

## Cystinosis Research Foundation Final Progress Report

**Title:** *Elucidating the role of aberrant macrophage activation in nephropathic cystinosis*

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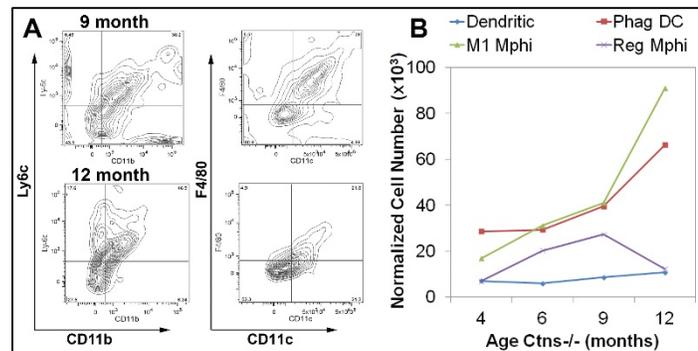
**Funding Period:** September 1, 2013 to December 31, 2015

### OVERVIEW

Overall, the project produced some insightful and intriguing results. The project was designed to further delineate the *Ctns*<sup>-/-</sup> macrophage phenotype both in vitro (Aim 1) and confirming these phenotypic findings in vivo in the context of chronic kidney injury (Aim 2). The project progressed as expected for Aim 1 but we were confounded by the results from Aim 2. As discussed below, we found that despite in vitro studies of a hyperphagocytic, hyperinvasive macrophage phenotype that there was less fibrosis in *Ctns* ko chimeric mice during chronic kidney injury. We are still investigating the mechanism behind this discrepancy but hypothesize that it is due to an impaired ability to respond appropriately to chemotaxis during kidney injury. Studies are ongoing to investigate this hypothesis.

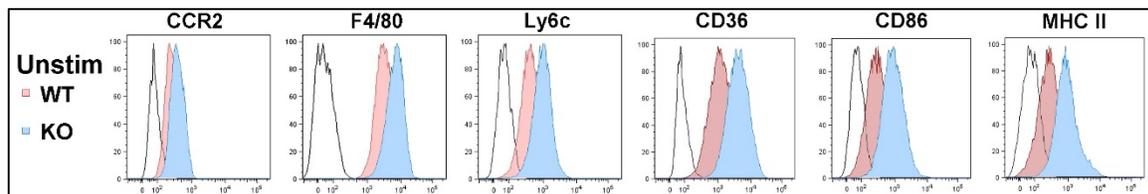
**Aim #1:** To determine the *Ctns*<sup>-/-</sup> macrophage phenotype in response to cytokine activation and the mechanisms that lead to its altered behavior

Over the past two years, we have been funded by the CRF to investigate the hypothesis that *CTNS*<sup>-/-</sup> macrophages (MΦ) are programmed to execute a more profibrotic phenotype. In our UUU model, *Ctns*<sup>-/-</sup> mice developed worse fibrosis (19% higher) with significantly more F4/80+ interstitial MΦ (63%) and suggested that *Ctns*<sup>-/-</sup> MΦ are important during chronic tubular injury. In order to investigate *Ctns*<sup>-/-</sup> MΦ behavior, we performed FACS analysis on macrophage subpopulations in nephropathic cystinotic kidneys at mild (6m) to moderate-severe fibrosis (12m). MΦ are a heterogeneous population and can be divided functionally by their phenotype: M1 – classically activated/pro-inflammatory; and M2 – reparative/wound healing, regulatory, and profibrotic. However, distinct M2 markers to correlate these three phenotypes in the kidney



**Figure 1: Macrophage subpopulations in Nephropathic Cystinosis.** FACS analysis was performed using the BD LSR II. Kidneys were digested with Liberase into a single cell suspension in FACS buffer and prepared for FACS. After gating for viable, CD45+ cells, analysis for MΦ subpopulations were performed. (A) Representative FACS plot in 9 and 12 mos old *Ctns*<sup>-/-</sup> mice; (B) Graph summarize cell populations after normalization. n=3-5/group; Dendritic – CD11c+ F4/80-; Phag DC (phagocytic dendritic) – CD11c+ F4/80+; M1 Mphi (M1 macrophage) – CD11b+ Ly6c<sup>med/hi</sup>; Reg Mphi (regulatory macrophage) – CD11b+ Ly6c<sup>lo</sup>.

