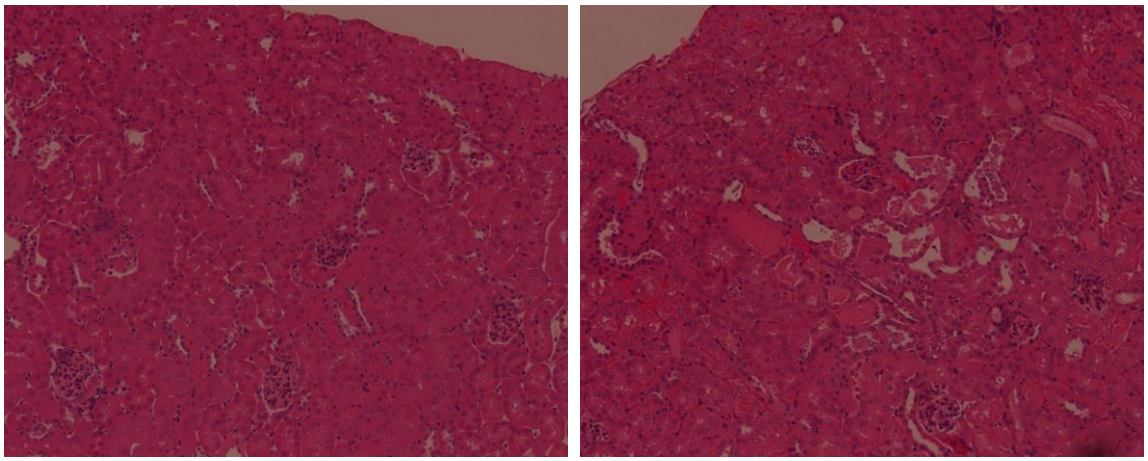
3.11 BALB/c mice are more resistant to renal IRI than C57BL/6

To confirm kidney injury, serum creatinine levels (Figure 15A) and H & E staining (Figure 15B) were analyzed to measure renal dysfunction, annexin V staining to measure cell apoptosis, and PI staining for cell necrosis in BALB/c mice following IRI (Figure 15C). H & E staining demonstrated more tubular and glomerular necrosis after renal IRI. Serum creatinine at 37 °C averaged 173 (± 43.84) μmol/L at 24 hours and died within 2 days (*n* = 2). When the IRI temperature and clamping duration were decreased to 33 °C and 45 min., serum creatinine level measured 30 μmol/L at 24 hours and 12 μmol/L at 48 hours (*n* = 1). Thus, the optimal conditions for BALB/c mice appear to be at 33 °C for 60 min. Similarly, serum creatinine levels (Figure 15D) and H & E staining (Figure 15E) were analyzed to measure renal dysfunction, annexin V staining to measure cell apoptosis, and PI staining for cell necrosis (Figure 15F) in C57BL/6 mice at 32 °C for 45 min. Here, we demonstrate that BALB/c mice are more resistant to renal IRI than C57BL/6 mice.

