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Inhibition of NK cell-mediated Cytotoxicity by Tubular Epithelial Cell Expression of Clr Proteins

Benjamin B. Fuhrmann
The University of Western Ontario

Supervisor
Jevnikar, Anthony M.
The University of Western Ontario Co-Supervisor
Zhang, Zhu-xu
The University of Western Ontario

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ABSTRACT

Cytotoxic effector cells can target and kill parenchymal cells of the kidney which results in injury and loss of function. Endogenous regulatory systems may exist to attenuate Natural Killer (NK) and other effector cell activation and cytotoxicity in diverse conditions, including ischemia-reperfusion injury associated with kidney transplantation. Understanding these mechanisms will direct new therapeutic strategies. Kidney tubular epithelial cells (TEC), the predominant cell type in kidneys, may negatively regulate NK cell activation by surface expression of C-type lectin-related proteins (Clr). Clr-*b* and -*f* were found to be expressed by wild type (WT) TEC. Clr-*b* was upregulated by TNFα+IFNγ *in vitro*. Elimination of both Clr-*b* and Clr-*f* expression with siRNA resulted in increased NK killing of TEC compared to individual silencing of Clr-*b* or Clr-*f* TEC ($p<0.01$), or WT control TEC ($p<0.001$). NK cells treated *in vitro* with soluble Clr-*b* and Clr-*f* reduced their capacity to kill Clr-*b*/-*f* $^{-/-}$ TEC as compared to untreated NK cells ($p<0.05$). NK cells therefore are regulated by proteins expressed by TEC and thus may represent an important endogenous regulatory system in the kidney to limit organ injury. As no current drugs exist to specifically target NK cells, Clr-*b* and Clr-*f* soluble proteins that bind to NK cells may represent a novel and clinically feasible strategy to protect organs from NK cell-mediated inflammation during ischemia-reperfusion and other kidney injury models.

KEYWORDS

Tubular epithelial cell, Natural Killer cell, Clr-*b*, Clr-*f,* Ischemia Reperfusion Injury

CO-AUTHORSHIP STATEMENT

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ACKNOWLEDGMENTS

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Chapter 1

1   INTRODUCTION

1.1   Challenges in Kidney Transplantation

Chronic Kidney Disease (CKD) has been increasingly recognized as a global health problem (1). It has been noted that patients suffering from CKD are also likely to develop hypertension, and cardiovascular disease with increased mortality (2). In the early stages of CKD, kidney function can be maintained or sometimes improved by commitment to improved diet and medications to control fluid accumulation, blood sugar in diabetes, calcium/phosphate levels, anemia, and blood pressure (3). However, once kidney function decreases to less than 10-15% of normal levels, End Stage Renal Disease (ESRD) exists with increased complications from hypertension, malnutrition, bone disease, and ultimately death without treatment (4). Currently, there are two viable treatment options for ESRD: dialysis and kidney transplantation (5). Many studies have indicated that kidney transplantation is associated with decreased mortality and an increased quality of life in comparison to chronic dialysis treatment in selected patients (5, 6). While highly variable depending on other morbidities, kidney transplant patients can gain 10 to 15 years of life compared to those remaining on dialysis (6). Despite the many benefits of renal transplantation, complications can arise after the procedure that lead to acute rejection or the greatest current challenge: the development of chronic allograft injury. This complex, multifactorial entity is poorly understood and has no defined therapy.

Nonimmunological (antigen-independent) factors that affect the success of a renal transplant include organ viability, drug toxicity, and recipient factors (7). The viability of a donor organ can be affected by many factors including age, prolonged cold ischemic time, donor brain injury, renal mass, delayed graft function, acute tubular necrosis, and whether the donor was living or deceased (7). Additionally, if the transplant recipient has a lipid disorder, diabetes, hypertension, or any kind of circulatory obstructions, the success of the graft may be shortened (7). The immunological (antigen-dependent) factors that can

promote allograft injury are related to antibody (humoral) and cellular immunity, as well as infection (7). Graft injury complications can arise from antibody-mediated rejection (ABMR), particularly if the recipient has been previously sensitized to donor antigens (7). Similarly, cellular immunity arising from direct or indirect allorecognition is influenced by prior antigen exposure, or by failure of immunosuppression to adequately subdue the recipient's immune responses (7). Lastly, despite immunosuppression being a critical element of a successful transplantation, unintended side effects of conventional immunosuppressants can create the opportunity for viral and other infections, particularly by human cytomegalovirus and BK (Bren nan-Krohn) polyomavirus (7).

The primary challenge in kidney transplantation remains to be allograft rejection mediated by the alloimmune response (8). This alloimmune response is multifaceted, and a complete understanding of its many components is required to guide research towards effective therapies. The principle targets of the immune response to allogeneic tissues are highly polymorphic major histocompatibility complex (MHC) molecules or human leukocyte antigens (HLA) as they are termed in humans (9). HLA matching between donor and recipient significantly reduces the risk of graft rejection and failure following solid organ transplantation (9). The alloimmune response involves components of both the innate and adaptive immune system, which are linked during inflammation. In both the graft and surrounding tissues, donor and recipient dendritic cells (DCs) become activated by inflammation and innate responses, and migrate into secondary lymphoid organs to present alloantigens to T cells with cognate T cell receptors (10). This interaction activates signal transduction pathways that trigger increased $CD4^+$ T helper cell expression of molecules like the growth factor Interleukin-2 (IL-2) and its high affinity receptor CD25, resulting in proliferation of $CD8^+$ effector T cells, and other cells (11, 12). Additionally, B cells are activated to generate alloantibodies against donor HLA and non-HLA antigens (10, 13, 14). In a matter of days, a robust alloimmune response consisting of alloantibodies and effector T cells can be initiated against the donor organ. A rare and more rapid form of rejection, termed Hyperacute Rejection, can occur if the recipient possesses unrecognized or undetected pre-formed antibodies against blood group or HLA antigens (15). This pre-sensitization can be facilitated by events such as pregnancy, a prior blood transfusion, or a previous transplant (16). Recipients with previous exposure to donor antigens also have

high affinity alloreactive memory T cells that represent an essential element of allograft rejection and a major barrier to tolerance induction in transplantation (17–20). Memory CD4^{+} T cells are resistant to current immunosuppressive therapies and can provide help in the generation of donor specific antibodies leading to alloantibody-mediated graft injury (21, 22). Direct contact of circulating memory CD8^{+} T cells with donor tissue can trigger reaction to donor MHC class-1, proliferation, and expression of IFNγ (23–26). As long-term kidney transplant survival has not appreciably increased over the past 20 years, it is intuitive that with so many components of the immune system contributing to allograft injury, new insights and knowledge are required to generate new strategies that can be translated into the clinic before we will achieve improved transplant outcomes.

1.2 Acute Allograft Injury and Ischemia Reperfusion Injury

Transplantation is invariably associated with some degree of acute allograft injury initially caused by ischemia reperfusion injury (IRI) (27). Kidney IRI (K-IRI) is a prominent cause of delayed graft function and under severe circumstances can lead to early graft loss via acute renal failure (ARF) (28). Ischemia occurs in the early stages of the surgical procedure when blood vessels in the recipient are clamped, removing oxygenated blood supply to the tissue, and thus creating a hypoxic and nutrient deficient environment (29). Reperfusion injury is the result of restoring blood flow to the resident tissue, and upon reintroduction with oxygen, reactive oxygen species (ROS) are formed (29). Free radical mediated oxidative stress causes an upregulation of pro-inflammatory cytokines and chemokines (30). Chemokines from the CC, CXC, and CX3C chemokines families are early mediators of inflammation in renal IRI (31, 32). These chemokines help to increase vascular permeability and expression of endothelial cell adhesion molecules that mediate leukocyte infiltration and activation (33, 34). Additionally, damage to host cells caused by oxidative stress can trigger activation of both the toll-like receptor (TLR) and Complement arms of the innate immune system (30). Endogenous ligands such as high-mobility box group 1 (HMGB1) released by damaged cells during K-IRI function as ligands for TLR4 receptors (35). Activation of the Complement system can be mediated through the impaired expression of complement regulatory proteins on the surface of tubular epithelial cells

(TEC) (36, 37). Both the Complement system and engagement of TLR4 can induce TEC to produce pro-inflammatory chemokines, macrophage inflammatory protein 2 (MIP-2), and cytokines IL-6 and TNF-α, augmenting immune responses (30).

A histological hallmark of IRI is neutrophil and macrophage infiltration into the graft (38–40). Neutrophils are potent effector cells which cause injury by obstructing renal microvasculature and releasing ROS and proteases. Macrophages secrete cytokines like IFN-γ, recruit neutrophils, and induce programmed cell death of resident tissue cells (40). While the role of NK cells in IRI has not been well defined, a study conducted by Zhang *et al*. has demonstrated the key role of NK cells in acute and chronic allograft kidney injury (41–43). NK cells can directly kill TEC following IRI, and adoptive transfer of NK cells into mice lacking all lymphoid immune cells worsened injury after IRI (41). It has become evident that the adaptive immune system also participates in K-IRI, with crucial contributions originating from CD4+ T cells. CD4+ T cells infiltrate the graft within 4 hours following ischemia but resolve after 24 hours in what has been termed as a "hit-and-run" phenomenon (38, 39, 44). In addition to T cells being recruited early following IRI, resident dendritic cells, CD19+ B cells, CD3- NK1.1+ NK cells, and CD4+ NK1.1+ NKT cells also infiltrate the kidney (39). These cells can further progress inflammation, IRI, and acute allograft injury.

TEC comprise more than 75% of renal parenchymal cells, and their susceptibility to acute injury contributes to the success or failure of kidney transplants, as tubular injury can be a primary cause of nephron loss (45). Basically, eventual graft failure can occur if TEC cell death exceeds the kidney's regenerative capacity. TEC located in the proximal tubules of the cortex are particularly sensitive to IRI (4, 37–39). Proximal TEC are responsible for reabsorption of solutes and water back into the bloodstream, and loss or disruption of these cells can lead to organ dysfunction (49). In acute renal allograft rejection, perforin and granzyme B lytic pathways as well as Fas-FasL interactions mediate TEC death (50). Several studies have identified that perforin and granzyme B pathways participate in CD56+ NK and CD3+ cytotoxic lymphocyte-mediated destruction of tubular cells (51–53). In addition, FasL, expressed by TEC, interacts with Fas to initiate apoptosis of immune cells (54, 55) as well as adjacent TEC, termed "fratricide" (56, 57).

Understanding the extent by which TEC influence the severity of IRI is crucial in developing therapeutic options to reduce acute kidney injury.

1.3 Preventing acute injury and chronic graft rejection

In the modern era, rates of acute allograft rejection have been reduced by the application of potent immunosuppressive drugs. Immunosuppressive drugs are utilized either to deplete lymphocytes, divert lymphocyte traffic, or block lymphocyte activation pathways (10). A combination of T and B cell depleting antibodies, calcineurin inhibitors (CNI) such as tacrolimus, anti-proliferative agents such as azathioprine, mTOR inhibitors such as sirolimus, and glucocorticoids are used in various combinations to suppress the recipient's immune responses (10). Despite these drugs, chronic allograft dysfunction remains the leading cause of late kidney graft loss (58). Chronic allograft injury which was previously termed chronic allograft nephropathy (CAN), is characterized by progressive tubular atrophy, interstitial fibrosis, and damage to the microvascular and glomeruli (7, 59). Chronic graft rejection as a result of CAN and other associated injuries typically occurs 5 to 10 years after transplantation with a slow decay in graft function (59). The incidence of CAN in transplant patients is as high as 60% of grafts 10 years post-transplant (60). Currently, no specific strategies have been implemented to combat the progression and incidence of chronic allograft injury or fibrosis. Chronic allograft injury is generally irreversible (59), and if kidney failure and subsequent graft loss should occur, the patient must either return to dialysis or receive another transplant.

