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Inhibition of NK cell cytotoxicity by tubular epithelial cell expression of Clr-b and Clr-f

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ABSTRACT

NK cells participate in ischemia reperfusion injury (IRI) and transplant rejection. Endogenous regulatory systems may exist to attenuate NK cell activation and cytotoxicity in IRI associated with kidney transplantation. A greater understanding of NK regulation will provide insights in transplant outcomes and could direct new therapeutic strategies. Kidney tubular epithelial cells (TECs) may negatively regulate NK cell activation by their surface expression of a complex family of C-type lectin-related proteins (Clrs). We have found that Clr-b and Clr-f were expressed by TECs. Clr-b was upregulated by inflammatory cytokines TNFα and IFNγ *in vitro*. Silencing of both Clr-b and Clr-f expression using siRNA resulted in increased NK cell killing of TECs compared to silencing of either Clr-b or Clr-f alone ($p < 0.01$) and when compared to control TECs ($p < 0.001$). NK cells treated *in vitro* with soluble Clr-b and Clr-f proteins reduced their capacity to kill TECs ($p < 0.05$). Hence, NK cell cytotoxicity can be inhibited by Clr proteins on the surface of TECs. Our study suggests a synergistic effect of Clr molecules in regulating NK cell function in renal cells and this may represent an important endogenous regulatory system to limit NK cell-mediated organ injury during inflammation.

1. Introduction

Transplantation is invariably associated with allograft injury caused by ischemia-reperfusion injury (IRI) initially followed by immune cell-mediated injury (Kouwenhoven et al., 2001). Kidney IRI is a prominent cause of delayed graft function (DGF which can lead to acute renal failure (ARF) and even early graft loss (Thadhani et al., 1996). In acute allograft rejection, perforin and granzyme B lytic pathways and Fas-Fas ligand interactions can mediate TEC death (Sharma et al., 1996). TECs are highly functional cells and comprise more than 75% of renal parenchymal cells. Thus, their susceptibility to acute injury can in large part, determine the long-term success or failure of a kidney transplant.

We have demonstrated that NK cells can directly lyse TECs and are capable of initiating and contributing to IRI in the absence of both B and T cells. (Zhang et al., 2008, 2010). More recent studies have implicated NK cells in chronic allograft vasculopathy, as they can mediate long-term kidney allograft injury. They can also enhance both T cell and antibody-mediated chronic cardiac allograft rejection. (Hirohashi et al., 2012; Uehara et al., 2005; Zhang et al., 2015). Though immunosuppression can influence NK cell phenotypes as well as cytokine production, current therapies are insufficient to block their cytotoxic capacity (Hoffmann et al., 2015). This may account for long-term failure of some grafts. Understanding the mechanism(s) of NK cell-mediated cytotoxicity of TEC that is associated with severity of IRI would be key in developing effective therapeutics to prevent NK-related kidney injury and transplant loss.

NK cell functions are regulated by their receptors and ligands

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Abbreviations

Clr	C-type lectin-related protein
IRI	ischemia-reperfusion injury
KLRB1	killer cell lectin like receptor B 1
MHC	major histocompatibility complex
NK	natural killer
NKR-P1	natural killer receptor-protein 1
siRNA	small interfering RNA
TECs	tubular epithelial cells

interactions. NK cells express NK cell receptor protein-1 (NKR-P1) family members in mice and killer cell lectin-like receptor B1 (KLRB1) family members in humans. Receptor interaction with C-type lectin-related proteins (Clrs) on target cells is well described (Carlyle et al., 2004; Iizuka et al., 2003; Rahim et al., 2015). Clrs are glycosylated transmembrane proteins expressed on the surface of a wide variety of cell types (Carlyle et al., 2004; Rahim et al., 2015). Different receptor-ligand pairs of NKR-P1:Clr family members result in stimulation or inhibition of NK cell cytotoxicity (Rahim et al., 2015).

Inhibitory Clr–b and Clr–f proteins are expressed in the kidney and their respective cognate receptors NKR-P1B/D and NKR-P1G are expressed on murine NK cells (Zhang et al., 2012). The broad expression of Clr-b has suggested that NKR-P1B/D:Clr-b interaction might act as a second "missing-self" mechanism, along with major histocompatibility complex class I (MHC-I) and NK cells engagement, the latter being a primary regulatory signal. A previous study demonstrated that ligation of NKR-P1G with Clr-f inhibits NK cytotoxicity(Leibelt et al., 2015). Importantly, intestinal epithelial cells utilize the NKR-P1G:Clr-f system for homeostasis. Downregulation of Clr-f with cellular stress, pathogen challenges, or mucosal injury may stimulate a proinflammatory response(Leibelt et al., 2015). Interestingly, Clr-f is expressed mostly in the intestine and kidney while Clr-b is more widely expressed(Leibelt et al., 2015). The expression of both Clr-b and -f suggests they may play an important role in immune modulation in tissues sensitive to inflammatory injury.

Collectively this supports the concept that Clr-b and Clr-f ligands expressed by TECs in the kidney may represent a potent and endogenous regulatory mechanism to protect cells from diverse forms of NK-mediated inflammation and cytotoxicity in kidney injury. This would also suggest that our limited success in altering long-term allograft outcomes may be related to the absence of effective current therapeutics to limit NK cytotoxicity. We therefore tested the capacity of inhibitory Clr-b and Clr-f expression in regulating TECs' susceptibility to NK killing. Our current study demonstrates that NK cell cytotoxicity of TEC can be regulated by Clr-b and Clr-f proteins. Our study suggests that Clrs may represent an important endogenous regulatory system to limit NK cell-mediated kidney injury, which might be exploited in clinical scenarios.