A

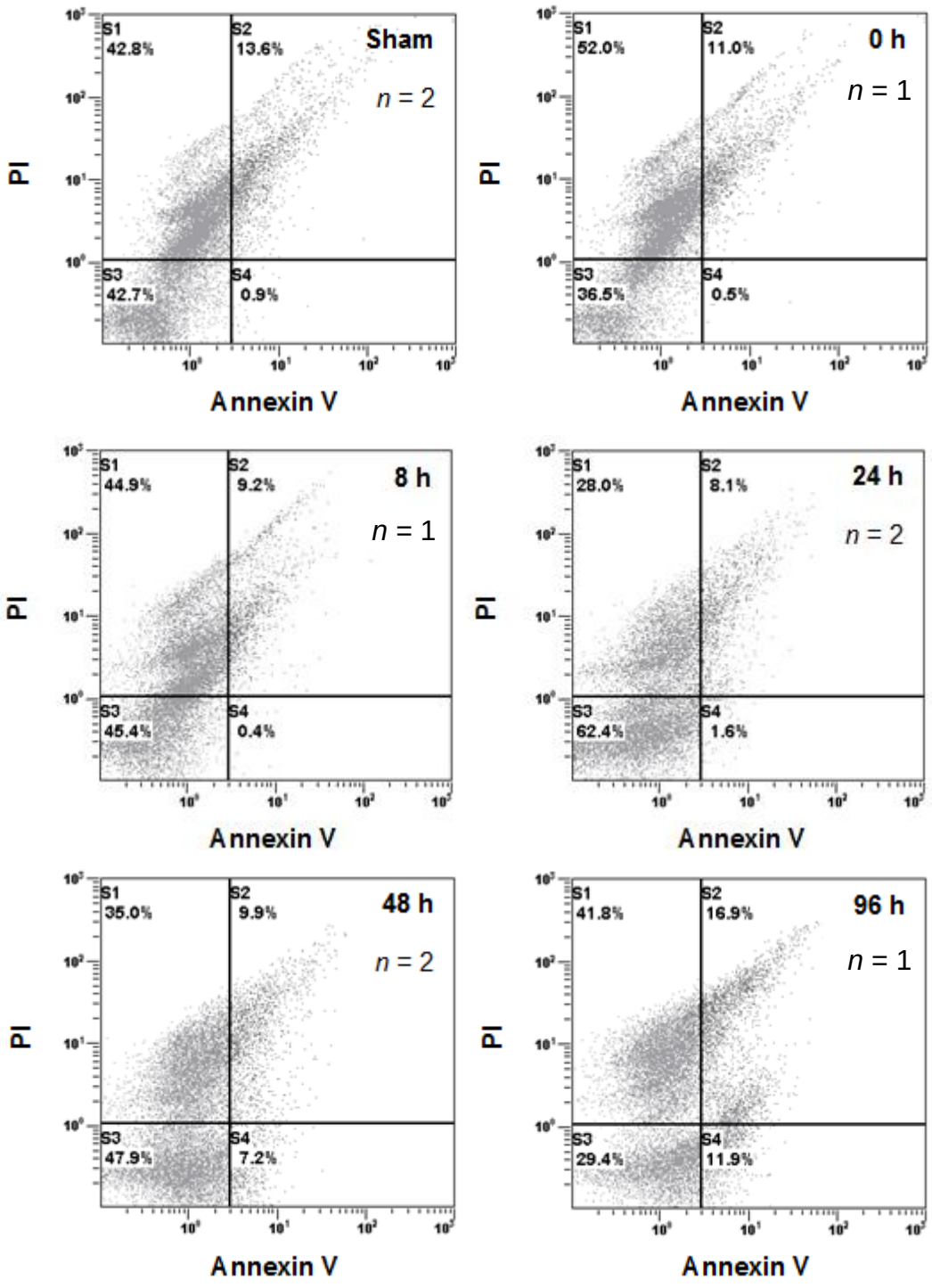


B

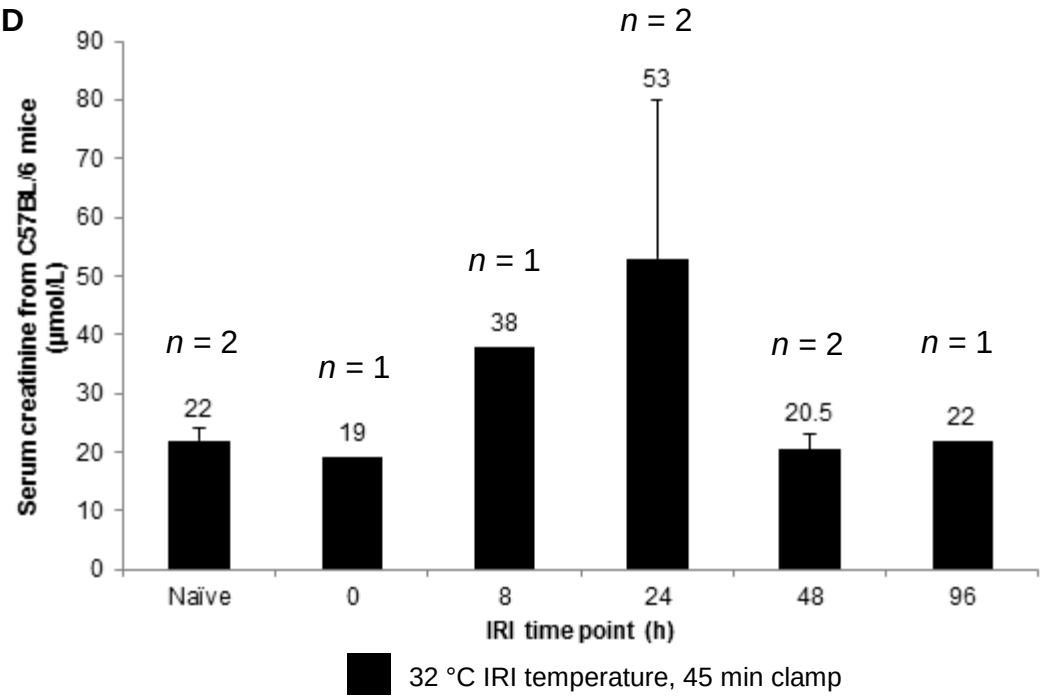


BALB/c Naïve **BALB/c 24 h Post-IRI**

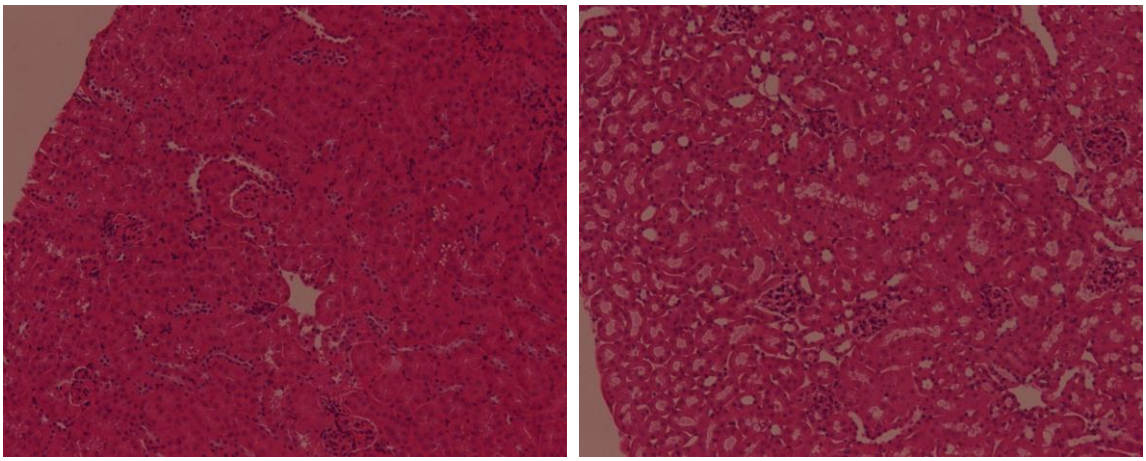
C


Sham
n = 2
S1 42.8%
S2 13.6%
S3 42.7%
S4 0.9%
0 h
n = 1
S1 52.0%
S2 11.0%
S3 36.5%
S4 0.5%
8 h
n = 1
S1 44.9%
S2 9.2%
S3 45.4%
S4 0.4%
24 h
n = 2
S1 28.0%
S2 8.1%
S3 62.4%
S4 1.6%
48 h
n = 2
S1 35.0%
S2 9.9%
S3 47.9%
S4 7.2%
96 h
n = 1
S1 41.8%
S2 16.9%
S3 29.4%
S4 11.9%
PI
Annexin V