is continuing to evolve. We characterized the macrophage subpopulations in the cystinotic kidney with several basic MΦ markers: F4/80 – phagocytic MΦ; CD11b – monocytes/ MΦ, neutrophils, and NK cells; CD11c – dendritic/resident tissue MΦ, monocytes, neutrophils, and some B cells; Ly6c – M1 type; CD45 – all myeloid cells. We found that the number of M1-type MΦ (Ly6c<sup>med/hi</sup>) almost doubled between 4 to 6 months and suggests that these cells may initiate an early pro-inflammatory response. During the progression from mild to moderate-severe fibrosis (12m) there was a significant increase in the M1 subset by 120% and is the predominant subpopulation within the cystinotic kidney. Phagocytic dendritic cells increased by 67% but regulatory macrophages decreased by 55% at 12 months (Figure 1). These results suggest that M1 MΦ and phagocytic dendritic cells are important in the progression of injury and fibrosis in nephropathic cystinosis.



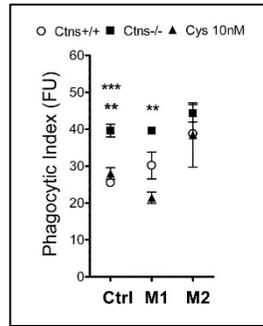
**Figure 2: Cell surface expression in Unstimulated *Ctns*<sup>-/-</sup> Macrophages.** Bone marrow harvested from wild type C57BL6 mice and *Ctns*<sup>-/-</sup> mice, 2-3 months of age, and placed in growth media containing M-CSF for 7 days to differentiate into macrophages (BMDM). Cells were seeded at 70-80% confluency and activated with M1 (LPS, IFN $\gamma$ ) or M2 (IL4). FACS analysis was performed. Representative histograms are shown. Pink—*Ctns*<sup>+/+</sup>; Blue—*Ctns*<sup>-/-</sup>

Based on these findings, we investigated the importance of *Ctns* to several important characteristics of baseline and activated macrophage function in the context of tissue injury: phagocytosis, migration, and invasion. Studies from our previous grant cycle demonstrated that at baseline, *Ctns*<sup>-/-</sup> bone marrow derived MΦ have a different expression pattern of cell surface markers (Figure 2). This demonstrated that CSF1 differentiated *Ctns*<sup>-/-</sup> macrophages had higher surface marker levels of CCR2 (receptor for CCL2/MCP1), CD36 (phagocytic receptor), MHCII and CD86 (antigen presentation), F4/80, and Ly6c.

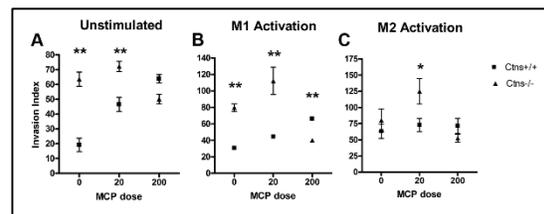
Since phagocytosis is a major function of macrophages and is one of the predominant subpopulations seen during nephropathic cystinosis progression, we determined the phagocytic index of *Ctns*<sup>-/-</sup> macrophages at baseline and with activation. We used pHrodo E.coli BioParticles as a measure of phagocytic index since it will only emit a fluorescent signal upon exposure to acidic pH, during phagosome fusion with lysosomes; A higher fluorescent value indicates an increase in BioParticle uptake and higher phagocytic index. There was a 155% increase in phagocytic index in *Ctns*<sup>-/-</sup> BMDM compared to wild type controls (Figure 3,  $P=0.0008$ ). In contrast to wild-type controls where phagocytic index increased with M1 and M2 activation, the loss of *Ctns* led to no change in phagocytic index with macrophage activation. However, when *Ctns*<sup>-/-</sup> BMDM were reated with cysteamine phagocytic behavior reverted to nearly the same as wild-type macrophages (Figure 3). This suggests that cystine accumulation is the key determinant of the hyperphagocytic behavior of baseline and activated *Ctns*<sup>-/-</sup> macrophages.