2. Materials & methods

2.1. Animals

Wild-type (WT) C57BL/6 (B6; H-2^{b}) mice were from the Charles River (St. Constant, QC). The B6 Clr-$b^{-/-}$ (*Ocil*$^{-/-}$) mice were generously provided by Dr. Matthew Gillespie (Monash University, Clayton, Australia)(Kartsogiannis et al., 2008). All animals were maintained in the animal facility at Western University using approved protocols and procedures.

2.2. Tubular epithelial cells (TECs) culture

TECs were isolated from C57BL/6 mouse kidneys after digestion with collagenase (Sigma-Aldrich, Oakville, ON, Canada) for 30 min. TECs were grown on 0.1% collagen-coated plates (Sigma-Aldrich) in complete K1 culture medium (ThermoFisher, Burlington, ON, Canada), supplemented with 5% bovine calf serum, hormone mix (5 μg/mL of insulin, 1.25 ng/mL of prostaglandin E1 (PGE1), 34 pg/mL of triiodothyronine, 5 μg/mL of transferrin, 1.73 ng/mL of sodium selenite, and 18 ng/mL of hydrocortisone), 25 ng/mL of EGF. 100 U/mL penicillin and 100 μg/mL streptomycin. Proximal tubular phenotypes of TECs were confirmed by morphology and flow cytometry through staining with anti-CD13, CD26, Cytokeratine, and E-cadherin antibodies. Surface expression of Clr-b was detected by anti-Clr-b (clone 4A6, kind gift from Dr. Carlyle, University of Toronto, Canada) and flow cytometry (Cytomics FC500 or CytoFLEX, Beckman Coulter) (Carlyle et al., 2004). Surface expression of major histocompatibility of complex (MHC) class-I was detected by anti-H-2K^{b} (ThermoFisher) and flow cytometry.

2.3. NK cell culture

NK cells were purified from the spleens of wild type (WT) and Clr-$b^{-/-}$ C57BL/6 mice using anti-CD3ε depletion of T cells and anti-CD49b (DX5) MACS beads selection of NK cells (Miltenyi BioTECs, Auburn, CA). NK cells were primed for 7 days in the presence of human IL-2 (2000 IU/mL, Proleukin®, Novartis, QC, Canada) in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 0.5 mM 2-mercaptoethanol. The purity of NK cells was confirmed by flow cytometry analysis to be >90% CD3^{-}CD49b^{+} NK1.1^{+}.

2.4. Kidney ischemia reperfusion injury

To induce ischemia, the renal artery leading to the left kidney was clamped for 45 min at 32 °C on a thermo-regulated pad. Body temperature was monitored by a thermometer. After 45 min, the clamp was released, and the right kidney was removed. Sham control mice were treated with the same operative procedure as the injured group except the left kidney was not clamped and the right kidney was not removed. Kidneys were collected at different time points for histological analysis. Serum was bused for creatinine detection by a Jaffe reaction method with an IDEXX Catalyst One (IDEXX Laboratories, Markham, ON, Canada).

2.5. Quantitative Real-time PCR

Total RNA was extracted from tissue or cells using a PureLink™ RNA Mini Kit (Invitrogen, USA). The concentration and purity of the isolated RNA were determined using a GENESYS™ 10S UV–vis spectrophotometer (ThermoFisher, Mississauga, ON, Canada). cDNA were generated from RNA using Superscript II (ThermoFisher). 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). Primers are: β-actin CCA GCC TTC CTT CCT GGG TA and CTA GAA CAT TTG CGG TGCA. Clr-f GAA TAT AGC AAC TTG GTT CTC and GGA TTT ACA ACT ACT GAC AAA C. Clr-b AGC TCC TCA GCT CTG AGA TGT GTG and 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.

2.6. OCIL (Clr-b) mouse genotype confirmation

Clr-$b^{-/-}$ B6 mice have a targeted deletion of exon-3 in the *Clec2d* gene, resulting in a non-functional ectodomain. Total RNA was isolated from the one cubic millimeter of liver tissue, RNA concentration and purity was determined, and RNA was used to synthesize cDNA as

described above. PCR was performed on the generated cDNA using the following primers: OCIL (exon2) TAG TCC CAC AGG CAG CCC GC and TGA CGA CTC TCT GTG CAG GCC A. Gel electrophoresis of PCR products was performed on a 1% agarose gel with 10 μL of SYBR Safe stain (ThermoFisher).

2.7. RNA silencing

Primary WT and Clr-b$^{-/-}$ TECs were grown to 80% confluency and transfected *in vitro* with Clr-f siRNA (ThermoFisher) using transfection reagent (Endofectin™ Max, Genecopoeia, Rockville, MD, USA). Successful silencing was confirmed at 24, 48, and 72 h by RT-PCR. Cells were harvested at 48 h for use in *in vitro* cytotoxicity assays.

2.8. ^{51}Chromium release assay

Target cell death was measured in NK cells and TECs co-cultures using the ^{51}Chromium (^{51}Cr) release assay. Briefly, untreated or siRNA-silenced TECs were labelled with ^{51}Cr, washed, and then used as targets. IL-2 primed NK cells were prepared and used at indicated effector NK cells ratios against ^{51}Cr-labelled TECs targets seeded at 10^4 cell/well in a V-bottom 96-well microplate. The NK:TEC ratio was optimized and a 30:1 ratio was selected for our studies.

In some experiments, 250 ng/mL of soluble human glutamic acid decarboxylase (hGAD65) control, 80 ng/mL of sClr-b, 80 ng/mL of sClr-f, or 80 ng/mL of both sClrs were added to wells containing only NK cells 30 min prior to co-culture. Following 4 h of co-culture at 37 °C, the plates were spun down and 100 μL 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 the 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 (MilliporeSigma).