D



E



C57BL/6 Naïve **C57BL/6 24 h Post-IRI**

F

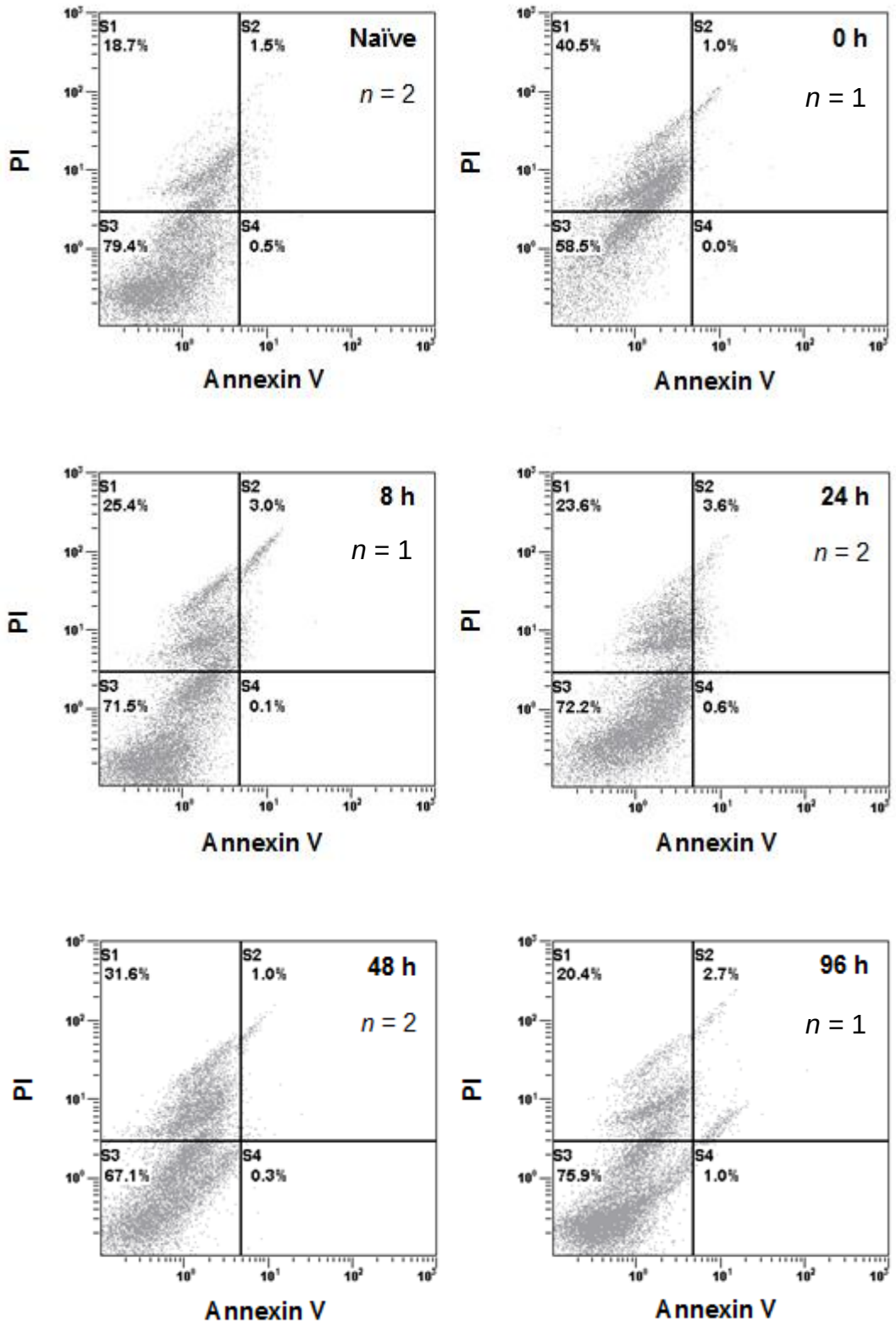

Naïve
n = 2
S1 18.7%
S2 1.5%
S3 79.4%
S4 0.5%
0 h
n = 1
S1 40.5%
S2 1.0%
S3 58.5%
S4 0.0%
8 h
n = 1
S1 25.4%
S2 3.0%
S3 71.5%
S4 0.1%
24 h
n = 2
S1 23.6%
S2 3.6%
S3 72.2%
S4 0.6%
48 h
n = 2
S1 31.6%
S2 1.0%
S3 67.1%
S4 0.3%
96 h
n = 1
S1 20.4%
S2 2.7%
S3 75.9%
S4 1.0%
PI
Annexin V

Figure 15. Ischemia-reperfusion injury model is optimal at 33 °C in BALB/c mice

A) BALB/c mice serum creatinine extracted as described in *Materials and Methods*; *n* = 1 - 12. **B)** BALB/c kidney sections were taken from either naïve (left) or 24 hours post-IRI (right) and were stained with hematoxylin and eosin. Pictures were taken in the medulla region of the kidney at 100x magnification. **C)** Cell death in BALB/c TEC, as measured by annexin V and PI staining of sham, 0 h, 8 h, 24 h, 48 h, and 96 h after IRI to detect apoptotic and necrotic death, respectively. BALB/c kidney TEC were extracted as described in *Materials and Methods*; *n* = 1 - 2. **D)** C57BL/6 mice serum creatinine; *n* = 1 - 2. **E)** C57BL/6 kidney sections were taken from either naïve (left) or 24 hours post-IRI (right) and were stained with hematoxylin and eosin. Pictures were taken in the medulla region of the kidney at 100x magnification. **F)** Cell death in C57BL/6 TEC, as measured by annexin V and PI staining of sham, 0 h, 8 h, 24 h, 48 h, and 96 h after IRI to detect apoptotic and necrotic death, respectively. C57BL/6 kidney TEC were extracted as described in *Materials and Methods*; *n* = 1 – 2.

3.12 Clr-b decreases in C57BL/6 to F1 kidney transplants

As ischemia-reperfusion injury is an unavoidable phenomenon during organ transplantation, we characterized Clr-b expression in kidney grafts to determine whether this signalling mechanism can play a role in transplants. A parent to F1 transplant model in which T cell tolerance is present was utilized for these studies. Real-time PCR data analysis of Clr-b in C57BL/6 to (C57BL/6 × BALB/c)F1 kidneys indicated that there was a reduction in Clr-b expression 60 days post-transplant (Figure 16). As Clr-b acts to protect the kidney against NK cell-mediated cytotoxicity, ligand down-regulation in these grafts may partly compromise its ability to defend against NK cell-mediated tubular death. Our data demonstrate for the first time a critical role for NK cells in mediating chronic kidney injury in transplantation, independent of T and B cells (submitted for publication).