To effectively develop drug strategies to combat chronic allograft rejection, an understanding of how acute injury events such as IRI might contribute to chronic injury or identifying effector cells that are not affected by current drugs is clearly essential. NK cells can directly lyse TECs and are capable of initiating and contributing to K-IRI in the absence of B and T cells (41, 61). Recent studies have demonstrated that NK can trigger chronic allograft vasculopathy, as well as enhance both T cell and antibody-mediated chronic cardiac allograft rejection (43, 62). Though current immunosuppression may influence recipient NK cell cytokine production and phenotype, it is insufficient to alter their

cytotoxic capacity (63). A study by Hoffmann *et al.* demonstrated that, IFN-γ production of NK cells of kidney transplant patients was retained after stimulation with PMA/Ionomycin, and coculture with HLA-class-1 deficient K562 human myelogenous leukemia cells (64). Additionally, degranulation and release of perforin and granzyme A/B was not impaired in kidney transplant recipients compared to healthy individuals (64).

1.4   NK cell Biology and Function

NK cells are bone marrow-derived innate lymphoid cells that develop from a common lymphoid progenitor cell capable of giving rise to all lymphocyte subsets (65). NK cells attempt to induce cytotoxicity in abnormal or foreign cells through the release of perforin and granzyme, and contribute to a full inflammatory immune response by releasing cytokines such as IFN-γ, TNF-α, and granulocyte-macrophage colony stimulating factor (GM-CSF) (66–68). NK cells express an array Type II integral membrane proteins with C-type lectin extracellular domains encoded by the Natural Killer Gene Complex (NKC) (69). These proteins act as receptors that interact with ligands on target cells, resulting either in the stimulation or inhibition of NK cell activity (69). NK cell activation occurs in the absence of inhibition by the binding of MHC class-1 to the inhibitory members of the murine Ly49 receptor family or their human analogues, the killer cell immunoglobulin-like receptors (KIRs). NK cell activation triggered by the absence of MHC class-1 binding to these inhibitory receptors has been termed the "Missing-self Hypothesis" (70). Absence of MHC class-1 is particularly relevant in initiating an immune response against viral infections and cancer, as pathologically transformed and virus-infected cells often downregulate class-1 MHC in an attempt to escape discovery by cells of the adaptive immune system (71, 72). A second method of NK cell activation is termed "Target Interference" where stimulatory signals are present while the major inhibitory signal from MHC class-1 is absent (70). Activation is triggered by stimulatory NK cell receptors (NKCs) such as NKp30, NKp44, and NKp46 that in the presence of MHC class-1 that would not normally be sufficient to cause activation (70). A third means of NK cell activation is termed "Induced Self" which arises when inhibitory signals are outcompeted

by signals from the activating receptor NKG2D (70). The NKG2D receptor present on NK cells interacts with stimulatory ligands Rae-1. MULT-1, and H60 in mice and ULBP and MICA/B in humans (70).

The role NK cells play in solid organ transplantation is of particular interest due to their constitutive and potent cytotoxicity, with a capacity to lyse target cells in the absence of pre-sensitization, as well as ability to distinguish between allogeneic MHC antigens (73). In addition to their direct cytotoxic activity, NK cells produce and release IFN-γ and TNF-α to stimulate a robust innate immune response (74, 75). It has been demonstrated that NK cells infiltrate kidney grafts after ischemia and participate in K-IRI by lysing TEC directly in a perforin-dependent manner (41, 61). NK cells can thus affect transplant outcomes due to their ability to promote alloreactivity responses of T cells as well as by killing antigen presenting cells that might contribute to tolerance (76). Finally, Hirohashi *et al.* demonstrated that NK cells play a necessary role in the development of antibody-mediated chronic rejection in an Fc-dependent and complement-independent manner (62). Evidently, NK cells have a significant effect on transplantation outcomes, and the mechanisms regulating NK function in the kidney should be clarified.

While donor-recipient HLA/MHC matching is important to prevent rejection mediated in-part by NK cells, it is clear that there are many other receptors expressed on the surface of parenchymal cells that can regulate NK cell activation. Within the past 20 years, a complex network of regulatory interactions that exist between NK cells and their targets has become more defined. Central to this regulation appears to be members of the NK receptor NKR-P1 family in mice and KLRB1 family in humans, which interact with C-type lectin-related proteins (Clrs) encoded by the *Clec2* genes on target cells (77–79). Clrs are glycosylated disulfide-linked homodimeric type II transmembrane proteins expressed on the surface of a wide variety of cell types across different tissues and species (78). Several different NKR-P1:Clr interactions have been identified to either promote the stimulation or inhibition of NK cell cytotoxic activity (79).

1.5 Clr Ligands and NKR-P1 Receptors

Like the major histocompatibility complex genes, the genes encoding the natural killer cell receptors possess significant interspecies genomic diversity (80, 81). The Ly49 and KIR innate pattern recognition systems are highly polymorphic and polygenic. The C57BL/6 mouse strain possesses a Ly49 genomic cluster that encodes two functional stimulatory Ly49 receptors (Ly49*d*, *h*) and eight functional inhibitory receptors (Ly49*a*, *c*, *e*, *f*, *g*, *i*, *j*, *q*) (81). On the other hand, the structurally and functional related Nkrp1 gene family possesses a highly conserved genomic organization within individual murine strains. This lack of diversity suggests a resistance to evolutionary diversification (81).

The NK1.1 alloantigen has long been used as marker for NK cell identification and purification (82, 83). Subsequently, the NK1.1 alloantigen was revealed to be a member of the NKR-P1 family of receptors in mice and shown to possess the capacity to produce an activation signal in NK cells (84–87). To date, the *Nkrp1a*, *-b*, *-c*, *d-*, *-e*, *-f*, and *–g* genes have been identified to code for their corresponding NKR-P1 receptor isoform, though studies have suggested that the *Nkrp1-e* is non-functional (79). It has been predicted that NKR-P1A, NKR-P1-C, and NKR-P1F receptors exert stimulatory effects on NK cells, while activation of NKR-P1B, NKR-P1D, and NKR-P1G cause inhibitory effects (77, 88–90). In 2001, Plougastel *et al*. identified a novel set of Clr genes that were subsequently named Clr-*a*, *-b*, *-c*, *d-*, *-e*, *-f*, and *–g*, where Clr-*b* was identical to the osteoclast inhibitory ligand (*Ocil*) and many others were similar to members of the *Ocil* gene family (91, 92).

Several screening studies in mice have identified ligands for different NKR-P1 receptors discovered an interaction between the NKR-P1 family members and the Clr proteins (81). These are very complex interactions. Despite the fact that many physiological functions of Clrs have yet to be discovered, the known mouse receptor:ligand interactions are NKR-P1B/D:Clr-*b*, NKR-P1F:Clr-*c*, *-d*, and *–g*, and NKR-P1G:Clr-*d*, *-f*, -*g* (79). In humans, NKR-P1A (KLRB1/CD161) expressed on NK cells shows inhibitory function upon binding the Clr-related ligand LLT1 (93, 94). Stimulatory cross linking of NKR-P1 receptors like NKR-P1A, -C, and –F results in phosphorylation of the FcRγ immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residues (88). This recruits spleen tyrosine kinases (Syk) and lymphocyte-specific protein tyrosine kinases

(lck), leading activation of NK cytotoxicity through downstream signalling cascades like the NF-κB, NFAT, and MAPK pathways (88). In contrast, the inhibitory receptors NKR-P1B, –D, and -G possess a conserved cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (62, 67). These inhibitory receptors bind Src homology 2 (SH2)-containing protein tyrosine phosphatase-1 (SHP-1) in a phosphorylation-dependent manner, which block downstream signalling cascade activation by inhibiting Syks (77, 89, 96).

1.6   Immuno-regulatory Role of Clr:NKR-P1 System

The different Clr proteins are expressed in a tissue- and organ-restricted manner (97). In the kidney, it has been identified that "inhibitory" Clrs –b and –f are strongly expressed, and "stimulatory" Clrs –c and –g are weakly expressed across different species of mice (97). The broad expression of Clr-b across many tissues and hematopoietic cells, and its inhibitory interaction with NKR-P1B/D has led to the hypothesis that the NKR-P1B/D:Clr-b system may act as a second "Missing-self" mechanism. Like Ly49, NKR-P1B is expressed on approximately 60% of B6 mouse NK cells (97). There is even some evidence to suggest that B6 mouse kidneys may contain resident CD11b$^+$ dendritic cells that express NKRP1B/D (97). In support of the missing-self-like hypothesis, similar to MHC class-1, Clr-b expression is reduced in many tumor cell lines and downregulated in response to genotoxic stress and viral infection (98–101). One study demonstrated a dominant effect of MHC class-1-dependent NK cell inhibition over the inhibition mediated by the NKR-P1B:Clr-b interaction (102). However, it was also demonstrated that hematopoietic cells lacking both MHC class-1 and Clr-b were subjected to enhanced rejection compared to MHC class-1 deficient or Clr-b deficient cells alone (102).

Clr-f has been identified via RT-PCR to be expressed very highly by both the intestinal epithelium and kidney TEC (97). Across B6, BALB/c, and 129S6 mouse strains, Clr-f and NKR-P1G amino acid sequences are highly conserved with a minimum of 99% homology (97). A study was conducted by Leibelt *et al.* that investigated the functional consequences of NKR-P1G ligation with Clr-f (103). It was observed that NKR-P1G$^+$

intestinal intraepithelial lymphocytes (IELs) readily degranulate when co-cultured with CHO cells, while no degranulation was detected by the same IELs when co-cultured with CHO cells ectopically expressing Clr-f (103). Degranulation towards Clr-f^+ CHO cells was restored in the presence of anti-Clr-f blocking mAb 10A8, providing further evidence that ligation of NKR-P1G with Clr-f promotes cytotoxic inhibition (103). It is hypothesized that IELs utilize the NKR-P1G:Clr-f system to survey the state of intestinal epithelial cells, and downregulation of Clr-f may indicate cellular stress, pathogenic challenge, or mucosal injury and stimulate a proinflammatory immune response (103). The constitutive expression of Clr-f by the kidney suggests it may be an important immune modulator for TEC.

NK cells are efficient cytotoxic effectors which are not targeted by current drug strategies in transplantation. Greater insight into the ability of proteins expressed by TEC to regulate NK cells may generate effective therapies for acute and chronic kidney injury.