2.9. Clrs protein expression and western blot analysis

Clrs sequence information was from Uniprot (Clr-b: Entry #Q91V08; Clr-f: Entry #Q8C1T8). The PCR product was cloned into binary plant transformation vector pBI101.1, generating vector pBI-pro according to our protocols (Alqazlan et al., 2019; Tremblay et al., 2011). We expressed soluble forms of 6X His-tagged Clr-b (sClr-b) and sClr-f ectopic domains in tobacco plants, a system which allows transient and high efficiency production of proteins of interest with free of endotoxin.

Clr-b topological (extracellular) sequence (amino acid, AA 63–207): LSATK TEQIP VNKTYAACPQNWIGVENKCFYFSEYPSNWTFAQAFCMA-QEAQLARFDNQDELNFLMRYKANFD-SWIGLHRESSEHPWKWTDNTEYNNTIPIRGEERFAYLNNNGIS-STRIYSLRMWICSKLNSYSLHCQTPFFPS.

Clr-f topological (extracellular) Sequence (AA 74–218): RNKIP AMEDR EPCYT ACPSGWIGFGSKCFYFSEDMGNWTFSQSSCVASNSH-LALFHSLEELNFLKRYKGTSDHWIGLHRASTQHPWIWTDN-TEYSNLVLTRGGGECGFLSDNGISSGRSYTHRK-WICSKFVSSCKSRVGSVPRHV.

Constructs were then transformed into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating(Ma et al., 2005). Primary transgenic plants were selected on MS medium containing 100 mg/L kanamycin. Total leaf protein was extracted, and protein was purified by His-Tag purification column (MilliporeSigma). Protein concentration was determined based on the Bradford dye-binding method (Bio Rad).

Following protein purification, soluble Clr-b and Clr-f proteins were detected using rabbit anti-6X His tag antibody (Abcam, Toronto, ON, Canada) in Western blot. Proteins were visualized using Goat anti-Rabbit HRP antibody (Seracare, Milford, MA, USA). Bands were visualized with a FluorChem Imaging System (ProteinSimple, Sane Jose, CA, USA).

2.10. NK cell viability assay

NK cells were cultured with soluble Clr proteins (sClr-f, sClr-b) and a control hGAD65 at different concentrations. NK cell viability was assessed with a flow cytometer (CytoFLEX flow cytometer, Beckman Coulter) and propidium iodide (PI, ThermoFisher) staining.

2.11. Statistical analysis

Experimental values were expressed as mean ± standard deviation (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.

3. Results

3.1. Clr-b mRNA expression increases on TECs in vitro and in vivo after kidney injury while Clr-f mRNA expression increases in vivo after kidney injury

We tested whether TECs can limit NK cell-mediated injury in a simulated inflammatory environment. We isolated primary TECs from the kidneys of B6 mice according to protocols (Du et al., 2003; Zhang et al., 2008) and exposed them *in vitro* to the pro-inflammatory cytokines TNF***α*** and IFN***γ*** for 24 and 48 h. RNA was isolated for expression of Clr-b and Clr-f analysis using PCR. Interestingly, the expression of Clr-b mRNA was significantly increased following treatment with proinflammatory cytokines (Fig. 1A). Mouse anti-Clr-f antibody is not available, and thus we were limited to testing Clr-f mRNA expression by PCR. Clr-f mRNA expression seemed to increase but was not significant after cytokine treatment (Fig. 1B). The cell surface protein level of Clr-b also increased 24 h after cytokine treatment (Fig. 1 C&D). However, the protein level of Clr-b returned to baseline untreated TEC levels after 48 h (Fig. 1C&D), suggesting the increase in expression is self limited and somewhat short. The increase of Clr-b and Clr-f in response to these pro-inflammatory cytokines which are well known to activate NK cells, suggest this is a protective response of TECs against NK cell-mediated cytotoxicity. TECs also express abundant surface MHC class-I but levels can be modestly increased with cytokine exposure (Fig. 1E).

Next, we analyzed Clr expression *in vivo*. Clr-b mRNA expression in B6 mouse kidney increased following kidney IRI 24–96 h (Fig. 2A). TECs were isolated after kidney IRI and analyzed for surface expression of Clr-b by flow cytometry. Clr-b surface expression was detected on kidney TECs before and increased after IRI compared to sham (Fig. 2B). Similarly, PCR analyses showed that Clr-f mRNA levels increased 24 h post kidney IRI and then gradually decreased (Fig. 2C). Hence, these data are consistent with the concept that Clr-b and Clr-f may play protective roles in the kidney against NK cell-mediated cytotoxicity.

3.2. Inhibition of both Clr-b and Clr-f expressions is required to increase NK cell-mediated TEC killing

Our previous studies have demonstrated that NK cells can directly mediate TEC death *in vitro,* and that NK cells are important participants in an *in vivo* model of kidney IRI (Zhang et al., 2008, 2010). We have also demonstrated that increased expression of Fas ligand and Fas receptors in TECs can be induced by IFN-γ, a pro-inflammatory cytokine secreted by activated NK cells (Du et al., 2005; Vujanovic, 2001). This finding suggests NK cells may induce TEC death indirectly through Fas-Fas ligand, as well as direct contact-dependent NK cytotoxicity. However, the role of Clr-related, MHC-independent inhibition of NK cells by TECs in kidney injury has remained undefined.