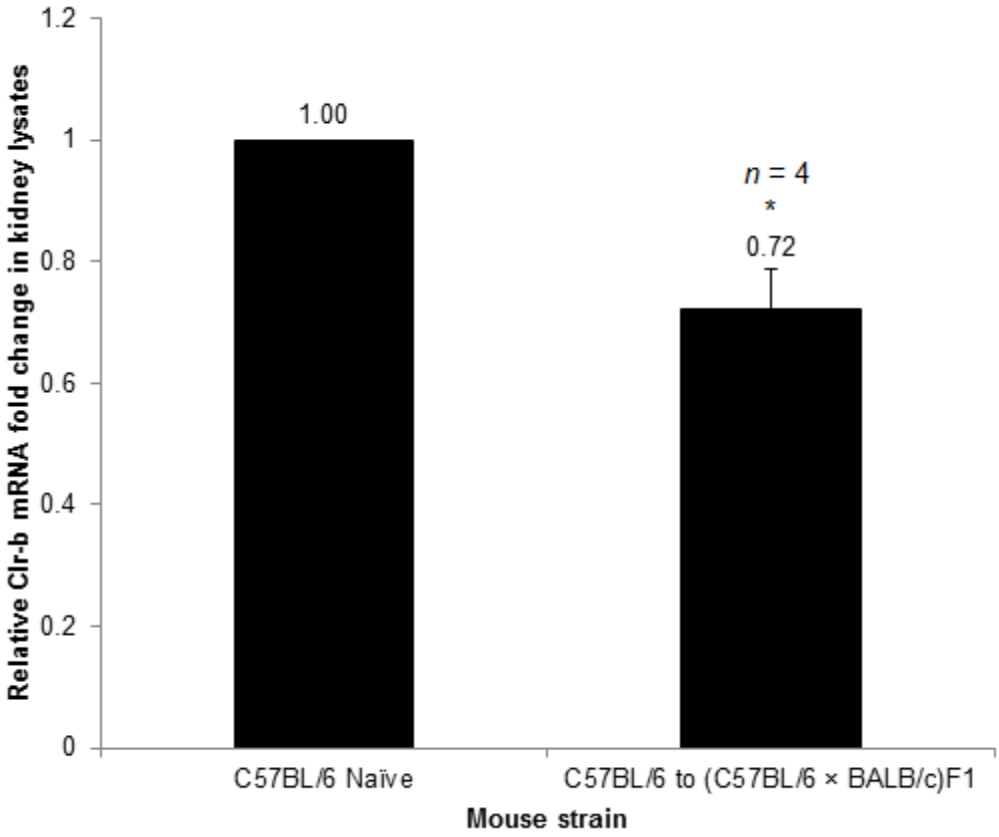

1.2
1
0.8
0.6
0.4
0.2
0
Relative Clr-b mRNA fold change in kidney lysates
1.00
n = 4
*
0.72
C57BL/6 Naïve
C57BL/6 to (C57BL/6 × BALB/c)F1
Mouse strain

Figure 16. Clr-b ligand in kidney decreases in C57BL/6 to F1 transplants

Quantitative RT-PCR of Clr-b RNA expression in transplanted kidneys. Kidney transplantations were performed as described in Materials and Methods; $n = 4$; * $p < 0.05$ compared with untransplanted naïve C57BL/6 kidney.

Chapter 4

4 Discussion

4.1 Summary of this study

In this study, we demonstrated at the mRNA and surface levels that Clr-b can be upregulated by TNF-α and IFN-γ in tubular epithelial cells from the BALB/c and C57BL/6 mouse strains (Figures 4 and 6). Clr-b up-regulation in BALB/c, C57BL/6 and C3H kidneys was also observed in our *in vivo* IRI model (Figures 14 and 15). Although many of the ligands' functions have not yet been discovered, mRNA expression of the *Clr* subtypes appears to be notably higher in C57BL/6 mice than BALB/c mice (Figure 9). More experiments, however, need to be conducted to determine whether *Clr* expression is strain-dependent. Differences between mouse strains were also observed in the expression profile of the *Nkr-p1* receptors (Figure 8). In support of published literature[34], we detected *Nkr-p1b* in BALB/c but not C57BL/6 NK cells. We also did not detect *Nkr-p1b* up-regulation upon IL-2 stimulation by NK cells (Figure 8). Using siRNA technology, we found that knocking down Clr-b on TEC significantly increased NK cell-mediated cytotoxicity (Figure 12). Furthermore, Clr-b in kidneys regressed in C57BL/6 to F1 transplants (Figure 16), demonstrating a possible compromised mechanism and reduction in TEC's ability to defend against NK cell-mediated tubular death. Manipulating the NKR-P1B-Clr-b pathway in the clinical setting may prevent NK cell-mediated TEC death in both acute and chronic allograft injury.

4.2 Regulation of NK cell ligand Clr-b in TEC and the kidney

In recent studies, we have found that NK cells can kill syngeneic TEC *in vitro*[265]. NK cells quickly infiltrate into the kidney after ischemia and reperfusion. NK cell depletion in wild type C57BL/6 mice was protective while adoptive transfer of NK cells worsened injury in NK, T and B cell null $Rag2^{-/-}\gamma c^{-/-}$ mice with IRI. Our

results demonstrated for the first time that NK cells can directly kill TEC and that NK cells contribute substantially to kidney IRI[265]. Although reduced, a significant amount of NK cells still remains in the kidney after IRI over a long period of time. As a measure to prevent prolonged NK cell-mediated injury, the kidney may have developed a mechanism to resist against NK cell-mediated cytotoxicity after IRI and transplantation.

As previously mentioned, NK cell function is regulated by their receptors and ligand interactions. The Ly49 receptors and their ligand MHC class I molecules are important for the prevention of NK cell-mediated self-destruction. However, these molecules are constitutively expressed on TEC and have a high degree of polymorphism[33]. Other molecules, therefore, should be regulated after kidney IRI to increase the resistance capability of TEC towards NK cell-mediated cytotoxicity. It is known that the Clr-b molecule delivers inhibitory signals to its receptor NKR-P1B on NK cells[31]. Similar to MHC class I molecules, Clr-b is highly expressed on hematopoietic cells, excluding erythrocytes[31]. Tumour cell lines in mice often demonstrate Clr-b downregulation[31], indicating a potential role for MHC class I-independent “missing self-recognition”[31]. A recent study has found Clr-b to be down-regulated after viral infections such as vaccine virus and ectromelia virus[255]. Rat cytomegalovirus infection also down-regulated host Clr-b expression while concurrently expressed its own ligand, rat cytomegalovirus C-type lectin-like (RCTL)[242]. RCTL was demonstrated to interact with NKR-P1B and protect infected cells from NK cell-mediated cytotoxicity[242]. Genotoxic and cellular stress induced by chemotherapeutic agents or irradiation were other mechanisms demonstrated to downregulate Clr-b and enhanced cytotoxicity mediated by NKR-P1B^{+} NK cells[74].