1.7 Hypothesis

Studies of Clr-b and Clr-f have demonstrated the capacity to engage receptors of the NKR-P1 family and play a protective role by attenuating cytotoxic response by innate lymphoid effector cells. As both ligands are expressed by TEC, expression of Clrs by kidneys and perhaps other organs may represent a potent and complex endogenous regulatory mechanism that protects organs from diverse forms of NK-mediated inflammation and cytotoxicity. We therefore tested the capacity of inhibitory Clr family member expression in regulating TEC susceptibility to NK killing. We hypothesize that the endogenous Clrs expressed by TEC play a critical role in regulating NK cell cytotoxicity. We tested whether the cytotoxic potency of mature NK cells could be attenuated in the presence of TECs expressing Clr-b and –f, as compared to TEC that either express low levels of these Clrs or lack them entirely. By manipulating the cellular expression of Clr-b and Clr-f, or exposing NK cells to soluble versions of these proteins, we believe we can alter the NK cell cytotoxic response against TEC and in K-IRI.

Chapter 2

2   MATERIALS AND METHODS

2.1 Mice

Wild-type (WT) C57BL/6 (B6; H-2^b) mice were purchased from the Charles River Canada Inc (St. Constant, QC). The B6 Clr-$b^{-/-}$ ($Ocil^{-/-}$) mice were generously provided by Dr. Matthew Gillespie (Monash University, Clayton, Australia). All animals were maintained in the animal facility at Western University using approved protocols and procedures.

2.2 Tubular Epithelial Cell (TEC) Culture

TEC were isolated from mouse kidneys after digestion with collagenase (Sigma-Aldrich, Oakville, ON, Canada) for 30 min. Enriched TEC were grown on 0.1% collagen-coated plates (Sigma Aldrich) in complete K1 culture medium (Life Technologies, Burlington, ON, Canada), supplemented with 5% bovine calf serum, hormone mix (5µg/mL of insulin, 1.25ng/mL of prostaglandin E1 (PGE1), 34 pg/mL of triiodothyronine, 5µg/mL of transferrin, 1.73 ng/mL of sodium selenite, and 18ng/mL of hydrocortisone), 25ng/mL of EGF. 100 U/mL penicillin and 100µg/mL streptomycin (Life Technologies). Proximal tubular phenotype of TEC was confirmed by morphology and flow cytometry through staining with anti-CD13, CD26, Cytokeratine, and E-cadherin antibodies. Cells were passaged a maximum of two times with 0.25% trypsin (Life Technologies).

2.3 NK Cell Culture

NK cells were purified from the spleens of WT and Clr-$b^{-/-}$ C57BL/6 mice. Spleens were mechanically processed through a 40 µm cell strainer (BD Biosciences, Mississauga, ON, Canada), and whole splenocytes were labelled with anti-CD3ε magnetic MACS beads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C in 300µL of MACS buffer (PBS, 1% FBS, 0.5% EDTA). Splenocytes were passed through a magnetic MACS bead column to deplete CD3ε$^+$ cells, and the column was washed three times with MACS Buffer. NK cells were then purified from the flow through by CD49b$^+$ selection on MACS beads column

using anti-CD49b magnetic MACS beads (DX5; Miltenyi). NK cells were primed for 7 days in the presence of human IL-2 (Proleukin®, Novartis, QC, Canada) (Day1 - 2000 IU/mL, Day 3 – 1500 IU/mL) in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100µg/mL streptomycin, 2mM glutamine, 1mM sodium pyruvate, 10mM HEPES, and 0.5mM 2-ME. Purity of NK cells was confirmed to be >90% CD49b⁺ NK1.1⁺, CD3⁻.

2.4 Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from tissue or cells using TRIzol reagent or a PureLink™ RNA Mini Kit (Invitrogen, USA). Concentration and purity of the isolated RNA was determined using a GENESYS™ 10S UV-vis spectrophotometer (Thermofisher, Mississauga, ON, Canada). Standardized quantities of cDNA were generated from RNA using Superscript II (Thermofisher, USA). Real-time quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kit (ABM, Vancouver, BC, Canada) and the CFX Connect (BioRad, Hercules, CA, USA) on cDNA. Primers are: β-actin F (CCA GCC TTC CTT CCT GGG TA), β-actin R (CTA GAA CAT TTG CGG TGCA), Clr-*f* F (GAA TAT AGC AAC TTG GTT CTC) Clr-*f* R (GGA TTT ACA ACT ACT GAC AAA C), Clr-*b* F (AGC TCC TCA GCT CTG AGA TGT GTG) Clr-*b* R (AGG GGA GAT GGT TTC CGT GCC TTT). β-actin amplification was used as the endogenous control. The normalized δ threshold cycle values and relative expression levels ($2^{\Delta\Delta Ct}$) were calculated.

2.5 OCIL (Clr-*b*) Mouse Genotype Confirmation

One cubic millimeter of liver tissue from both WT and Clr-*b*⁻/⁻ B6 mice was homogenized in TRIzol reagent (Invitrogen, USA). Total RNA was isolated from the tissue, RNA concentration and purity was determined, and 5µg of RNA was used to synthesize cDNA as described above. PCR was performed on the generated cDNA using the following primers: OCIL F (exon2) (TAG TCC CAC AGG CAG CCC GC), OCIL R (exon4) (TGA CGA CTC TCT GTG CAG GCC A). The following PCR protocol was used: initial denaturation (94˚C for 5 min); 35 cycles (94˚C for 30s, 60˚C for 30s, 72˚C for 45s); final extension (72˚C for 4 min). Gel electrophoresis of PCR products was performed on a 1% agarose gel with 10µL of SYBR Safe stain (Life Technologies) added.

2.6   SiRNA Silencing

Expression of Clr-*b*/-*f* was confirmed in WT and Clr-*b*[-/-] TEC using RT-PCR. Primary WT and Clr-*b*[-/-] B6 TEC were grown to 80% confluency and transfected *in vitro* with Clr-*f* siRNA targeting exon 5 (ThermoFisher, siRNA ID 85821) using Endofectin™ Max Reagent (Genecopoeia, Rockville, MD, USA). Successful silencing was confirmed at 24, 48, and 72 hrs by RT-PCR. Cells were harvested by trypsin at 48 hrs for use in *in vitro* cytotoxicity assays.

2.7 ^{51}Chromium Release Assay

Target cell death was measured in NK:TEC co-cultures using a standard ^{51}Chromium (^{51}Cr) Release Assay. Briefly, untreated or silenced TEC were labelled with [75µCi] ^{51}Cr at 37°C for 1.5 hrs, washed, and then used as targets. IL-2 activated NK cells were prepared and used at indicated effector:target ratios against ^{51}Cr-labelled TEC targets seeded at 10^4 cell/well of a V-bottom microplate in RPMI 1640 supplemented media. In some experiments, 250ng/mL of soluble hGAD65, 80ng/mL of sClr-*b*, 80ng/mL of sClr-*f*, or 80ng/mL of both sClrs was added to wells containing only NK cells thirty minutes prior to co-culture. Following 4 hrs of co-culture at 37°C, the plates were spun down for 5 min at 1500 rpm and 100µL of supernatant was harvested from each well. The release of target cell ^{51}Cr was determined in counts per minute (cpm) by a γ counter. The following formula was used to calculate specific percent cell death of target cells: % Cell Death = [(ER - SR)/(TR – SR)] x 100, where ER (experimental release) is the cpm from wells containing both effector and target cells, SR (spontaneous release) from wells containing targets and culture media, and TR (total release) from wells containing targets and 1% Triton™ X-100 non-ionic surfactant (Sigma Aldrich).

2.8   Western Blot

Following protein purification, the soluble proteins were loaded into a 10% SDS-PAGE Gel (30% Acrylamide, Gel buffer 1.5 ph8.8, 10% SDS, 10% APS, TEMED) and electrophoresis was performed. After electrophoresis, the glass plates were removed from the electrophoresis apparatus, and the gel was incubated in cold Transfer Buffer (10% methanol, 24nM Tris, 194 mM glycine) for 10 minutes to remove detergent. A Bio-Ice

Cooling Unit (Bio-Rad, USA) was filled with water and chilled to -20˚C prior to use. The gel, membrane, filter paper, and fiber pads were soaked for 15 minutes in transfer buffer and stacked in the transfer cassette. The cassette was placed inside the cooling unit that was then filled with transfer buffer. A standard stir bar was added to help maintain even buffer temperature and ion distribution in the cooling unit. The transfer was run for 1.5 hrs at 80V. After the transfer, the membrane was removed and blocked with 5% non-fat dried milk in PBS for 1 hr or overnight at 4˚C on a standard analogue shaker (VWR, Mississauga, ON, Canada).

The membrane was washed (PBS, 0.1% Tween) three times for 10 minutes each on the shaker. The membrane was then incubated with rabbit anti-6X His tag antibody (Abcam, ab137839, Toronto, ON, Canada) in 2.5% non-fat dried milk in PBS for 1 hr at room temperature while shaking. The membrane was washed in the same washing solution three times for 10 minutes each. The membrane was then incubated with the secondary Goat anti-Rabbit HRP antibody (Seracare, Milford, MA, USA) at 1:5000 dilution for 1 hr at room temperature while shaking. The membrane was washed three final times with wash buffer, and was developed to allow for protein visualization with a FluorChem M Imaging System (ProteinSimple, Sane Jose, CA, USA)

2.9   NK Cell Viability Assay

NK cells cultured with IL-2 for 7 days were collected, spun down, re-suspended in RPMI, and 300,000 cells/well were pipetted into wells of a 96-well plate. The final volume of each well was raised to 200µL with RPMI. Soluble Clr proteins (sClr-*f*, sClr-*b*) were added into each well at the desired concentrations. Soluble human GAD65 (hGAD65) was created using the same plant protein expression and purification method, and was used as an irrelevant plant protein control. NK cells were co-cultured with these soluble proteins for 4 hrs before plates were spun down at 1500 RPM 4˚C. The supernatant was removed, and cells were re-suspended in 150µL of Flow Buffer (PBS, 2% FBS, 0.001% NaN$_3$) and 1µL of Propidium Iodide (PI) (BD Pharmingen™, Canada) to stain for loss of plasma membrane integrity. A Cytoflex S (Beckman Coulter, Mississauga, ON, Canada) flow cytometer was used to quantify cell death as defined by PI$^+$ cells.

NK cell viability was also measured kinetically using an IncuCyte® Live-Cell Analysis System (Essen Bioscience Inc, Ann Arbor, MI, USA). Cells were added to a 96-well plate and cultured with sClr and hGAD65 as described above. Each well also contained 100 nM of Sytox™ Green Nucleic Acid Stain (Thermofisher) that is impermeant to live cells. The 96-well plate was placed inside a tray in the IncuCyte®, and fluorescent images of each well was taken periodically at a 4x magnification. The IncuCyte® software was used to count the total number of cells, and the number of green Sytox™ positive cells in each well. The following formula was used to calculate specific percent cell death of NK cells in each well: % Cell Death = [(ER - SR)/(TR – SR)] x 100, where ER (experimental release) is number of Sytox™ positive cells in wells with soluble proteins, SR (spontaneous release) from wells containing untreated NK cells, and TR (total release) from wells containing NK cells and 1% Triton™ X-100 non-ionic surfactant (Sigma Aldrich).

2.10 Statistical Analysis

Experimental values were expressed either as mean + SD. Data was analyzed using the Student's t-test for paired values, and one- and two-way ANOVA with a Bonferonni post-hoc corrections test.