To study the possible inhibitory effects of Clr-b and Clr-f expressed by TECs, we examined changes of NK cell-mediated TEC killing with inhibition by Clr-b and Clr-f. The genotype of Clr-*b*$^{-/-}$ mice was confirmed

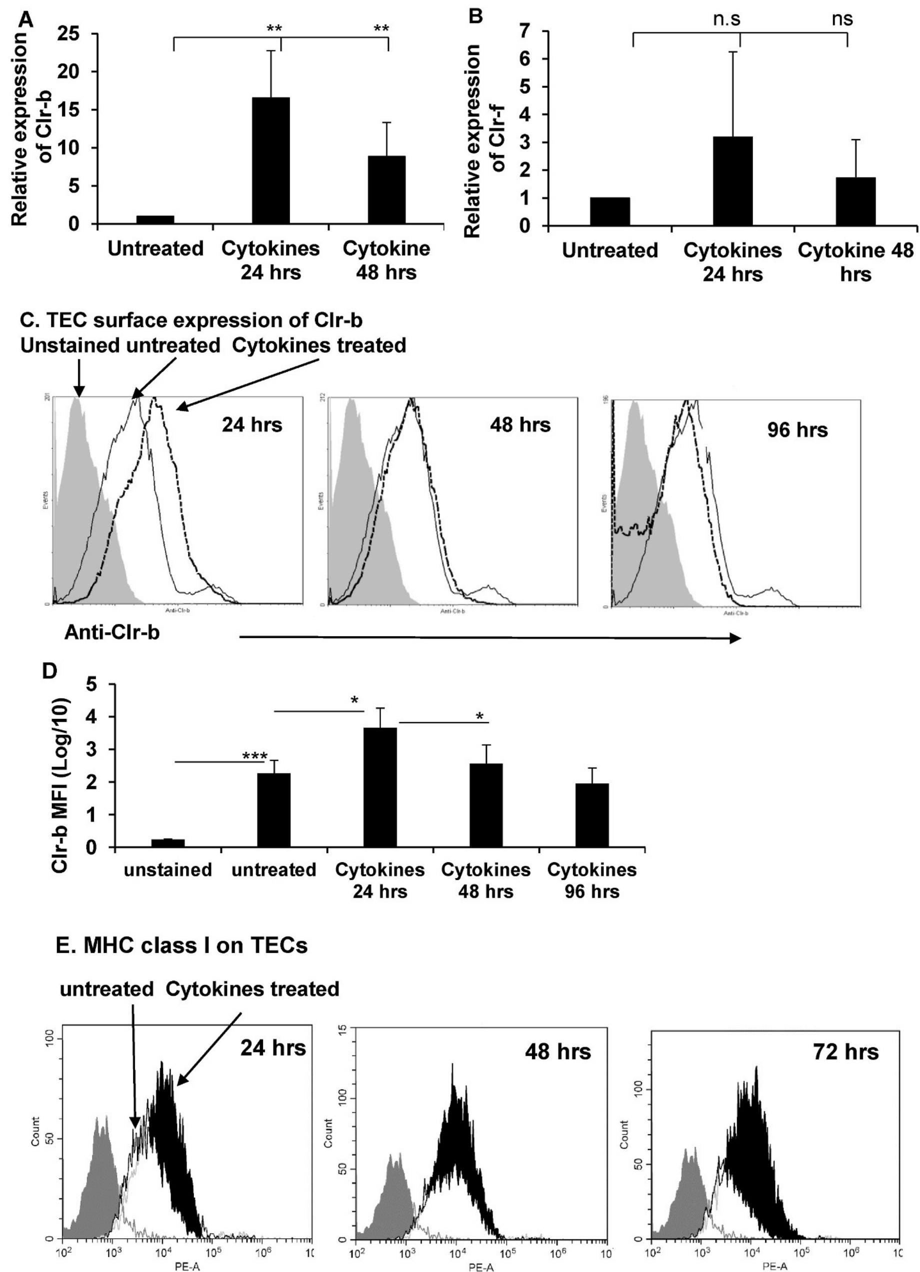

Fig. 1. Clr-b expression is increased in murine TECs following pro-inflammatory and hypoxic stress *in vitro*.
(A&B) Primary TECs were prepared as detailed in the Methods and treated *in vitro* for 24 and 48 h (hrs) with 25 ng/mL of IFNγ and 25 ng/mL of TNFα. RNA was isolated from cultured cells and cDNA was generated. Relative fold change of Clr-b and Clr-f mRNA expression before and after cytokine treatment was determined by qPCR. B-actin was used as reference. Data is shown as Mean ± standard deviation (SD) (Clr-b, n = 4 repeated experiments. Clr-f, n = 5 repeated experiments), $^{**}p < 0.01$, not significant (ns), Student's t-test. **(C)** Surface Clr-b expression after cytokines treatment was confirmed by anti-Clr-b and flow cytometry analysis. Data are representative of 3-repeated experiments. **(D)** Mean fluorescent intensity (MFI) in a logarithmic scale was averaged from 3-repeated experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, Student's t-test. **(E)** Surface expression of MHC class-I with and without cytokines treatment was detected by anti-H-2K^b and flow cytometry. Data are representative of two repeated experiments.

using PCR as described in the methods (Fig. 3A). We utilized siRNA to silence Clr-f in WT and Clr-b$^{-/-}$ primary TECs *in vitro* (Fig. 3B). NK cell-mediated TECs death was then measured in co-culture killing assays using a standard ^{51}Chromium (^{51}Cr) release method. Interestingly elimination of either Clr-b or Clr-f alone in TECs did not alter NK cell-mediated killing (Fig. 3C). However simultaneous absence of both Clr-b and Clr-f expression resulted in significantly increased NK killing of TECs compared to the Clr-b$^{-/-}$, or Clr-f silenced, or WT control groups (Fig. 3C).