In this study, we found that Clr-b expression is up-regulated in the kidney after IRI based on real-time quantitative PCR and FACS analysis (Figures 13 and 14). Similarly, expressions of Clr-b mRNA and protein were significantly enhanced in primary-cultured TEC after treatment with pro-inflammatory cytokines TNF-α and IFN-γ, well-known to be factors involved in inflammation and renal IRI[68] (Figures

4 and 6). The primary role of TNF-α is to regulate immune cells and is involved in systemic inflammation. Among other cells, TNF-α is predominantly released by macrophages and T cells after infection[224], while resident dendritic cells appear to be the predominant source in the early-stages of renal IRI[62]. TNF-α may interact with either TNFR1 or TNFR2; TNFR1 is broadly expressed and can be activated by both membrane-bound and soluble TNF homotrimers, while TNFR2 is limited to immune cells and respond to the membrane-bound form[224]. Upon receptor-ligand interaction, TNF-α may activate the MAPK pathways to mediate cell differentiation, proliferation, and pro-apoptotic events[224]. TNF-α may also activate the NF-κB pathway, leading to transcription of proteins involved in cell survival, proliferation, inflammation, and anti-apoptosis[224]. Among the genes mediated by NF-κB, it is possible that Clr-b is one of the factors upregulated to promote self-survival. IFN-γ, on the other hand, is predominantly produced by NK and T cells and can interact with heterodimer IFN-γ receptor 1 (IFNGR1) and receptor 2 (IFNGR2) to activate the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway[105]. IFN-γ not only promotes cellular and humoral immunity, such as NK, T and B cell activation, but also stimulates MHC class I and II expression on normal cells[204]. While Clr-b initiates an MHC-independent pathway, it may also be up-regulated during the process of inflammation to prevent NK cell-mediated death of healthy cells.

Natural killer cells are the earliest defense against tumours and viral infections. As one of the mechanisms to counter foreign intruders, NK cells secrete the pro-inflammatory cytokine TNF-α. TNF-α is an important regulator of both adaptive and innate immune responses by promoting inflammation and has been associated with malignancy[152]. Both circulating and urinary TNF-α levels have been found to be up-regulated in inflammatory chronic kidney diseases such as diabetic nephropathy[170, 257]. In the early stages of many kidney diseases, variations of urinary fluid flow induces shear stress, which was demonstrated to increase mononuclear chemoattractant protein-1 (MCP-1), TNF-α, vascular cell adhesion molecule-1 (VCAM-1), and adhesion of monocytes in human endothelial cells. Interestingly, when exposed to short-term fluid shear stress,

TNF-α mainly came from endothelial production. However, long-term exposure to stress induced the release of TNF-α directly from tubular cells[165], demonstrating interactive paracrine signalling between these cells. A recent study also showed that TNF-α can play a crucial role in nephritis by inducing MCP-1, and cell surface adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression via the MAPK signaling pathway and promote macrophage and lymphocyte infiltration[93].

The TNF-α converting enzyme (TACE) is a metalloproteinase responsible for cleaving TNF-α into a 26 kDa membrane form and a 17 kDa soluble peptide, and also membrane shedding of TNFR1 and TNFR2[248, 250]. In acute rejecting kidneys, TACE was demonstrated to be upregulated mainly in TEC, where it co-localizes with TNFR2 and leads to TNFR2 release[250]. TACE suppression, such as by TNF-α protease inhibitor, can block TNFR2 release and suppress the pro-inflammatory effect of TNF-α in acute renal injury[250]. In addition, TNF-α-induced activation of the extracellular-signal-regulated kinase (ERK)/guanine nucleotide exchange factor H1 (GEF-H1)/Ras homolog gene family member A (RhoA) signalling pathway in tubular cells was demonstrated to be mediated through TACE-dependent epidermal growth factor receptor (EGFR) activation[107]. Such a mechanism could act as both an inflammatory and proliferative stimuli and thus, TNF-α may play an important role in the regulation of not only cell death, but also wound healing and fibrogenesis in TEC during chronic injury.

Interferons are another class of cytokines commonly released by cells in response to pathogens, and named after their ability to "interfere" with viral replication. Interferons activate immune cells such as NK cells and macrophages, increase recognition of infection or tumour cells by upregulating antigen presentation to lymphocytes, and increase the ability of cells to resist against infections[204]. In a study, mice deficient of type I interferons, which includes IFN-γ, showed significantly reduced necrosis of tubules in the outer medulla, loss of the brush border, cast formation and tubular dilatation[75]. Type 1 interferons were also shown to increase neutrophil and macrophage infiltration in the outer medulla,

and promote the expression of pro-inflammatory TNF-α, IL-1, IL-6 and CXCL-2[75]. Interferons are important chemical mediators in kidney injury and inflammation, and are one of the cytokines secreted by NK cells to induce in cell death.

In our study, we demonstrate that the inhibitory ligand Clr-b is upregulated in response to the cytokines TNF-α and IFN-γ in primary-cultured TEC (Figures 4 and 6). While at the mRNA level, *clr-b* expression in BALB/c TEC increased quickly after cytokine induction and peaked at 48 h, Clr-b was found to be upregulated on the tubular cell surface only at the 24 h time-point. Similarly, in C57BL/6 TEC, mRNA levels were relatively high at the 48 h time point in comparison to basal level, yet Clr-b upregulation was again only detected at the 24 h time point on the surface of primary-cultured TEC. It appears that the early onset of gene upregulation may temporarily induce Clr-b expression, but other post-transcriptional or -translational factors may be involved to inhibit further induction, as Clr-b surface expression returned to normal physiological levels by 48 h. In fact, these factors may be upregulated at a later onset than Clr-b, as not only did the ligand revert back to basal levels, but Clr-b was observed to be downregulated 48 h and 72 h in BALB/c and C57BL/6 TEC, respectively. Similarly in MULT-1 regulation, protein expression is post-translationally controlled by cytoplasmic lysine residues associated with polyubiquitination of the protein[171]. Another plausible explanation may be that protein shuffling within cells resulted in the decrease in Clr-b detection. Nonetheless, both these modification factors and Clr-b appear to be transiently regulated by TNF-α and IFN-γ, as surface Clr-b protein was detected to match basal levels by 96 h. While these pro-inflammatory cytokines are known to induce necrosis and apoptosis, we demonstrate here for the first time that they upregulate the inhibitory Clr-b, a ligand that may prevent NK cell-mediated cytotoxicity.

Since renal IRI also involves oxygen deprivation, we subjected these primary-cultured TEC to hypoxic conditions. In BALB/c TEC, we did not detect any significant changes of Clr-b expression on the cellular surface (Figure 5A). Conversely, in C57BL/6 TEC, we detected a positive shift in Clr-b on the tubular

cell (Figure 7A). A plausible explanation is that BALB/c TEC are more adaptable to hypoxia or the hypoxia time was not sufficient, as there were no indications of cell death with annexin V and propodium iodide staining (Figure 5B), whereas there were clear apoptotic and necrotic cell populations in C57BL/6 TEC (Figure 7B). A more severe condition may be required for BALB/c TEC to match the amount of hypoxic injury as the C57BL/6 mouse strain.