Macrophage chemotaxis and invasion was assessed using a laminin coated invasion chamber. Laminin is a principle component of tubular basement membrane (TBM) and therefore serves as a model of macrophage invasion through TBM to an injured tubular cell against a chemoattractant gradient with CCL2 (0-200ng/mL). We found that unstimulated wildtype macrophages and with M1 activation had higher invasion indices with increasing concentrations of CCL2, however, both M2 wildtype and *Ctns*<sup>-/-</sup> macrophages do not respond to CCL2 chemoattraction. The loss of *Ctns* in both unstimulated and M1 activated macrophages led to a two to three-fold increase in the invasive index in the absence of CCL2 compared to wildtype (Figure 4A, B). In contrast to wildtype macrophages, there was no change in the invasion index with increasing concentrations of CCL2 with the loss of *Ctns* in unstimulated and with M1 activation. Interestingly, at high doses of CCL2 (200ng/mL) there was a significant decrease in the invasive index from baseline (no CCL2) in unstimulated and M1 activated *Ctns*<sup>-/-</sup> macrophages by 22% and 51%, respectively. Taken together, these data suggest that although unstimulated and M1 activated *Ctns*<sup>-/-</sup> macrophages are hyperinvasive at baseline, they are less migratory than wild-type macrophages at higher levels of CCL2.

Previous studies from last grant period demonstrated that there were many differentially expressed genes upon both M1 and M2 activation. Therefore, we performed a microarray in unstimulated, M1, and M2 activated wildtype and *Ctns*<sup>-/-</sup> macrophages. We also treated all *Ctns*<sup>-/-</sup> macrophages with cysteamine bitartrate to determine the effect of cystine accumulation on *Ctns*-dependent gene expression. This was performed at the University of Washington Microarray and Bioinformatics Core Laboratory using the Affymetrix Mouse Gene 1.0 ST arrays. Data was background corrected, normalized, and then summarized using a robust multi-array average. A weighted ANOVA model was then fit to these data and



**Figure 3. *Ctns*-deficiency leads to increased phagocytosis that normalizes with cysteamine.** Bone marrow harvested from wild type C57BL6 mice and *Ctns*<sup>-/-</sup> mice, 2-3 months of age, and placed in growth media containing M-CSF for 7 days to differentiate into macrophages (BMDM). 100,000 cells were seeded in 96 well plate in media with M1 (LPS, IFN $\gamma$ ) or M2 (IL4) for 24hours. In a group of *Ctns*<sup>-/-</sup> BMDM, treatment with cysteamine bitartrate 10nM concurrent with M1/M2 activation. The following day, pHrodo E.coli BioParticles were added and incubated for 4 hours at 37°C and fluorescence (488) was measured. n=3/group. ○ *Ctns*<sup>+/+</sup> ● *Ctns*<sup>-/-</sup>; ▲ *Ctns*<sup>-/-</sup> + Cysteamine 10nM; FU— fluorescent light units. \*\* P<0.01, *Ctns* ko vs wt.



**Figure 4. Dysregulated chemotaxis in unstimulated and activated *Ctns*<sup>-/-</sup> macrophage.** Bone marrow harvested from wild type C57BL6 mice and *Ctns*<sup>-/-</sup> mice, 2-3 months of age, and placed in growth media containing M-CSF for 7 days to differentiate into macrophages (BMDM). BMDMs were activated M1 (LPS, IFN $\gamma$ ) or M2 (IL4) for 24hours. The following day 2.5x10<sup>5</sup> BMDM were seeded in the upper chamber of a laminin invasion chamber in Optimem, no FCS. The bottom chamber contained media with CCL2/MCP1 at various concentrations. After 24h migrated cells were fixed, stained, and released into the lower chamber and read in a spectrophotometer. Graphs summarize semi-quantitative results of normalized readings to unstimulated wild type BMDM. n=3/group. \* P<0.05; \*\*P<0.01

Comparisons	Number significant
<a href="#">KO cysteamine vs WT cysteamine</a>	177
<a href="#">KO vehicle vs WT vehicle</a>	586
<a href="#">Interaction baseline</a>	8
<a href="#">KO - M1 cysteamine vs WT - M1 cysteamine</a>	483
<a href="#">KO - M1 vehicle vs WT - M1 vehicle</a>	682
<a href="#">Interaction M1</a>	38
<a href="#">KO - M2 cysteamine vs WT - M2 cysteamine</a>	561
<a href="#">KO - M2 vehicle vs WT - M2 vehicle</a>	342
<a href="#">Interaction M2</a>	112

Comparisons and number of significant genes at an unadjusted p < 0.05 and 1.25-fold change.