To further demonstrate that the absence of Clr-b and Clr-f was required to observe an increase in NK cell killing, we co-cultured TECs deficient in either one or both Clrs along with NK cells from either WT or Clr-b$^{-/-}$ mice. As NK cells from Clr-*b*$^{-/-}$ B6 mice would not have developed in the presence of Clr-b, we hypothesized that these NK cells may not possess fully competent Clr-b inhibitory function. However, there was no significant difference in Clr-b$^{-/-}$ TEC death using either WT and Clr-b$^{-/-}$ NK cell groups (Fig. 3D). However, TECs lacking both Clr-b and Clr-f were killed significantly when co-cultured with WT NK cells (Fig. 3D). Collectively our data suggest that co-expression of Clr-b and Clr-f is required to significantly inhibit NK cells-mediated TEC killing.

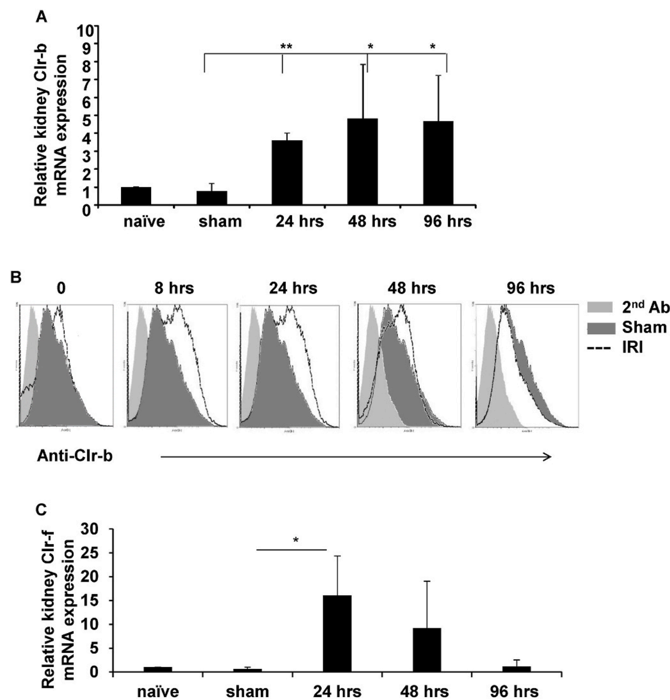

Fig. 2. Clr-b and -f mRNA expression increased in murine TECs following kidney IRI *in vivo*.
(A) Kidneys from B6 mice were collected 0–96 h after IRI for Clr-b RNA analysis as detailed in the Methods. Naïve and sham-treated kidneys were used as controls. Data is shown as Mean ± SD (n = 4 kidneys), *p < 0.05, Student's t-test. **(B)** TECs were purified from B6 mice and used for flow cytometry analysis after α-Clr-b staining. Surface expression of Clr-b was detected 0, 8, 24, 48, and 96 h after IRI. **(C)** Kidney Clr-f RNA expression 0–96 h after IRI. Data is shown as Mean ± SD (n = 6), *p < 0.05, Student's t-test.

3.3. Soluble Clr-b and Clr-f proteins reduce NK-mediated killing of TECs in vitro

As NK cells are not functionally affected by current immunosuppression in clinical transplantation or IRI, we tested whether soluble versions of Clr-b and Clr-f proteins could alter killing by their effect on NK cells. Clr-b and -f proteins were faithfully expressed in transgenic plant leaf biomass according to our published methods (Alqazlan et al., 2019; Ma et al., 2004, 2005). Proteins were extracted and purified using His-Tag purification columns. We confirmed the expected size of Clr-b (~20kD) and Clr-f (~18kD) respectively by Western blot (Fig. 4A and B). GAD65 is a membrane associated enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2. Human GAD65 would not be expected to interact with murine NK cells, thus serving as the control. GAD65 was generated by the same plant expression method (Wang et al., 2008) and was used as a soluble protein control in assays.

To exclude sClr-induced NK cell death as a confounding variable, NK cells were cultured with sClr proteins alone to confirm lack of effect on NK cell viability. NK cell death after 4 h of culture was quantified by flow cytometry and dead cells were defined as propidium iodide positive (Fig. 4C). Compared to vehicle controls, 80 ng/mL of sClr-b and sClr-f had no effect on NK cell viability (Fig. 4C&D, p < 0.01). However much higher (240 ng/mL and 400 ng/mL respectively) concentrations of soluble sClr did reduce NK viability by 16%–21% (Fig. 4C&D). Therefore, all assays were conducted using 80 ng/mL to treat NK cells in co-culture experiments.

NK cells were treated with Clr proteins for 30 min before co-culture with WT or Clr-b/f deficient TECs. Interestingly, significant attenuation of NK cell-mediated killing required exposure to both soluble Clrs when compared to untreated NK cells (Fig. 4E). As expected, hGAD65 soluble protein control had no effect on NK cell-mediated killing (Fig. 4E).