As suspected, BALB/c mice were resistant against renal IRI when compared to the C57BL/6 mouse strain, since there were no indications of kidney injury under our IRI protocol. We therefore subjected these mice to a more severe condition than that of C57BL/6 mice. Twenty-four hours after surgery, there were marked upregulation of Clr-b on the cellular surfaces in kidneys, which continued even after 96 h in BALB/c mice (Figure 14B). Subsequently, in C57BL/6 mice, we once again observed ligand upregulation at 24 h and regression to physiological levels afterwards, similar to our *in vitro* studies (Figure 15B). However, it should be noted that these strains underwent different IRI conditions, thus making it difficult to interpret the differences between these two mouse strains.

Based on these data, we conclude that the inhibitory Clr-b ligand is up-regulated on tubular cells and the kidney as a consequence of renal IRI. NK cells are observed in kidney post-transplant patients long-term after surgery[47]. Thus, Clr-b may act as an inhibitor against NK cells to protect kidney grafts from the immune system. A balance between stimulatory and inhibitory signals on both tubular and NK cells may dictate the amount of cytotoxicity caused by these infiltrating immune cells, where a minor preference for stimulatory signals may ultimately lead to chronic allograft rejection.

4.3 Clr-b expression in TEC inhibits NK cell activity

There are a plethora of receptors on NK cells, including activating receptors such as NKG2D, Ly49 subtypes which can either promote or inhibit activity, or the

NKG2A/CD94 complex which inhibits cytotoxicity. These receptors bind to target cells to form an immune synapse and rearrange actin within the NK cell. This results in the reorganization of the golgi complex and microtubule organizing centre to polarize lytic granules toward the synapse, and the release of granzyme B or perforin towards the target cell. NK cells are also known to mediate inflammatory responses by secreting cytokines, including TNF-α and IFN-γ. The degree of cytotoxicity depends on the balance between activating and inhibitory receptors.

We have previously demonstrated that NK cells can injure kidneys and TEC in a perforin-dependent manner[265]. Following IRI, we noted increased expression of the NK cell ligand Rae-1 on TEC, as well as stimulatory receptor NKG2D expression by NK cells. This receptor-ligand interaction was demonstrated to act as an NK cell activation signal to lyse TEC[265]. Fas-FasL pathway may also be another mechanism involved in acute ischemic kidney injury, as blockade of Fas ligand on leukocytes attenuated kidney IRI in mice[67].

In this study, we silenced the inhibitory ligand Clr-b in BALB/c tubular cells by using siRNA technology to determine whether NK cell-mediated lysis is affected by the NKR-P1B-Clr-b molecular pathway. As suspected, *clr-b*-silenced cells had a higher level of cell death (Figure 12). We demonstrate that, without the inhibitory function of Clr-b, kidney TEC becomes more susceptible against NK cell-mediated cytotoxicity. This indicates that Clr-b plays a dominant role to control NK cell-mediated cytotoxicity even in the presence of other activating molecules on NK cells. While Clr-b blockade with anti-Clr-b antibody did not alter NK cell-mediated lysis in our hands (Figure 11), we suspect that we either did not use a sufficient quantity of antibody or the antibody does not have neutralizing capabilities. We have nonetheless demonstrated with siRNA that removing *clr-b* proves to be very effective in promoting TEC death.

Interestingly, another study done in rat models found that NKR-P1B$^+$ NK cells with high NKR-P1B expression killed YAC-1 cells at a higher efficiency than

either NKR-P1B$^-$ Ly49s3$^+$ NK cells or NK cells with low NKR-P1B expression[100]. These NKR-P1B$^+$ NK cells, however, have been demonstrated to act as mature effector NK cells. Similar to effector CD8$^+$ T cells, the NKR-P1B$^+$ NK cells expressed low levels of CD62L, CCR7 and CD27, and had limited proliferative capacity[212]. A plausible explanation, therefore, is that other activating receptors on rat NKR-P1B$^+$ NK cells in this scenario outbalanced the inhibitory effect of NKR-P1B, resulting in the higher cytotoxic efficiency. Another study that introduced genetically engineered chimeric NKR-P1B into primary mouse NK cells resulted in NKR-P1B overexpression and increased sensitivity against inhibitory Clr-b by NK cells[234]. In NS0 cells, NKR-P1B$^+$ NK cells demonstrated reduced cytotoxic efficiency against targets transfected with Clr11, the rat equivalent of Clr-b, than untransfected NS0 cells[100]. These studies support the inhibitory role of NKR-P1B-Clr-b interaction and our finding that Clr-b protects TEC against NK cell-mediated cytotoxicity.

In this study, we also noted that NKR-P1B is not affected by IL-2 stimulation (Figure 8). Similarly in rats, where subsets of NK cells with either high or low NKR-P1B expression were isolated and cultured *in vitro*, cytokine activation could not induce NKR-P1B[100]. However, while NKR-P1B may not be regulated with IL-2 stimulation, we were able to induce its ligand Clr-b with the cytokines IFN-γ and TNF-α in TEC. It may be that NKR-P1B is superfluous in nature, and expression of Clr-b may be the main dictator in this molecular pathway. In support of this hypothesis we found that C57BL/6 to (C57BL/6 × BALB/c)F1 transplantation resulted in Clr-b down-regulation in kidney grafts (Figure 16). Hence NK cell-mediated rejection may be mediated by the down-regulation of Clr-b in kidney grafts, shifting the balance towards NK cell activation. Taken together, we demonstrate that NKR-P1B-Clr-b interaction serves as an important mechanism in regulating NK cell function in kidney tubular cells. The inhibitory NKR-P1B receptor may play a tolerogenic role as an MHC Class I-independent mechanism to prevent NK cell-mediated lysis of healthy tubular cells.

Sequence analyses have determined that the NKR-P1 receptor subtypes consist of either the classical ITAM or ITIM motif[10, 29, 83, 91, 139]. NKR-P1A, NKR-P1C and NKR-P1F contain the ITAM motif and have been proposed to act as stimulatory receptors, while NKR-P1B and NKR-P1D contain ITIM motifs and are inhibitory. Interestingly, studies have suggested that the signalling pathway of these stimulatory receptors involve an interaction between the ITAM motif and the Fc receptor γ-chain (FcRγ) adaptor molecule[9]. In antibody-dependent cell-mediated cytotoxicity (ADCC), specific antibodies recognize and bind to membrane antigens found on target cells. NK cells bind to the Fc region of these antibodies through its FcγIII receptors and release cytokines and cytotoxic granules toward the target cells, triggering apoptosis. It has been revealed that the FcγIII receptors on human NK cells can either mediate lysis against immunoglobulin (Ig)G antibody-coated target cells or induce NK cell inhibition upon interaction with monomeric IgG[221]. Similar to the stimulatory NKR-P1 receptors, FcγIII receptors also signal through intracellular recognition between the FcγIII ITAM motif and the FcγR adaptor molecule. Therefore, it remains possible that these stimulatory NKR-P1 receptors act additively or synergistically with FcγIII receptors to promote NK cell activity.