Chapter 3

3   RESULTS

3.1   TEC Clr-*b* expression is increased *in vitro* following stimulation with IFN-γ and TNF-α

Our previous work has demonstrated that NK cells can directly mediate TEC death *in vitro,* and that NK cells are important participants in an *in vivo* model of K-IRI (41). We have also demonstrated TEC apoptosis and increased expression of Fas receptor can be induced by IFN-γ, a pro-inflammatory cytokine secreted by activated NK cells (75, 104). This finding suggests NK cells may indirectly induce TEC death in addition to initiating direct contact-dependent cytotoxicity. To date, the role of Clr-based, MHC-1 independent inhibition of NK cells by TEC has not been investigated.

To investigate the interaction between TEC expressing different levels of inhibitory Clrs and NK cells, we isolated primary TEC and NKs from both B6 mice. Primary NK cells were isolated from the spleens of B6 mice and cultured for at least seven days in the presence of human IL-2. Using a Nikon Eclipse 90i microscope, phase-contrast images of cultured NK cells were taken before and after stimulation with IL-2 (Figure 1A and 1B). IL-2-primed NK cells appeared larger in size with an aberrant morphology compared to untreated NKs (Figure 1A and 1B). Previous work from our lab has demonstrated that culturing NK cells with IL-2 greatly enhances their ability to kill TEC *in vitro* (41). We isolated primary TEC from the kidneys of mice and cultured these cells in full K1$^{+/+}$ media prior to experiments. Our TEC displayed a cobblestone morphology that is consistent previously published TEC morphology analysis (Figure 2A) (105, 106).

We tested whether TEC might attempt to limit NK injury in an inflammatory environment as found in IRI, by increasing surface expression of inhibitory Clr-*b* and Clr-*f*. We treated primary TEC *in vitro* with sublethal amounts of pro-inflammatory cytokines TNFα and IFNγ for 24 hrs. RNA was isolated from both treated and untreated TEC, cDNA was generated, and fold change of expression of Clr-*b* and Clr-*f* was quantified using qPCR.

Interestingly, only expression of Clr-*b* was significantly increased following treatment with proinflammatory cytokines, while Clr-*f* did not display a consistent increase in expression (Figure 2B). Previous unpublished work from our lab has also demonstrated that kidney mRNA and protein expression of Clr-*b* increases in a time-dependent manner in an *in vivo* model of IRI. Thus, we believe that TEC increase expression of Clr-*b* in an attempt to protect themselves from NK-mediated cytotoxicity.

A.

B.
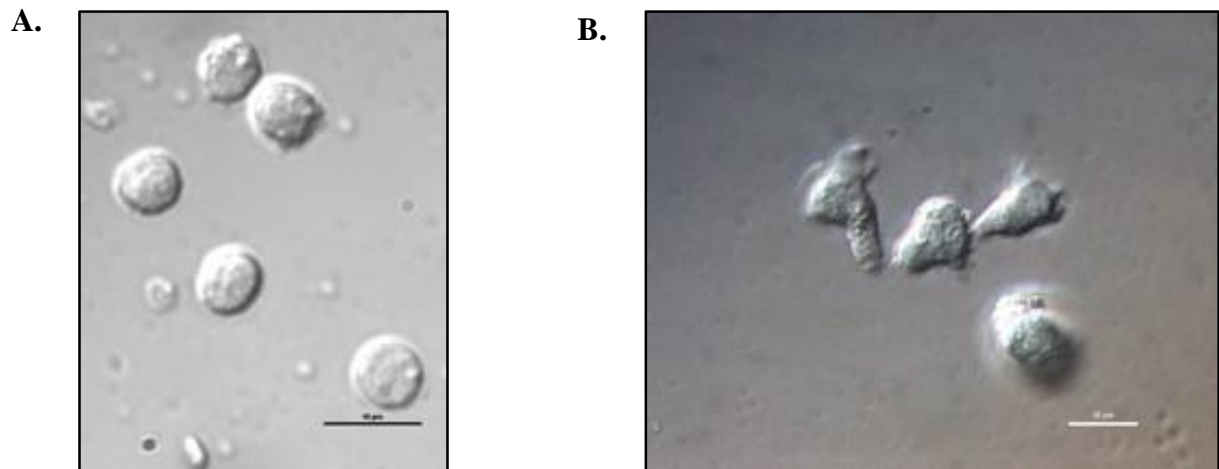
Figure 1. NK cell before and after IL-2 priming.

Splenocytes labeled with biotin-conjugated CD3ε primary antibodies and an anti-biotin magnetic MACS bead were passed through a magnetic MACS bead column to deplete $CD3\varepsilon^+$ cells. NK cells were then purified from the flow through by $CD49b^+$ selection on MACS beads column using anti-CD49b magnetic MACS beads. NK cells were cultured with human IL-2 for 7 days (Day1 - 2000 IU/mL, Day 3 – 1500 IU/mL). Images were captured at a 100X magnification **(A)** before treatment with IL-2 and **(B)** after 7 days of treatment. NK cells show a typical change in shape with activation.
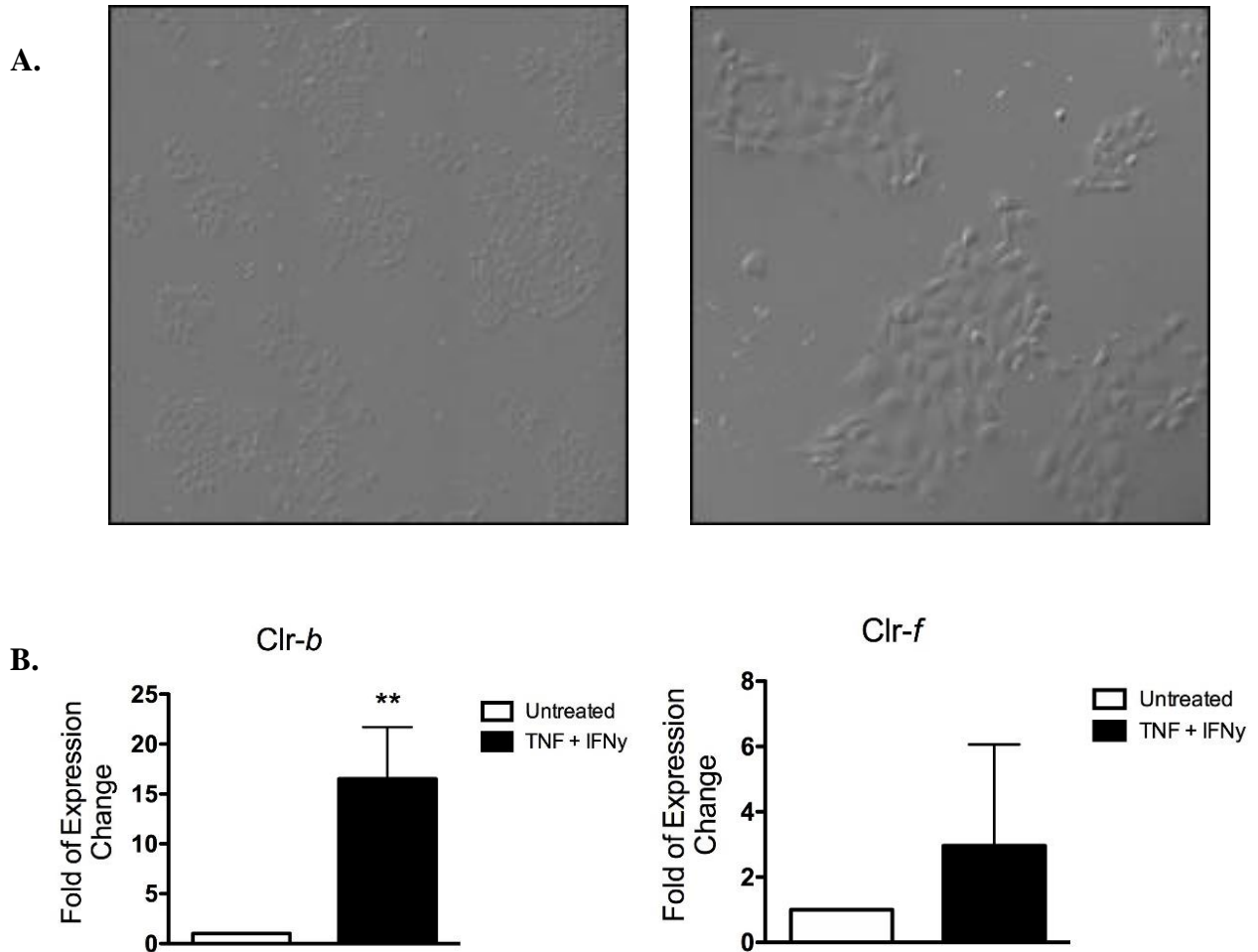
Figure 2. TEC Clr-*b* expression is increased *in vitro* following stimulation with IFNγ and TNFα.

(**A**) Tubular epithelial cells (TEC) were isolated from mouse kidneys after digestion with collagenase. A stitched image (left) and single image (right) were captured at 20X magnification using differential Interference microscopy (DIC). (**B**) Primary TEC were treated *in vitro* for 24 hrs with 25ng/mL of IFNγ and 25ng/mL of TNFα in full K1$^{+/+}$ media. RNA was isolated from cultured cells and cDNA was generated. Relative fold change of Clr-*b* and Clr-*f* mRNA expression by primary TEC before and after cytokine treatment was determined by qPCR. Data is shown as Mean + SD (Clr-*b;* n=4, Clr-*f* n=6). **p<0.01, Student's t-test for paired values.

3.2 Inhibition of Clr-*b* and Clr-*f* expression in C57BL/6 primary TEC

To study the possible inhibitory effects of Clr-*b* and Clr-*f* expressed by TEC, we sought to observe any changes in the magnitude of NK cell-mediated killing in against TEC deficient in these proteins. Dr. Matthew Gillespie from the University of Monash, Australia generously provided our group with Clr-*b*$^{-/-}$ (*Ocil*$^{-/-}$) B6 knockout mice. The Clr-*b*$^{-/-}$ B6 mice have a targeted deletion of exon-3 in the *Clec2d* gene, resulting in a frameshifted transcript, and a non-functional ectodomain (107). Both WT and Clr-*b*$^{-/-}$ transcripts were detected using PCR, however the mutated transcripts were smaller than WT, confirming the deletion of exon-3 and the genotype of our Clr-*b*$^{-/-}$ mice (Figure 3A).

To inhibit expression of Clr-*f*, we utilized siRNA to silence Clr-*f* mRNA transcripts in both WT and Clr-*b*$^{-/-}$ B6 primary TEC *in vitro*. Using a GFP fluorescent siRNA and live-cell imaging analysis, we confirmed a high efficiency of transfection in cultured primary TEC, indicating siRNA would be suitable method for manipulating protein expression of Clr-*f* (Figure 3B). We designed siRNA for Clr–*f* that targeted exon-5 of the mRNA transcript and created primers to detect decrease in transcript via qPCR. A visualization of this design was created using SnapGene (Figure 3C). Using both vehicle and scrambled siRNA controls, we successfully silenced Clr-*f* transcripts at 24, 48, and 72 hrs (Figure 3D). Despite extensive searching, no functional anti-mouse Clr-*f* antibody for the purposes of confirming a decrease in protein levels of Clr-*f* via western blot or flow cytometry could be procured.