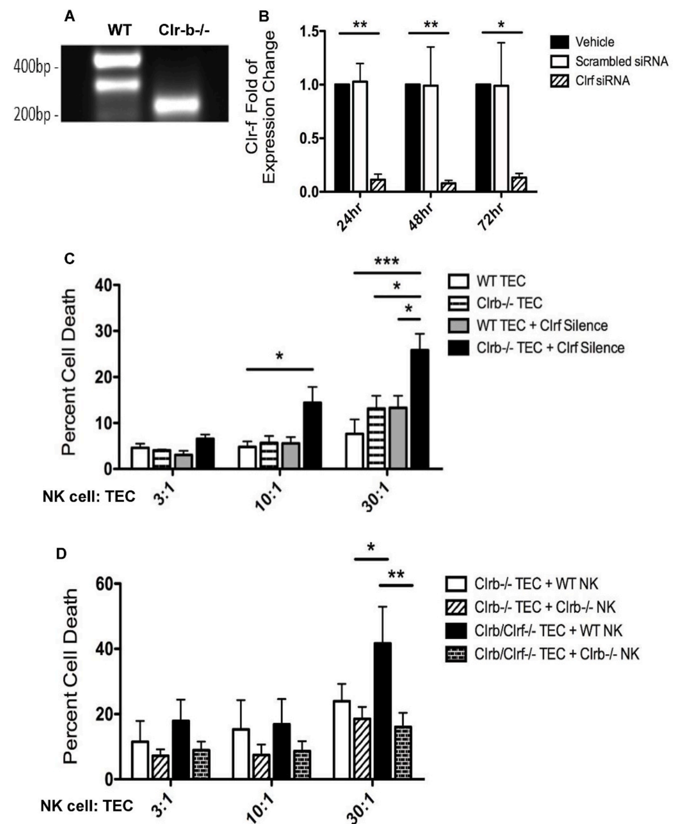

Fig. 3. Inhibition of both Clr-b and Clr-f, but neither protein individually, significantly increases NK cell-mediated TECs killing.
(A) Primary TECs were prepared as detailed in the Methods. Clr-b transcript expression in wild type and Clr-$b^{-/-}$ TECs was determined by PCR using primers specific for exons-2 through −4 for the coding sequence of Clr-b. The smaller band observed in the Clr-$b^{-/-}$ mice is indicative of deletion of exon-3 of the Clr-*b* transcript, rendering the ectodomain of the protein non-functional. **(B)** TECs were transfected with Clr-f siRNA. RNA was isolated at different time points 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. Bonferonni corrections test. **(C)** NK cell-induced TECs death. siRNA-mediated Clr-*f* silencing was performed 48 h prior to the cell death assay. TECs were labelled with ^{51}Cr and co-cultured with NK cells for 4 h. Free ^{51}Cr in 100 μL of supernatant from each well was counted and percent death was calculated using spontaneous and total ^{51}Cr release controls. Values are presented as mean ± SD (n = 3–4), *p < 0.05, ***p < 0.001, 2-way ANOVA, Bonferonni corrections test. **(D)** ^{51}Cr labelled TECs deficient in Clr-b as in Fig. 3A and/or Clr-f as in Fig. 3B were co-cultured for 4 h with NK cells purified from either WT or Clr-$b^{-/-}$ mice. Percent cell death was calculated as described above. 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 injection did not alter kidney IRI in vivo

We investigated whether Clr proteins could alter renal IRI *in vivo* using a wild type B6 mouse model. A mixture of sClr-b (1 μg/mouse) and sClr-f (1 μg/mouse) proteins or control PBS were injected into mice intravenously 2 h before kidney ischemia was induced. Clrs protein-injected mice did not show improvement in injury 24 h after IRI when compared to control mice, as measured by serum creatinine levels were modestly decreased (control 225 ± 62 vs. treated 179 ± 46 μmol/L, n = 6/group, p = 0.09, Fig. 5A). Histological assessment of kidneys did not show differences in injury scores (4 ± 0 vs. 4 ± 0, n = 6/group,