4.4 Knock down of Clr-b using siRNA technology

RNA interference refers to post-transcriptional gene silencing by degrading or blocking the translation of the target RNA of interest. SiRNAs show great promise in both biological applications and clinical therapy as it has the ability to knock down any gene of interest. In addition to deciphering biological mechanisms at the molecular level, siRNAs can be therapeutic by turning off the responsible genes in many diseases.

However, much work remains to apply siRNA as a therapeutic tool. One of the greatest challenges for achieving RNAi with siRNA is that many cells are difficult to transfect. Another hurdle is the limited duration of post-transfection effects,

typically with gene silencing activities peaking around 24 hours, and diminishing within 48 hours[189]. Target specificity is also of concern, as studies have suggested that non-target genes with sequence homology as short as 9 nucleotides may be affected[101]. In addition to non-specificity, siRNA efficacy must also be considered because different siRNA sequences targeting the same gene of interest have different potencies. Furthermore, as siRNA is quickly degraded by the blood stream, the dose and delivery to the body needs to be optimized. In New Zealand mice, there is spontaneous development of antibodies to double-stranded RNA and DNA, which can be accelerated by the administration of synthetic double-stranded RNA[186]. Therefore, the cell target, synthetic gene sequence, and immune response need to be considered in order to optimize siRNA specificity and efficacy.

As RNAi blocks further protein synthesis, there is still an initial pool of protein prior to gene interference. Hence, another factor that alter siRNA efficacy is the kinetics of protein degradation. Here, we show that while there is significant mRNA knockdown by 24 h, Clr-b protein down-regulation is not visible on the cell surface until 96 h (Figure 12). siRNA efficacy also varies depending on the cell type, where certain cells may have high gene knockdown while others show no knockdown despite efficient transfection. One plausible explanation is the dilution of siRNA within cells due to rapid cell division. For every cell division, the intracellular siRNA is also divided, and cells that multiply will lose their initial pool of siRNA. If cells, such as TEC, undergo rapid cell division, the dose of siRNA within these cells may become insufficient to silence the gene of interest.

While siRNA application poses technical challenges, there are also limitations to other therapeutic tools. Antibodies pose the issues of specificity, efficacy, cost, and their inability to target intracellular proteins[163, 172]. Antibodies have a short circulating half-life and require large quantities for administration[219]. There are also issues with limited penetration and antigen internalization by tumour cells[219]. Knockouts are technically challenging and limited to *in vitro* embryonic manipulations of animal cells. Antisense oligonucleotides are single-stranded

RNA or DNA of approximately 18-30 base pairs in length and complementary to target mRNA, resulting in either the cleavage of the resulting dsRNA by RNase H enzyme activity or blockade of the ribosomal assembly[139]. However, unlike siRNA, antisense oligonucleotides are not amplified by an existing endogenous gene silencing pathway. High intracellular concentrations are required due to its low stability, resulting in higher cost and toxicity, and sufficient time is needed to recognize and bind to the target mRNA[19, 209, 228, 233]. Ribozymes are catalytic RNAs that can perform sequence-specific mRNA cleavage. Ribozymes mediate the hydrolysis of self-phosphodiester bonds and hydrolysis of phosphodiester bonds in other RNAs and can catalyze the aminotransferase activity of the ribosome[29, 87, 160, 203]. Nevertheless, ribozymes require considerable optimization for efficiency and specificity to serve as a therapeutic tool. Finally, while microRNAs serve as powerful endogenous post-transcriptional regulators, they act by binding to complementary sequences on families of mRNA targets, resulting in multiple gene targeting functions[14, 91, 134, 138, 140, 143].

Extensive studies currently exist to optimize siRNA efficacy for clinical applications. Short-hairpin RNA (shRNA) can be used for prolonged and enhanced gene knockdown. Short-hairpin RNA is delivered via viral vectors and translocates to the cell nucleus. It is then exported for DICER to process the shRNA into functional siRNA. The process of converting shRNA into functional siRNA involves an endogenous RNAi machinery that naturally processes miRNA responsible for gene regulation[96]. There are reports, however, implicating shRNA to cause lethal toxicity[154, 158, 159]. In a human hepatitis B virus mouse model, Grimm and colleages studied the effects of 49 shRNAs, distinct in length and sequence, using a viral vector directed toward the liver in mice. They found that 36 of the tested shRNAs caused dose-dependent liver injury, and 23 shRNAs had killed their hosts within two months independent of the viral vector[85]. However, weakly-expressed shRNAs at low doses decreased serum viral DNA without signs of toxicity[85]. Furthermore, toxic shRNA levels were shown to inhibit the microRNA *miR-122* processing in the liver, whereas nontoxic shRNA doses did not[85]. It is possible that shRNA toxicity may be the result of oversaturating the

cellular miRNA processing machinery, preventing vital miRNAs from accessing those proteins. Therefore, shRNA sequence and dose are imperative in controlling endogenous shRNA expression for therapeutic potential.

Nanoparticles are also particularly of interest, as they may be a solution to siRNA's low stability in the bloodstream and provide a method for cell-specific delivery. Biodegradable poly(ester amine), a cationic polymer, conjugated to a kidney targeting peptide has been demonstrated to show good physicochemical properties as a gene delivery carrier, with DNA condensation ability, protection from enzyme degradation, and formation of nanosized complexes with spherical shapes. Its efficacy was demonstrated with the HGF gene in unilateral ureteral obstruction rat models to recover renal functions and treat kidney fibrosis[119]. Nanoparticles, consequently, also have promising potential to overcome current obstacles in siRNA delivery and gene therapy.

4.5 IRI susceptibility and strain-dependent genetic differences

C57BL/6 mice are the most commonly used inbred strain for general purpose and for the generation of congenic mutations. Although this strain is refractory to many tumours, it allows maximal expression of most mutations. BALB/c mice are also among the top 3 most widely used inbred strains. This strain is well-known for the production of plasmacytomas after injection with mineral oil, which is used for generating monoclonal antibodies.