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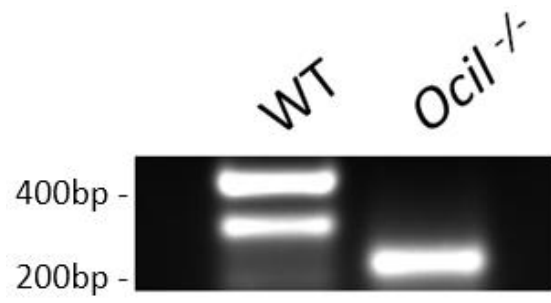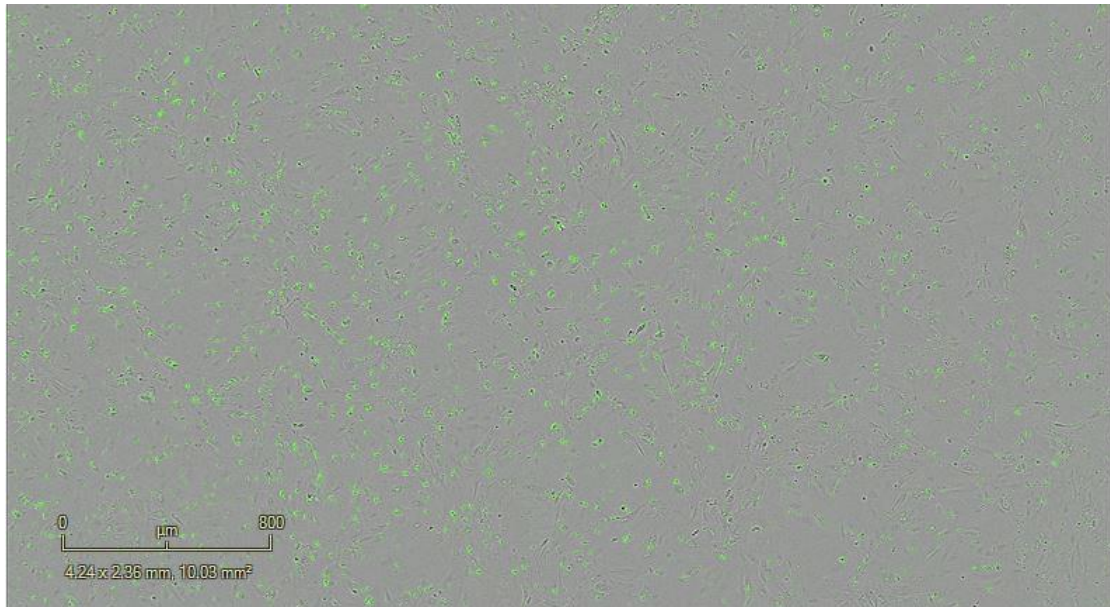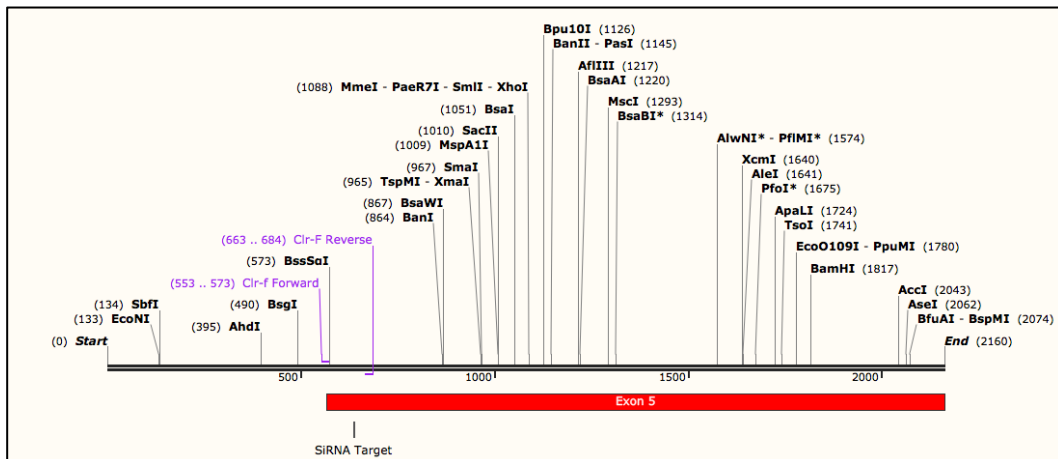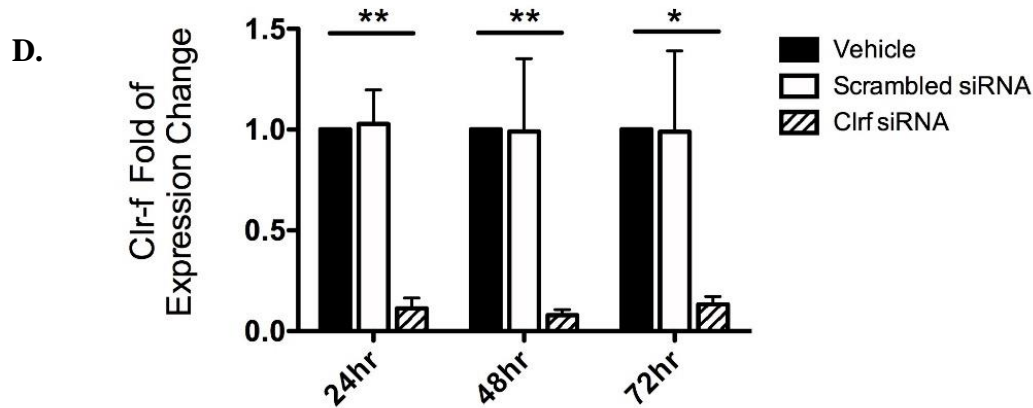
Figure 3. Inhibition of Clr-*b* and Clr-*f* expression in C57BL/6 primary TEC.

TEC were isolated from the kidney of WT B6 mice and transfected with siRNA. **(A)** PCR analysis of Clr-*b* transcript expression using primers specific for exons-2 through -4 for the coding sequence of Clr-*b*. The smaller band observed in the Clr-*b*$^{-/-}$ (*Ocil*$^{-/-}$) mice is indicative of deletion of exon-3 of the Clr-*b* transcript, rendering the ectodomain of the protein non-functional. **(B)** A scrambled fluorescent siRNA control was used to confirm transfection efficiency. Image was taken using an IncuCyte® Live-Cell Imager at 4x magnification. **(C)** Visual representation of Clr-*f* siRNA that targets exon-5 and the primers used in qPCR to confirm transcript silence. **(D)** TEC were transfected with siRNA that targets the transcripts for Clr-*f*. RNA was isolated using Trizol at different time points, converted into cDNA and qPCR was performed to compare the relative fold of expression change compared to a vehicle control at each time point. Data at each time point were shown as mean + SD (n=4). *p<0.05, **p<0.01, 2-way ANOVA.

3.3 Inhibition of both Clr-*b* and Clr-*f*, but neither protein individually, significantly increases NK cell-mediated TEC killing.

NK cell-mediated TEC death was measured at three different NK:TEC ratios in co-culture using a standard ^{51}Chromium (^{51}Cr) Release Assay as described in *Materials and Methods*. Forty-eight hours prior to co-culture with NKs, primary TEC from WT and Clr-$b^{-/-}$ kidneys were either silenced with Clr-*f* siRNA or treated with transfection reagent alone as a vehicle control. All cells were isolated from a B6 background to control for the primary NK cell inhibitory molecule MHC class-1. The inhibition of either Clr-*b* or Clr-*f* expression on TEC did not significantly increase NK cell-mediated killing (Figure 4A). However, at a ratio of 30:1 NK:TEC, the simultaneous absence of both Clr-*b* and Clr-*f* expression resulted in significantly increased NK killing of TEC compared to the Clr-$b^{-/-}$, Clr-*f* silenced, or WT control TEC groups (Figure 4A).

To further demonstrate that absence of the signal from both Clr-*b* and Clr-*f* is sufficient to illicit the observed increase in NK cell-mediated killing, we co-cultured TEC deficient in one or both inhibitory Clrs with NK cells from either WT or Clr-$b^{-/-}$ mice. As NK cells from Clr-$b^{-/-}$ B6 mice have not developed in the presence of Clr-*b*, we hypothesized that these NK cells would not possess fully competent NKR-P1B receptors. At a ratio of 30:1 NK:TEC, there is no significant difference in Clr-$b^{-/-}$ TEC death between WT and Clr-$b^{-/-}$ NK cell groups (Figure 4B). This result was consistent with our previous observation there appears to be no significant difference in NK cell-mediated killing against TEC when Clr-*b* alone is absent compared to WT TEC (Figure 4A). However, Clr-$b/f^{-/-}$ TEC were killed significantly more when co-cultured with WT NK cells compared to the same TEC co-cultured with NK cells from Clr-$b^{-/-}$ mice (Figure 4B).

It is our hypothesis that NKR-P1B on Clr-$b^{-/-}$ NK cells are functionally tolerant to Clr-$b^{-/-}$ targets, and therefore cannot sense the absence of Clr-*b* on Clr-$b/f^{-/-}$ TEC. As no loss of inhibitory signal is detected, the NK cells do no initiate increased cytotoxicity against the Clr-$b/f^{-/-}$ TEC. Instead, we report a decrease in the magnitude of TEC death that is more consistent with groups lacking a single inhibitory Clr protein, like the Clr-*f* silence group from Figure 4A. We conclude that inhibition of both Clr-*b* and Clr-*f* protein

expression, but neither protein individually, is sufficient to significantly increases NK cell-mediated TEC killing.