Table 1: Microarray of Unstimulated, M1, and M2 activated Ctns-/- Macrophages

- Inflammasome (Nlrp1)
- Transcriptional regulation (Gas5, ribosomal proteins, Hmgb2)
- Cell cycle (Cdkn1b, Impdh2, Pcdcd10, Cinp)
- Cellular Metabolism (Ggh, Pagr1a, Gstp1, Alg14)
- Cell signaling (Pten, Nfkb1a, Acta2)
- Oxidative stress (Prdx4, Txnip, Prdx3)

Table 2: Genes Down-regulated by Ctns in Macrophages

- Membrane trafficking (Rab4a, Acap1)
- Cell cycle (Anxa6, Dnmt3a0s, Xaf1)
- Metabolism (Apobec3, Fabp4, Dpep3, Osgin1)
- Transporters/Channels (Slc03a1, Slc15ac2)
- Inflammation (Ccl7, Mirlet7c-1, Mir150, Tnfaip8)

Table 3: Genes Up-regulated by Ctns in Macrophages

A surprising candidate gene common to both M1 and M2 activation was the down-regulation of Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain-1 (Nlrp1), especially since cystine crystals lead to activation of Nlrp3. These results need further investigation but the cystine independent effects on gene regulation strongly suggest that cystinosin affects inflammatory and cell signaling pathways (Table 4).

Our initial hypothesis was that aberrant response to cytokine activation in Ctns-/- MΦ resulted in more severe injury, however, our preliminary data below suggests that they attenuate injury. It is not clear if this is due to other factors such as proliferation/apoptosis or chemotaxis. Therefore, we will further characterize the in vivo data to help focus our in vitro investigations on mechanism.

- Transporter/Channels (Slc22a7, Slc5a4a, Slc4a10, Cldn13, Slc9a7)
- Inflammation/Cell signaling (Ptk6, Megf6, Mmp7, Fcgr2a, Cdh18, Cdhr2, IL10, Tnfsf10, Gas5, Ifi44)

Table 4: Genes Independent of Cystine Accumulation in Ctns-/- Macrophages

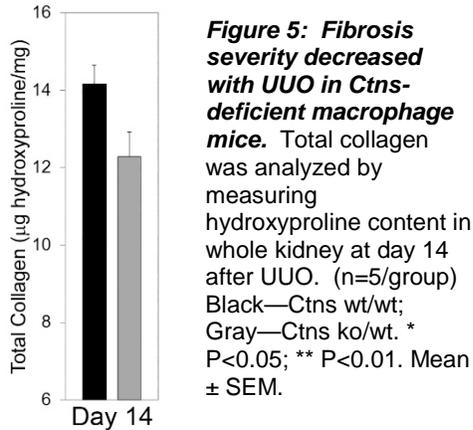
comparisons were performed with empirical Bayes adjusted contrasts.

The interaction analysis shows what genes are differentially expressed after treatment with cysteamine and thus are independent of cystine accumulation. We found more genes that were down-regulated by the loss of Ctns in macrophages than up-regulated (Tables 2 and 3).

**AIM #2.** To investigate the functional impact of the Ctns-/- macrophage phenotype on regeneration and fibrosis after renal injury.