BALB/c and C57BL/6 mice are well established to have different immune responses under physiological and pathological states, due to interstrain differences in their genetic background[146]. When compared to C57BL/6 mice, the BALB/c strain preferentially develops T helper type 2 cells over T helper type 1, is more resistant to autoimmune diseases, and is more susceptible to intracellular parasite infection and tumorigenesis. IFN-γ production by C57BL/6 T lymphocytes activates macrophages to produce NO and initiates inflammation,

while BALB/c T lymphocytes produce more IL-4 that suppresses macrophages[90, 205]. A previous study demonstrated that C57BL/6 macrophages also produce higher levels of TNF-α and IL-12 than those from BALB/c mice after stimulation of TLR-2 or TLR-4[252]. The resulting IL-12 production by C57BL/6 macrophages increases IFN-γ while decreases IL-13 production by $CD4^+$ T cells[252]. Studies have demonstrated that C57BL/6 mice are more susceptible to the induction of autoimmune diseases such as myasthenia gravis[83] and uveitis[13, 36, 222], while BALB/c mice display increased susceptibility to mammary[162, 237] and colon tumours[132]. In addition to immunological differences between mouse strains, there are also variations between the sexes. A Recent study showed that male BALB/c mice infected with *coxsackievirus B3* resulted in severe myocarditis and gave predominantly a T helper cell type 1 immune response. Conversely, females gave predominantly a T helper cell type 2 response. It was further demonstrated that the sex hormones testosterone and estradiol may be involved in the different T helper immune responses[95]. These discrepancies between mouse strains and the sexes offer significant distinctions in their immune responses for scientists to take into account and manipulate.

We have demonstrated that C57BL/6 mice are more susceptible to renal IRI than BALB/c mice (Figure 15), which may be due to not only the preferential dominance of T helper type 2 cells, but also the lack of MHC Class II Eα antigens. The MHC Class II antigens are surface glycoproteins involved in controlling immune responses and known to inhibit NK cell-mediated cytotoxicity[104, 120, 148, 149, 161]. A recent study demonstrated that NK cells can acquire MHC Class II antigens by intercellular membrane transfer, or trogocytosis, with dendritic cells and thereby competitively compete with T helper cells for antigen presentation[168]. C57BL/6 is an $H\text{-}2^b$ haplotype mouse strain that lack the MHC Class II E_α molecules, whereas BALB/c is haplotype $H\text{-}2^d$ and does not[155]. Cells from mouse strains with haplotypes $H\text{-}2^b$, $H\text{-}2^s$, $H\text{-}2^q$, and $H\text{-}2^f$ fail to express one of the antigen complexes, the E complex, on the surface[106]. The presence of the E_α gene's promoter region allows the expression of I-E molecules responsible for the autoimmune inhibitory effect[97, 157]. While E_α mRNA was found

to be 1.25 kb in length, E_α probes detected mRNA 2.8 kb in length from H-2^f mice, and mice of the *q* haplotype have defective RNA processing causing mRNA instability[157]. Mice of haplotypes *b* and *s,* including C57BL/6, were found to bear a deletion in the E_α gene. Thus, NK cell activation by MHC Class II antigens, along with other NK cell signalling mechanisms, may explain the variable susceptibility of renal IRI between mouse strains.

Here, we detected Clr-b on tubular cells regardless of whether it is of the BALB/c or C57BL/6 phenotype. However, strain variability comes into play on NK cells, as NKR-P1B is expressed differently depending on the strain of mouse. In a previous publication, cross-linking of NKR-P1B and NKR-P1C receptors on (C57BL/6 × Sw)F1 NK cells revealed a dominance of NKR-P1B inhibitory response over NKR-P1C stimulatory signalling[32]. Here, we confirm that NK cells from BALB/c mice express NKR-P1B, while C57BL/6 NK cells do not express this inhibitory receptor (Figure 8)[34]. This may provide additional insights for the variable susceptibilities to injury and NK cell-mediated immunological responses of different mouse strains. In our transplant experiment, kidney *clr-b* expression was significantly down-regulated after 60 days (Figure 16), providing a possible mechanism for NK cell-mediated injury in kidney grafts. The expression of inhibitory receptor NKR-P1B and its ligand Clr-b may therefore be one of the mechanisms that protect BALB/c mice against NK cell-mediated renal injury and explains the increased resistance against renal IRI.

4.6 Conclusions

Based on our findings, Clr-b expression on TEC and the kidney is found to be up-regulated after renal injury. As Clr-b-NKR-P1B interaction serves to protect cells from NK cell-mediated cytotoxicity, blockade of Clr-b may enhance TEC death and kidney injury caused by NK cells. We also found that the common pro-inflammatory cytokines TNF-α and IFN-γ, known to be associated with kidney allograft injury[68], induce Clr-b expression in TEC and the kidney (Figures 4 and

6). Cytokine-induced Clr-b expression, therefore, may prevent NK cell-mediated injury in TEC and the kidney. Furthermore, we also found that there were significant variations in NKR-P1 receptor subtype expression between mouse strains (Figure 8). It is possible that NKR-P1 polymorphisms in humans may explain the different susceptibilities of patients against ischemia-reperfusion injury. Up-regulating inhibitory Clr-b in transplant patients thus may protect the kidney from NK cell-mediated cytotoxicity in acute and chronic allograft injury.

4.7 Future directions

We will study whether increased Clr-b expression by gene delivery techniques can inhibit NK cell-mediated acute and chronic kidney injury, which may render a rationale for future clinical therapy to overcome NK cell-mediated lysis. We will examine whether enhancing expression of Clr-b before IRI can prevent kidney cells from NK cell-mediated injury. Clr-b expression will be amplified *in vivo* through Clr-b-coded vectors and renal injury will be measured by serum creatinine. Mice injected with Clr-b-coded vectors will be compared with its control vector with no Clr-b insertion (Origene, Rockville, MD).

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Curriculum Vitae

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EDUCATION & TRAINING

MSc in Pathology, Western University, London, ON 2010 – Present

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RESEARCH EXPERIENCE & PUBLICATIONS

Regulation of NK cell-mediated tubular epithelial cell death and kidney ischemia-reperfusion injury by the NKR-P1B receptor and Clr-b, Western University, London, ON 2010 – Present

In vitro endothelial differentiation of infantile hemangioma-derived CD133-positive stem cells Western University, London, ON 2008 – 2009

Intrinsic regulation of hemangioma involution by platelet-derived growth factor (published in Cell Death and Disease (2012)) Western University, London, ON 2008 – 2009

PRESENTATIONS

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RELATED WORK EXPERIENCE

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