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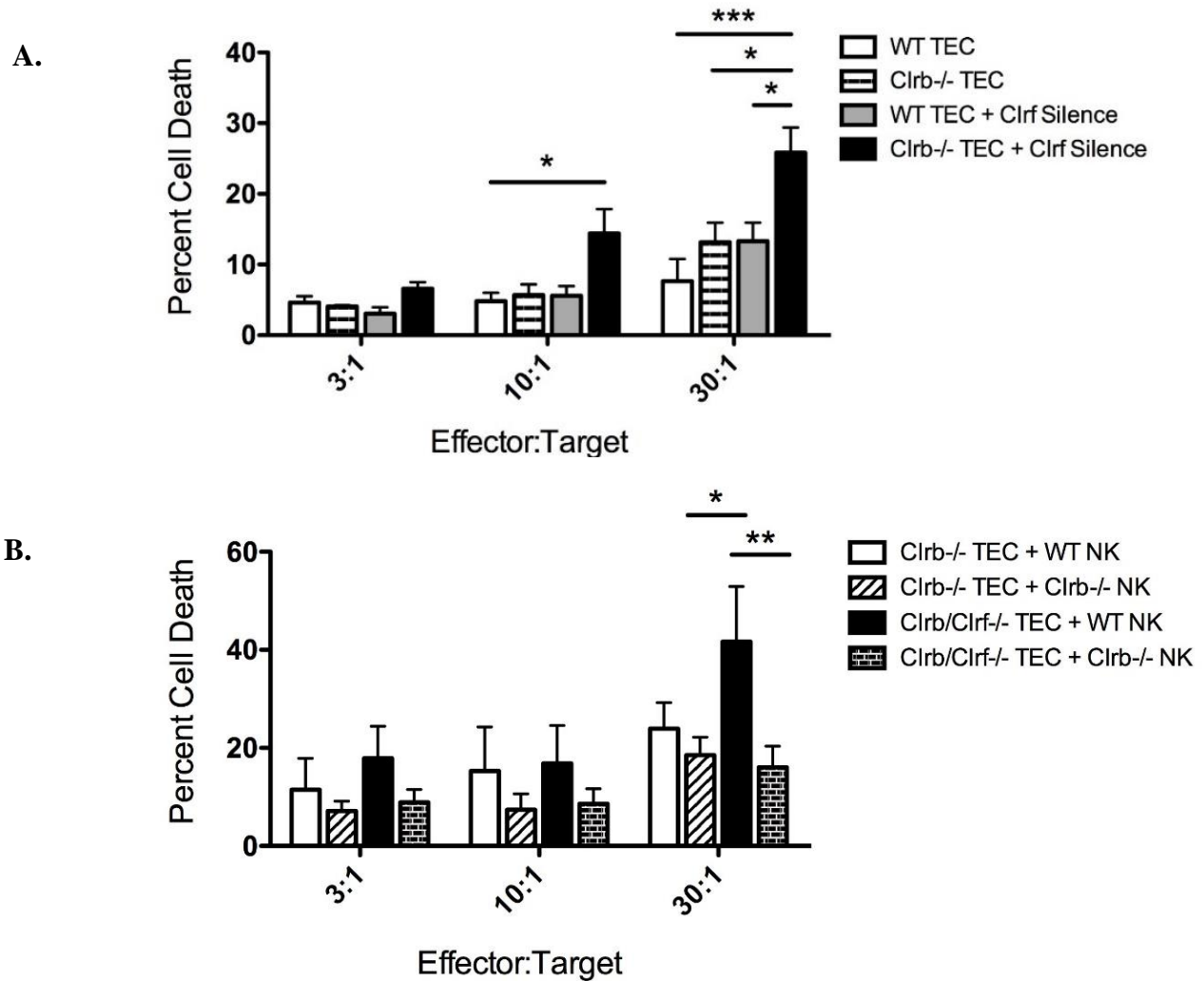
Figure 4. Elimination of both Clr-*b* and Clr-*f*, but neither protein individually, significantly increases NK cell-mediated TEC killing.

(A) NK cell-induced cell death was compared between TEC expressing different levels of Clr-*b* and Clr-*f*. SiRNA-mediated Clr-*f* silencing was performed 48 hrs prior to the cell death assay. TEC were labelled with ^{51}Cr and co-cultured for 4 hrs at 37˚C, 5% CO_2. Free ^{51}Cr in 100µL of supernatant from each well was counted and percent death was calculated using spontaneous and total ^{51}Cr release controls. Each group was plated in duplicate for each ratio. Values are presented as mean + SD (n=3-4), *p<0.05, ***p<0.001, 2-way ANOVA, Bonferonni corrections test. **(B)** ^{51}Cr labelled TEC deficient in one or both inhibitory Clrs were co-cultured for 4 hrs (37˚C, 5% CO_2) with NK cells from either WT

or Clr-$b^{-/-}$ mice. Percent cell death was calculated as described above. Each group was plated in duplicate for each ratio. Values are presented as mean + SD (n=5-6), *p<0.05, **p<0.01, 2-way ANOVA, Bonferonni corrections test.

3.4 A combination of soluble Clr-*b* and Clr-*f* reduces NK-mediated killing of TEC *in vitro*

As NK cells are not explicitly targeted with immunosuppressants in kidney transplantation and IRI, we tested whether soluble versions of Clr-*b* and Clr-*f* could reduce injury by an effect on NK cytotoxicity. Using sequence information from Uniprot (Clr-*b*: *Entry* #Q91V08; Clr-*f*: Entry #Q8C1T8), we expressed soluble forms of 6X His-tagged Clr-*b* (sClr-*b*) and Clr-*f* (sClr-*f*) ectopic domains in tobacco plants, a system which allows transient and high efficiency production of proteins of interest, that is free of endotoxin. Proteins were extracted and purified from leaf mass. We confirmed expected size of our sClrs using a Western blot: ~20kD and ~18kD for sClr-*b* and sClr-*f* respectively (Figure 5A and B). This work was done in collaboration with Dr. Shengwu Ma of Lawson Health Research Institute.

GAD65 is a membrane associated enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2 (108). Human GAD65 would not be expected to interact with murine NK cells, thus serving as a useful control. Human recombinant GAD65 (hGAD65) was generated with the same method as sClr-*b* and sClr-*f*, and used as a soluble protein control in assays. NK cells were cultured in a wide range of concentrations of soluble Clr proteins to define concentrations that could contribute to any direct effect on NK viability. This was essential to exclude sClr-induced NK cell death as a confounding variable in our assessment of the capacity of sClr-*b* and sClr-*f* to protect TEC from NK-mediated cytotoxicity. Percent NK cell death after 4 hrs of culture was quantified by flow cytometry and defined as propidium iodide positive cells (Figure 5C), and by live-cell imaging (Figure 5D). Compared to PBS vehicle controls, 80ng/mL of each sClr-*b* and sClr-*f* did not induce NK cell death (Figure 5C). However, compared to vehicle and hGAD65 controls, much higher (240ng/mL and 400ng/mL) concentrations of soluble sClr could reduce NK viability (by 16% and 21% of NK cells respectively) (Figure 5C). Therefore, all assays were conducted using 80ng/mL to treat NK cells in co-culture experiments.

To investigate if soluble Clr proteins possessed any capacity to inhibit NK cells, we treated NK cells with Clr proteins and co-cultured these cells with either WT or Clr-*b*/*f* deficient TEC. Thirty minutes prior to co-culture, hGAD65, sClr-*b*, sClr-*f*, or both sClrs

were added to wells containing only NK cells. TEC were labelled with ^{51}Cr and co-cultured with treated or untreated NK cells for 4 hrs. Interestingly, only NK cells treated with both soluble Clrs killed significantly less Clr-*b*/-*f* $^{-/-}$ TEC than untreated NK cells (Figure 5D). As expected, hGAD65 soluble protein control did not significantly affect NK cell-mediated TEC killing (Figure 5D). Notably, our results suggest that treatment with sClr-*f* alone might attenuate NK cell activation, however this trend did not achieve statistical significance (Figure 5E).

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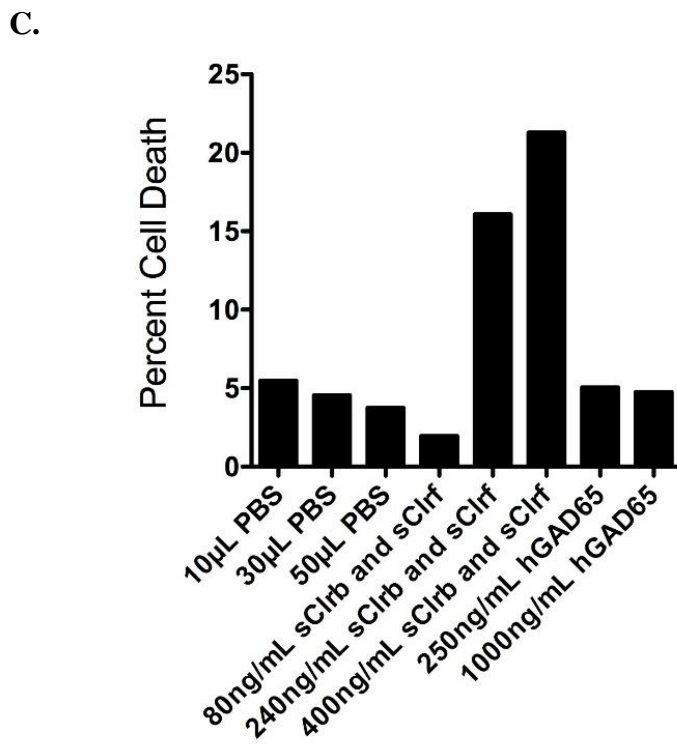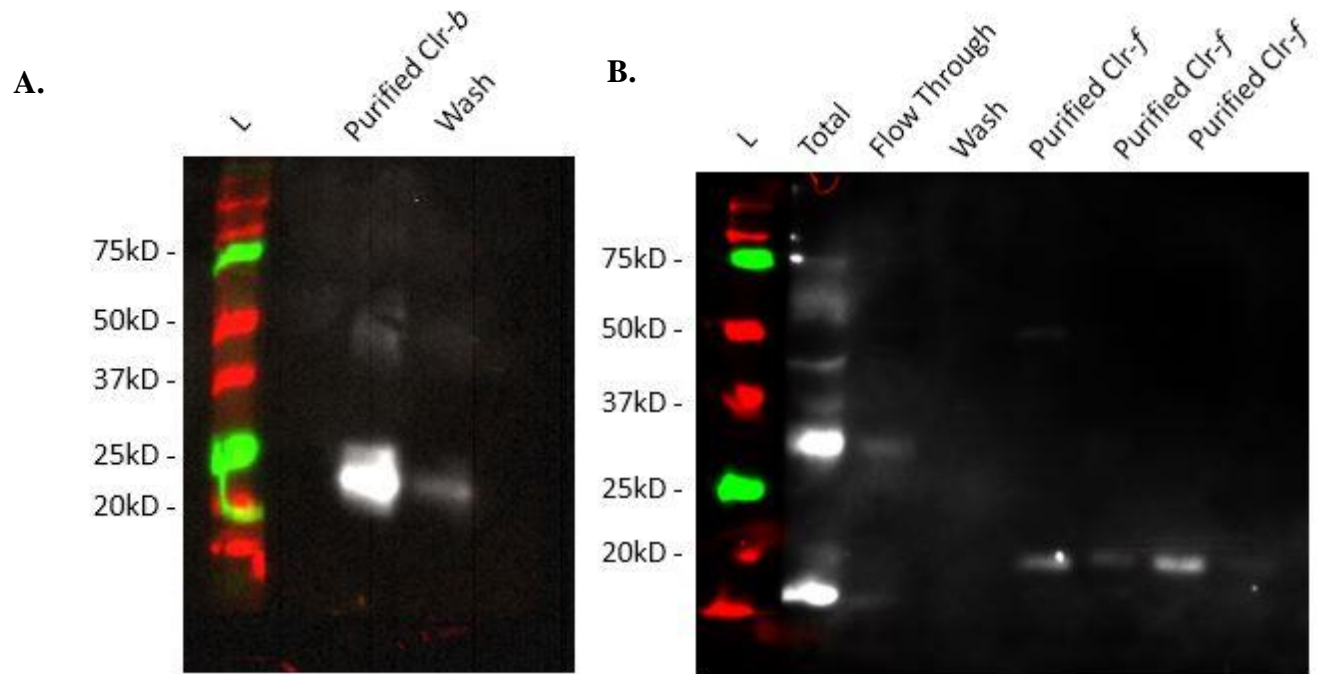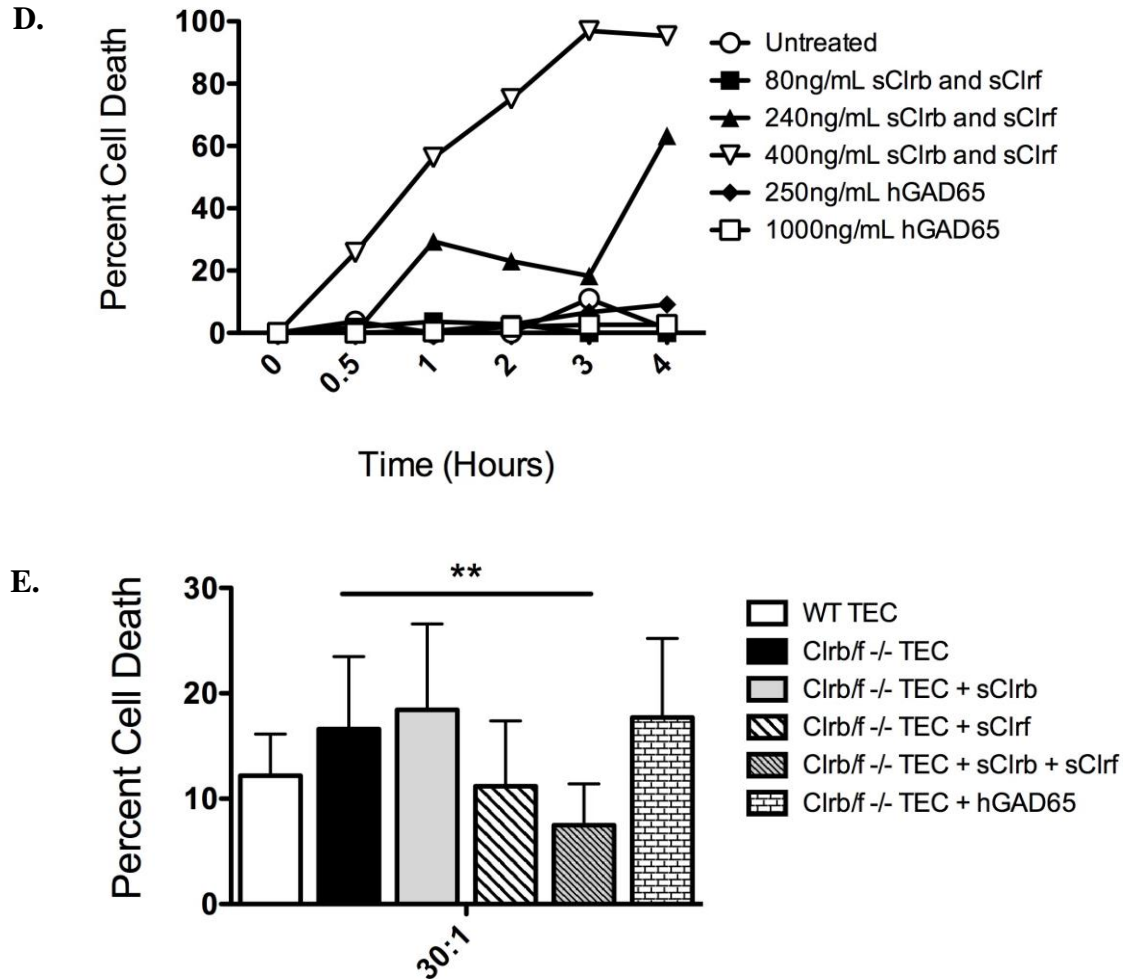
Figure 5. A combination of soluble Clr-*b* and Clr-*f* reduces NK-mediated killing of TEC *in vitro*.