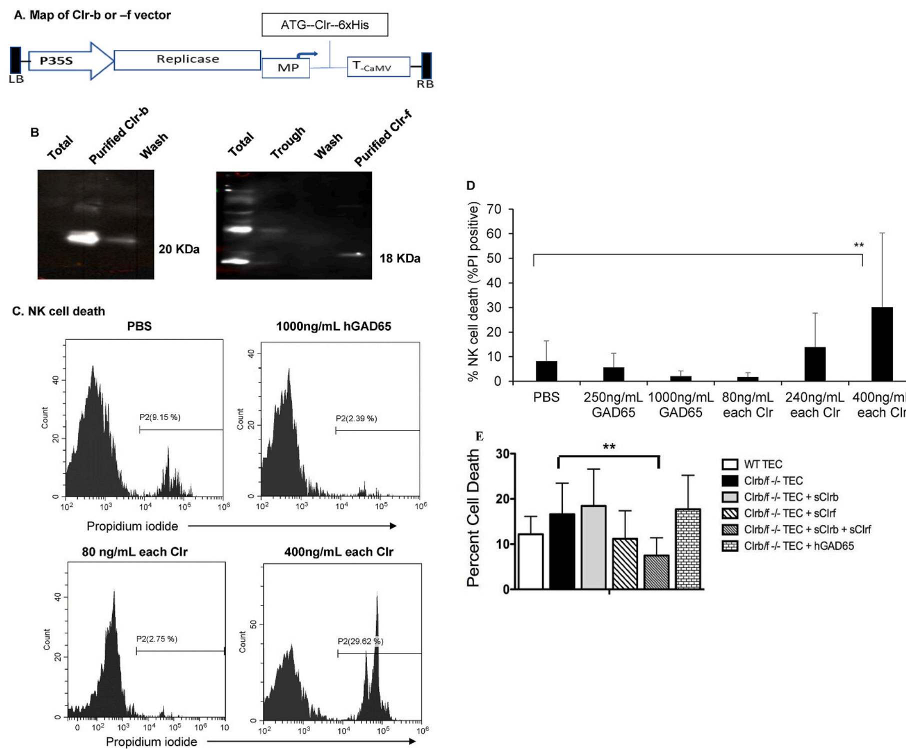

Fig. 4. A combination of soluble Clr-b and Clr-f reduces NK cell-mediated killing of TECs.
(A) The Clr-b or Clr-f ectopic domains were selected as detailed in the Methods. The PCR product of Clr-b or Clr-f were cloned into binary plant transformation vector pBI101.1 respectively. Constructs were then transformed into *Agrobacterium tumefaciens* strain LBA4404. (**B**) Total leaf protein was extracted, and sClr-b or sClr-f purification was performed on a Nickel affinity chromatography column, and sClr-b and sClr-f protein molecular weight was confirmed by Western blot using rabbit anti-6X His tag antibody. 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. (**C**) NK cell viability was tested after 4 h of culture with selected concentrations of sClr-b and sClr-f. hGAD65 protein was used as control. Percent cell death was quantified by flow cytometry after propidium iodide (PI) staining. Histograms are representative of 3-repeated experiments. (**D**) NK cell death was defined as propidium iodide positive and averaged from 3-repeated experiments. $^{**}P < 0.01$, *t*-test. **(E)** Thirty minutes prior to co-culture, 250 ng/mL of soluble hGAD65, 80 ng/mL of sClr-b, 80 ng/mL of sClr-f, or both sClrs were added to NK cells. TECs were labelled with ^{51}Cr. NK cells were co-cultured with either WT or Clr-b/f deficient TECs (30:1) for 4 h. Each group was plated in triplicate. Values are presented as mean ± SD (n = 5 or 6), $^{*}p < 0.05$, 1-way ANOVA, Bonferonni post-hoc corrections test.

Fig. 5B&C). In the doses administered here, sClr-b and sClr-f did not provide benefits for protecting kidney injury *in vivo*.

4. Discussion

The role of kidney Clr expression *in vivo* nor their participation in renal injury has not been well explored. It is known that Clr-b is broadly expressed across many tissues and hematopoietic cells(Zhang et al., 2012). In contrast, Clr-f exhibits a more specific tissue expression in the kidney, liver, and intestinal tract of mice. In the kidney, it remains unknown whether Clr participates in injury or given their ability to interact with NK cells, whether they might represent an endogenous mechanism by which TECs can resist NK cell cytotoxicity (Lau et al., 2014). Our current study has demonstrated that augmented killing of TECs occurs *in vitro* following loss of Clr-b and Clr-f, clearly supporting that NKR-P1:Clr binding represents an important inhibitory pathway in NK-mediated kidney injury that may be operative *in vivo*.

We have reported here that expression of Clr-b is enhanced by cytokines associated with IRI *in vitro* and by IRI *in vivo* while Clr-f expression is increased by IRI *in vivo* (Figs. 1 and 2). It has been reported that enhanced Clr-f expression on the surface of intestinal epithelial cells (IECs) *ex vivo* occurs with TLR3 stimulation (Leibelt et al., 2015). Interestingly, TLR3 stimulation also corresponded with a decreased frequency of NKR-P1G^{+} intraepithelial lymphocytes (IELs) and NKR-P1G protein expression on IELs (Leibelt et al., 2015). It is plausible that IELs and NK cells downregulate expression of NKR-P1G to decrease their ability to respond to inhibitory signals from Clr-f. In kidney, this may represent a similar mechanism that limits potentially excessive NK mediated injury during kidney IRI. Our data indicate that the absence of inhibitory Clr-f:NKR-P1G signalling alone is insufficient to significantly increase NK cell killing of TECs *in vitro* (Fig. 3). However, the inflammatory *in vivo* environment of the kidney during IRI may facilitate NK cell's cytotoxic killing of TECs. The absence of Clr-f:NKR-P1G-mediated inhibition appears to be insufficient despite sufficient levels of Clr-b expression. Thus, enhancing inhibition via Clr-f by preventing downregulation of NKR-P1G might represent a therapeutic strategy to reduce acute kidney from NK cell activation.

In our study, elimination of either Clr-b or Clr-f expression alone on TECs did not significantly increase NK cell-mediated killing (Fig. 3C&D). However, the combined absence of both Clr-b and Clr-f resulted in significantly increased NK cell killing of TEC (Fig. 3C&D). One possibility is that increased killing of TECs occurs at the level of the NK:TECs lytic synapse(Orange, 2008). MHC class-I engagement is a primary mechanism of NK cell inhibition, and its absence induces NK cell activation. Importantly, MHC class-I molecule expression was not decreased and remained high on TEC after cytokine treatment in *vitro* (Fig. 1E), suggesting that the removal of both Clr-b and Clr-f can override inhibition from MHC class-I molecule expression.

Clearly, MHC class-I engagement during NK cell development is necessary to produce functional NK cells (Elliott et al., 2010; Wu and Raulet, 1997). As NKR-P1B serves a similar function to receptors that recognize MHC class-I, it is similarly possible that interaction with Clr-*b*, possibly during development, is a requisite to create fully competent NK cells. While NK cells from Clr-$b^{-/-}$ mice are hyporesponsive to NK1.1