Soluble forms of 6X His-tagged **(A)** Clr-*b* (sClr-*b*) and **(B)** Clr-*f* (sClr-*f*) were generated. Purification was performed on a Nickel affinity chromatography column, and molecular weight was confirmed via western blot. NK cell viability was tested after 4 hrs of culture with selected concentrations of our soluble proteins. hGAD65, a protein not predicted to interact with NK cells, was generated and purified with the same method as the sClrs, and was used as a soluble protein control for our system. **(C)** Percent cell death was quantified by flow cytometry and defined as propidium iodide positive NK cells (n=1). **(D)** NK cell viability was also measured kinetically over a 4 hr time period using an IncuCyte® live

cell fluorescent imaging machine. Data is shown as mean of wells plated in triplicate for a single experiment. **(E)** To investigate if our soluble proteins possessed the capacity to inhibit NK cells, we treated NK cells with our proteins and co-cultured these cells with either WT or Clr-*b/f* deficient TEC. Thirty minutes prior to co-culture, 250ng/mL of soluble hGAD65, 80ng/mL of sClr-*b*, 80ng/mL of sClr-*f*, or 80ng/mL of both sClrs was added to wells containing only NK cells. TEC were labelled with ^{51}Cr and co-cultured with treated or untreated NK cells for 4 hrs at 37˚C, 5% CO_2. Each group was plated in triplicate for each ratio. Values are presented as mean + SD (n=5-6), *p<0.05, 1-way ANOVA.

Chapter 4

4   DISCUSSION

4.1   Regulation of NKR-P1:Clr Recognition System during IRI

The augmented killing of TEC *in vitro* following loss of Clr-*b* and Clr-*f* strongly supports that NKR-P1:Clr binding is an important inhibitory pathway in NK-mediated kidney injury. A central question remains: given that NK cells have a wide spectrum of receptors with which they interact, what purpose does Clr expression serve by targeted cells *in vivo*? It has been demonstrated that Clr-*b* exhibits broad expression across many tissues and hematopoietic cells (97). As Clr-*f* exhibits a highly specific tissue expression pattern restricted to the kidney, liver, and intestinal tract in B6 mice, it is likely that Clr-*f* serves a non-redundant function to Clr-*b*. We treated TEC *in vitro* with IFN-γ and TNF-α (Figure 2B), two potent pro-inflammatory cytokines that are both expressed in large quantities in the kidney during IRI. While Clr-*b* was significantly upregulated by this treatment, it remains unknown whether this represents an endogenous mechanism by which TEC decrease their susceptibility to NK cell cytotoxicity or a general upregulation of surface receptors found during inflammation. Unlike Clr-*b*, "inhibitory" Clr-*f* was not consistently upregulated by pro-inflammatory cytokine treatment in TEC (Figure 2B). While Clr-*f* may not be induced by IFNγ in TEC, previous studies have provided insight into the regulation of Clr-*f*. Steinle *et al.* have reported enhanced Clr-*f* expression on the surface of BALB/c mouse intestinal epithelial cells (IECs) *ex vivo* sixteen hours following intraperitoneal injection with poly(I:C), a synthetic analogue of double-stranded RNA (103). Interestingly, the challenge with poly(I:C) also corresponded with a decreased frequency of NKR-P1G[+] intraepithelial lymphocytes (IELs) and NKR-P1G protein expression on IELs (103). It is plausible that IELs and NK cells downregulate expression of NKR-P1G in the presence of poly(I:C) to decrease their ability to respond to inhibitory signals from Clr-*f*, so that they may more efficiently clear viral infection. Poly(I:C) is a ligand for toll-like receptor 3 (TLR3) which is expressed by splenic murine and human NK

cells (109). Upon simultaneous culture with poly(I:C) and suboptimal doses of IL-12, human NK cells become capable of producing IFN-γ and TNF-α, express high levels of activating markers CD69 and CD25, and acquire cytolytic activity against immature dendritic cells (iDCs) (110). Given that expression of activating markers is increased following TLR3 stimulation, a simultaneous decrease in inhibitory receptors is consistent with enhanced viral clearance.

TLR3 stimulation during IRI may decrease expression of NKR-P1G on infiltrating NK cells, while simultaneously promoting NK cell production of IFN-γ and TNF-α. While our data seems to indicate that the absence of inhibitory Clr-*f*:NKR-P1G signalling alone is insufficient to significantly increase NK killing of TEC *in vitro* (Figure 4A), the inflammatory *in vivo* environment of kidney IRI may tip the scales in favor of NK cells initiating direct cytotoxicity against TEC. Interestingly, TLR3-mediated induction of NK cell IFN-γ release would increase the expression of Clr-*b* by TEC. We have previously demonstrated that kidney Clr-*b* is increased in a time-dependent manner following an *in vivo* model of IRI (111). NK cells may therefore initiate direct toxicity against TEC during IRI due to stimulation by NKG2D ligands like Rae-1 and absence of Clr-*f*:NKR-P1G-mediated inhibition, despite sufficient levels of Clr-*b* expression. Thus, restoring the inhibitory signal from Clr-*f* by preventing possible TLR3-mediated downregulation of NKR-P1G may prove to be an effective therapy for reducing acute kidney from NK cell activation.

4.2 NKR-P1B/G signaling during engagement with TEC Clr-*b*/-*f*

To study the possible inhibitory effects of Clr-*b* and Clr-*f* expressed by TEC on NK function, we assessed NK cell-mediated killing against TEC deficient in these proteins. Elimination of either Clr-*b* or Clr-*f* expression on TEC did not significantly increase NK cell-mediated killing (Figure 4A). However, combined loss of both Clr-*b* and Clr-*f* expression resulted in significantly increased NK killing of TEC (Figure 4A). The mechanism by which loss of Clr expression enhances NK killing has not been elucidated.

One possibility is that increased killing of TEC occurs at the level of the NK:TEC lytic synapse. Following cell-to-cell contact, firm adhesion is mediated by the integrin family of adhesion molecules, and inhibitory/stimulatory NK receptors cluster at the synapse (112). Whether an NK cell proceeds to actin reorganization and cytotoxic degranulation seems to depend on the presence or absence of sufficient inhibitory signalling from the target cell (113). Our data suggest that elimination of both Clr-*b* and Clr-*f*, but neither protein individually, is sufficient to override inhibition from MHC class-1, and induce significant direct NK activation against TEC.

TEC deficient in both Clr-*b* and Clr-*f* were more susceptible to death when co-cultured with WT NK cells compared with NK cells from Clr-*b*$^{-/-}$ mice (Figure 4B). As NK cells from Clr-*b*$^{-/-}$ B6 mice have not developed in the presence of Clr-*b*, we considered whether this was an impairment related to NKR-P1B not being a fully competent receptor on Clr-*b*$^{-/-}$ NK cells. The concept of NK receptor education and NK cell licensing has support other models of NK activation. For example, NK cells from MHC class-1-deficient (β_2M$^{-/-}$) mice demonstrate poor killing of MHC class-1-deficient targets even though are unchanged in number, tissue distribution, and expression of activating receptors (114, 115). WT B6 NK cells readily produced IFNγ upon crosslinking of the activating NK1.1 receptor, however NK cells derived from β_2M$^{-/-}$ mice produce almost no IFNγ following NK1.1 stimulation (116). This observation seems to indicate NK cells from β_2M$^{-/-}$ mice are functionally defective, and that education through MHC class-1 is required to achieve the functional competence of NK cells (117). NK cells developed in an irradiated chimeric B6 β_2M$^{-/-}$ mouse model reconstituted with WT B6 bone marrow (BM) cells were compromised in their ability to subsequently reject β_2M$^{-/-}$ BM (118). Conversely, such "unlicensed" mature MHC class-1-deficient splenic NK cells show a gain of function and acquire a licensed phenotype after adoptive transfer in wild type hosts (119). These cells produce IFNγ at the same level as WT NK cells, and gain equivalent ability to degranulate as indicated by CD107a staining (119). Clearly, MHC class-1 engagement during NK cell development is necessary to produce functional NK cells. As NKR-P1B serves a similar function to receptors that recognize MHC class-1, it is similarly possible that interaction with Clr-*b*, possibly during development, is a requisite for fully competent NK cells. Such data also highlights the complex biology of NK cells and that interaction with Clr proteins

at sites distant to the eventual target tissue can have a profound effect on NK function.

Interestingly, NK cells that do not express functional inhibitory receptors for MHC class-1 are not autoreactive but rather are hyporesponsive against $\beta_2M^{-/-}$ targets (120). Work by Chen *et al.* has demonstrated that while NK cells from Clr-*b*$^{-/-}$ mice are hyporesponsive to NK1.1 stimulation and IL-12/IL-18 cytokine priming, the inhibitory capacity of the NKR-P1B receptor remains intact (121). We observed that combined Clr-*b/-f*-deficient TEC were killed significantly more when co-cultured with WT NK cells as compared with NK cells from Clr-*b*$^{-/-}$ mice (Figure 4B). Despite NKR-P1B on Clr-*b*$^{-/-}$ NK cells possessing a capacity to be inhibited by the Clr-*b* protein, it appears that these NKs are "tolerant" to Clr-*b*$^{-/-}$ targets. Clr-*b*$^{-/-}$ NK cells do no initiate increased cytotoxicity against TEC lacking Clr-*b/-f* expression, highlighting that change in signalling from a single inhibitory Clr protein is not sufficient to affect NK cell activation against TEC. Our findings provide further evidence that the NKR-P1B receptor must interact with its cognate ligand Clr-*b* at some point, in order to efficiently sense a decrease of Clr-*b* expression, and then augment NK cell-mediated killing of target cells.

4.3   Soluble Clr-*b*/-*f* as a new K-IRI therapeutic

Following our demonstration of enhanced NK killing of TEC in the absence of surface expression of Clr-*b* and -*f in vitro*, we sought to determine whether our understanding of NK regulation by TEC via Clr proteins could be exploited to decrease TEC death during IRI. As the different forms of Clr proteins are highly similar, antibodies meant to bind inhibitory NKR-P1 receptors *in vivo* may induce undesirable agonist effects by binding to and stimulating NKR receptors. Alternatively, genetic alteration to increase the expression of Clr-*b*/-*f* in kidneys could induce NK cell hyporesponsiveness and loss of viral clearance. It was demonstrated that sustained transgenic expression of NKG2D ligands Rae-1 in mice results in reduced expression of NKG2D and impairment of NKG2D-mediated NK cell activation (122). In the same manner, systemic transgenic overexpression of Clr-*b*/-*f* may cause a downregulation of NKR-P1B/G receptors *in vivo*. Therefore, we created soluble forms of the ectopic domains of Clr-*b* and Clr-*f*. Drug

delivery of additional Clr-*b* and Clr-*f* molecules may clinically feasible to promote the inhibitory potential of these molecules against NK cells. The ability of such soluble Clr proteins to inhibit NK cells may be related to perturbation of the NK-TEC synapse, or by inhibition of NK cell activation capacity even prior to engagement with target cells. This remains for future studies.

We repeated NK:TEC co-culture experiment comparing the cytotoxicity of sClr-*b*/-*f* treated and untreated NK cells. Of note, the concentrations of Clr proteins added to assays were approximated as no standards are currently available for precise determinations. Thus, we used total protein levels obtained in affinity purified eluates. While sClr-*b* and sClr-*f* appear to be toxic to NK cells when combined at very high concentrations of each Clr (240 and 400ng/mL of each) (Figure 5C), concentrations of 80ng/mL did not induce NK cell death as compared to vehicle and soluble protein controls. Similarly, in a previous experiment by Zhou *et al.*, a recombinant murine OCIL (Clr-*b*) protein significantly inhibited osteoclast formation *in vitro* at a concentration of 50ng/mL (123). Our results provide guidance into concentrations that might be required for effective *in vitro* treatment with soluble Clrs. NK cells treated with a combination of sClr-*b* and sClr-*f* killed significantly less Clr-*b*/-*f* $^{-/-}$ TEC than untreated NK cells (Figure 5D). Interestingly, treatment with sClr-*f* alone appeared to reduce NK cell killing of TEC. However statistical significance was not achieved, but perhaps optimization of the model would have clarified its effects more definitely (Figure 5D). Future experiments from our group will seek to further demonstrate the capabilities of sClr-*f* in protecting transformed targets with compromised MHC class-1. Additionally, we will investigate the potential for sClr-*b*/-*f* to protect from NK cell-mediated injury during IRI, using an *in vivo* mouse model of kidney IRI.

Interestingly, the NKR-P1A receptor in humans, thought to be a homologue of NKR-P1B in mice, is expressed across a wide variety of immune cell types including NK cells, αβ and γδ T cells, mucosal associated invariant T (MAIT) cells, invariant CD1d specific NKT cells, Th17 cells, Tc17 cells, and CD4$^+$CD25^{high}FoxP3$^+$ regulatory T cells (124–131). While NKR-P1A ligation with its ligand LLT1 is reported to be inhibitory in NK cells, many studies have demonstrated a co-stimulatory function of NKR-P1A ligation

in T cells (94, 125, 132–135). As NKR-P1A in humans appears to possess opposing anti-inflammatory/pro-inflammatory functions depending on the cell type it is expressed on, future studies will need to carefully examine the side effects of using a NKR-P1 agonist such as soluble Clr-*b/-f* or soluble LLT1 to inhibit NK cells.

4.4   Consequences of Genetic Variability for IRI Severity

In human kidney transplant patients, there is a substantial amount of variability in the severity of delayed graft function (DGF) and acute kidney injury (AKI). DGF following transplant is thought to be primarily a consequence of IRI (136). Factors such as donor age and genetic factors have been implicated in the severity of injury (137, 138).We do not, however, have a comprehensive database on the specific genomic characteristic of donors and recipients, and how genetic variation in donor organs may impact transplant outcomes. For example, inter-individual variations exists in many of the genes that regulate cytokine and chemokine expression, and this is being increasingly recognized as an important variable in allograft outcomes (139). Hutchinson *et al.* has correlated a $G_{308}A$ polymorphism in the recipients TNFα promoter region with an increased rate of rejection and an increased rate of steroid-resistant rejection in kidney transplant recipients (140, 141) Hoffmann *et al.* demonstrated that donor TGF-β haplotypes associated with increased protein production are associated with a development of acute cellular rejection in transplanted grafts (139). Similarly, genetic variation in the expression of inhibitory molecules Clr-*b/-f,* or NK receptors NKR-P1B/G may likely have an impact on IRI severity. Carlyle *et al.* have reported a high degree of divergence observed for the NKR-P1B/D alleles between the 129S1/BALB/c and B6 mouse strains (84). Notably, the NKR-P1B[129] receptor appears to respond more robustly than NKR-P1B[B6] to titrated doses of 239T Clr-*b* stimulator cells (142). The 129S1 and BALB/c NKR-P1G allele are identical, yet both differ from the B6 allele by a single nucleotide resulting in an amino acid substitution (143). Similarly, 129S1 and BALB/c Clr-*f* alleles are identical, but differ B6 by three amino acid substitutions (143). Our group has previously demonstrated that B6 mice are more susceptible than BALB/c mice to glomerular necrosis, decreased kidney function, and tubular cell death following IRI (111). It may be possible that polymorphisms

in the NKR-P1:Clr recognition system confer decrease susceptibility to IRI in B6 mice.

Consequently, variations in the homologue human NKR-P1:LLT1 recognition system may account for some of the DGF and IRI variability observed between patients. A study done by Rother *et al.* demonstrated that a c.503T>C SNP results in an amino acid transversion (Ile168Thr) in the extracellular domain of the NKR-P1A (CD161) receptor which is involved in the binding of LLT1 (144). This mutation resulted in diminished binding of LLT1 to NKR-P1A, and ligation of the mutated NKR-P1A receptor with LLT1 or anti-NKR-P1A antibodies inhibited NK cell activation to a lesser degree as defined by CD107 staining (144). Future studies no doubt will assess for potential genetic variability in genes coding for human NKR-P1/LLT1 proteins in DGF and non-DGF patients.

4.5 Conclusions

The Clr family of proteins and their ligands represent a highly complex and conserved mechanism by which NK cells can interact with diverse cell types, leading to inhibition and dampening of inflammation, or activation with potent destruction. While these receptors likely evolved to be yet another process by which hosts deal with 'danger' presented by cancer and infection, NK cells and their receptor interactions create a formidable challenge in organ transplantation. TEC may increase their expression of Clr-*b* with ischemia-reperfusion renal injury as an attempted mechanism for self-protection from overactive NK cells during inflammation. The augmented killing of TEC *in vitro* following loss of Clr-*b* and Clr-*f* strongly supports that NKR-P1:Clr binding is an important inhibitory pathway in NK mediated kidney injury. No current drugs target NK cells during acute kidney injury or following transplantation. Therefore, greater knowledge is required to generate novel strategies to block NK participation in IRI for example. In our work, we have demonstrated that manipulation of Clr-*b* and Clr-*f* expression can alter TEC injury. Alternatively, as alteration of organ expression of any protein presents considerable challenges, we have demonstrated that soluble Clr proteins that bind to NK cells bearing NKR-P1 receptors can alter their cytotoxic capacity and thus may represent a clinically feasible strategy to protect organs from diverse forms of NK cell-mediated inflammation

and injury. Future detailed work is required to expand on the findings presented here.

4.6   Future Directions

Now that we have provided some evidence for the inhibitory capabilities of a Clr-*b*/-*f* soluble protein combined treatment, we will use a previously established *in vivo* model of mouse kidney IRI to investigate the capacity of these proteins to protect mice from kidney injury during ischemia-reperfusion.

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Curriculum Vitae

Name: Benjamin Fuhrmann

Post-secondary   Western University
Education and   London, Ontario, Canada
Degrees: 2012-2016. BSc – Honors Double Major Microbiology &
                        Immunology, and Biology

                        Western University
                        London, Ontario, Canada
                        2016 – Present. MSc - Immunology

Honors and   Infection and Immunity Research Forum Best Poster
Awards: Presentation (Graduate Students > 6 months)
                        2017

                        All-Ontario University Athletics Academic Scholar
                        2016/2017

Publications:

- Mitochondria Permeability Regulates Cardiac Endothelial Cell Necroptosis and Cardiac Allograft Rejection – submitted for review March, 2018
- Intracellular pH Regulates TRAIL-induced Apoptosis and Necroptosis in Endothelial Cells – Aug, 2017 – Journal of Immunology Research

Conferences:

Abstract Title: Regulation of NK cell-mediated Cytotoxicity by Tubular Epithelial Cell Expression of Clr Proteins in Kidney Ischemia Reperfusion Injury - *Benjamin Fuhrmann, Ingrid Gan, Xuyan Huang, Hong Diao, Shengwu Ma, Zhu-Xu Zhang, and Anthony M. Jevnikar*

- Department of Surgery Research Day 2018 – London, Ontario
- American Transplant Congress 2018 – Seattle, WA
- Medical Resident Research Day 2018 – London, Ontario
- London Health Research Day 2018 – London, Ontario
- Infection and Immunity Research Forum 2017 – London, Ontario
- Canadian Society for Transplantation 2017 – Halifax, Nova Scotia

Abstract Title: Intracellular pH Regulates TRAIL-induced Apoptosis and Necroptosis in Endothelial Cells – *Zhu-Xu Zhang, Ingrid Gan, Alexander Pavlovsky, Xuyan Huang, Benjamin Fuhrmann, and Anthony M. Jevnikar*

- Medical Resident Research Day 2017 – London, Ontario
- London Health Research Day 2017 – London, Ontario