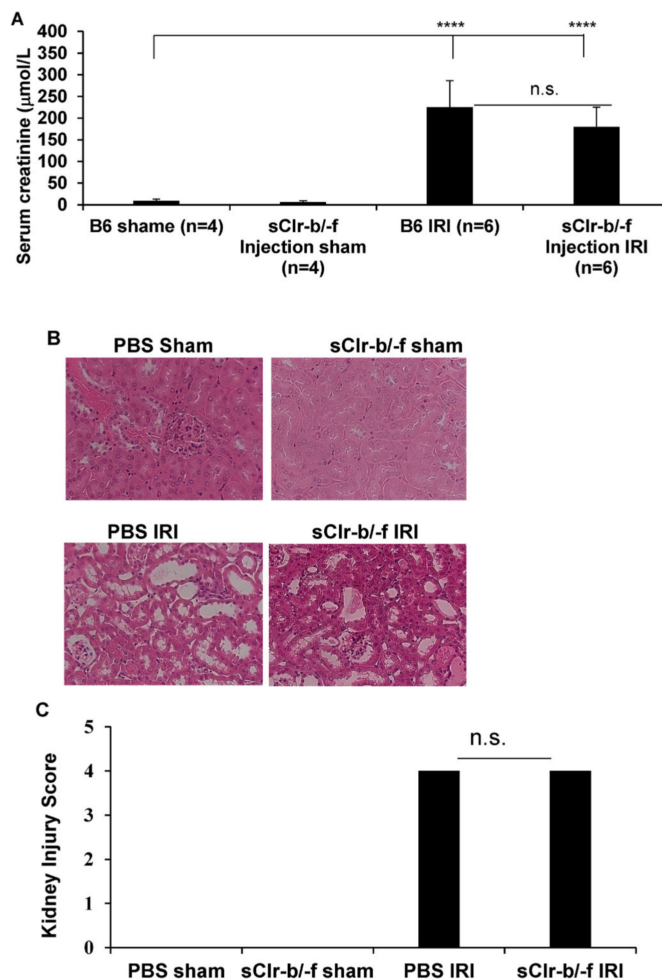

Fig. 5. A combination of soluble Clr-b and Clr-f does not provide additional benefits over control in renal IRI.
sClr-b/-f (1 μg/mouse) or PBS was intravenously injected into B6 mice. A renal clamp was applied to the right kidney pedicle and released after 45 min, and the left kidney was removed. The body temperature was maintained at 32°C. Kidney and serum were collected 24 h later. **(A)**. Serum creatinine levels were averaged from 6 mice in the treatment group and 4 mice from the sham group. **(B)** kidney sections were used for H&E staining. Histological pictures are shown at 200-fold magnification. **(C)**. Slides were scored double-blindly on a scale from 0 to 5 by a pathologist. (0: no change, 1: <10% area change, 2: 10–25% area change, 3: 25–50% area change, 4: 50–75% area change, 5: >75% area change). Kidney injury levels were averaged from 4 to 6 mice in each group.

stimulation and cytokine priming, the inhibitory capacity of the NKR-P1B receptor remains intact(Chen et al., 2015). Clr-b$^{-/-}$ NK cells do not initiate increased cytotoxicity against TECs lacking Clr-b/-f expression (Fig. 3), highlighting that change in signaling from a single inhibitory Clr protein is not sufficient to affect NK cell activation against TECs. Our findings further support that the NKR-P1B receptor must interact with its cognate ligand Clr-b to attenuate NK cell-mediated TEC killing.

As the different forms of Clr proteins are highly similar, monoclonal antibodies intended to bind inhibitory NKR-P1 receptors *in vivo* may induce undesirable agonist effects by binding to and stimulating NKR receptors. Therefore, we created soluble forms of the ectopic domains of Clr-b and Clr-f (Fig. 4). sClr-b/f inhibited NK killing of TECs (Fig. 4). Similarly, a previous study demonstrated that a recombinant murine OCIL (Clr-b) protein significantly inhibited osteoclast formation *in vitro* at a concentration of 50 ng/mL(Zhou et al., 2001). The ability of such soluble Clr proteins to inhibit interaction with NK cells may be related to a 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.

Despite clear results of protection *in vitro*, injection of mice with sClr-b/f did not provide benefit in preventing kidney IRI (Fig. 5). A number of variables may be involved including inadequate dosing, timing or rapid protein degradation *in vivo*. Future studies may require full dose-response experiments. 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, NKT cells, Th17 cells, Tc17 cells, and CD4^{+}FoxP3^{+} regulatory T cells(Exley et al., 1998; Lanier, 2005; Lanier et al., 1994). NKR-P1A ligation with its ligand LLT1 is reported to be inhibitory in NK cells. However, many studies have demonstrated a co-stimulatory function of NKR-P1A ligation in T cells (Buckle and Guillerey, 2021; Exley et al., 1998). As NKR-P1A in humans appears to possess opposing anti-inflammatory as well as pro-inflammatory functions, future studies as well will require a detailed assessment of potential side effects of using a NKR-P1 agonist such as soluble Clr-b/-f or soluble LLT1 to inhibit NK cells.

5. Conclusions

Augmented killing of TECs *in vitro* following loss of Clr-b and Clr-f strongly supports that NKR-P1:Clr binding is an important inhibitory pathway in NK cell-mediated tissue injury. More detailed understanding of NKR-P1: Clr interactions will be required to develop novel and clinically feasible strategies to block NK cell-mediated organ injury.

CRediT authorship contribution statement

Benjamin Fuhrmann: Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, preparation, Visualization. **Jifu Jiang:** Methodology. **Patrick Mcleod:** Methodology, Visualization. **Xuyan Huang:** Methodology, Visualization. **Shilpa Balaji:** Methodology. **Jaqueline Arp:** Methodology. **Hong Diao:** Methodology. **Shengwu Ma:** Methodology. **Tianqing Peng:** Methodology. **Aaron Haig:** Methodology, Validation, Visualization. **Lakshman Gunaratnam:** Writing – review & editing. **Zhu-Xu Zhang:** Conceptualization, Software, Validation, Formal analysis, Investigation, Resources, Writing – original draft, preparation, Visualization, Supervision, Project administration, Funding acquisition. **Anthony M. Jevnikar:** Conceptualization, Software, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Data availability

Data will be made available on request.

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