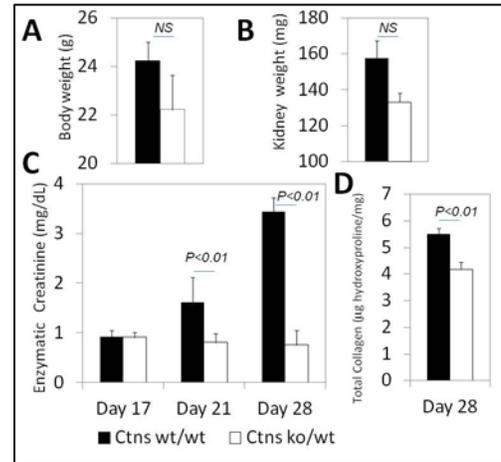
In order to determine the functional phenotype of Ctns-/- macrophage in response to kidney injury, we produced chimeric mice by bone marrow transplantation. Wild-type mice were irradiated with 900 rads and injected with bone marrow cells from Ctns -/- mice retroorbitally. After 8-10 weeks to allow complete engraftment of the monocyte/macrophage population, Ctns ko/wt mice and Ctns wt/wt chimeric controls underwent unilateral ureteral obstruction and were sacrificed at 14 days (n=5/group). Surprisingly, despite our microarray data demonstrating aberrant responses to M1 and M2 activation and functional studies demonstrating hyperphagocytic and hyperinvasive behavior, we found that fibrosis severity was mildly

decreased by 13% (Figure 5, n=5/group, P=0.049). However, this model is limited by the inability to determine kidney function due to the contralateral kidney.



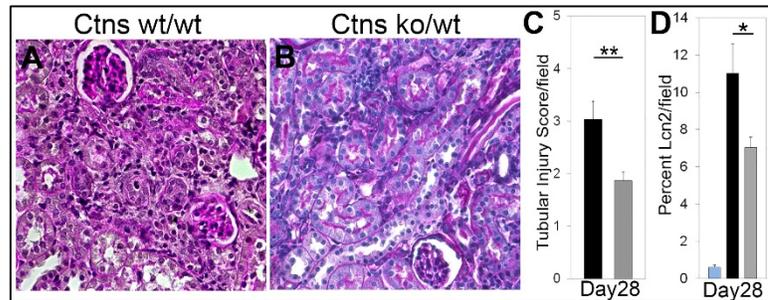
In order to confirm our findings, we generated chimeric mice as above and performed ischemia reperfusion injury (IRI) on these chimeric mice. After 14 days, both groups underwent contralateral nephrectomy to investigate kidney function of the IRI kidney. Each mouse underwent blood draws at days 17, 21, and 28 after IRI injury. Mice were sacrificed at day 28 and kidneys analyzed for fibrosis severity and kidney function was determined. There was no difference in both body weight and kidney weight at time of sacrifice in Ctns ko/wt mice compared to chimeric controls (Fig 6A, B). Although there was no difference in creatinine at day 17 (3 days after nephrectomy) between Ctns ko/wt and chimeric controls, there was a continued progression and deterioration of kidney function in the chimeric control mice but not in the Ctns ko/wt mice. There was approximately a 450 percent increase in creatinine between Ctns ko/wt and chimeric control mice at day 28 (Figure 6C). Analysis of fibrosis severity at time of sacrifice on day 28, confirmed our functional studies with a reduction in total collagen in Ctns ko/wt mice compared to chimeric controls. Interestingly, there was only a 24% reduction in total collagen compared to the much larger decrease in serum creatinine (Figure 6D).

We also examined the effect of Ctns -/- macrophages on tubular injury during chronic IRI. Periodic-acid Schiff (PAS) stains were performed on paraffin sections. Tubular injury was analyzed on 6-8 randomly selected PAS stained images. The following tubular injury scores were assigned, in a blinded manner, based on the estimated percent area affected with tubular dilatation, tubular cell flattening, interstitial inflammation, and loss of intact tubular architecture in the field: 1 (<10%); 2 (10-25%); 3 (26-50%); 4 (51-75%); or 5 (>75%). We found a significant reduction in tubular injury scores in Ctns ko/wt mice compared to chimeric control mice at day 28 after IRI (Figure 7A-C). Lipocalin2 (Lcn2) is the mouse homologue of Neutrophil gelatinase associated lipocalin (NGAL), an established marker of distal tubular injury that correlates with



kidney dysfunction. Lcn2 was expressed at low levels in distal tubules in normal kidneys and was significantly up-regulated immediately after kidney injury. Consistent with tubular injury scores, we found a significant reduction in Lcn2 expression in *Ctns ko/wt* mice by 36% at day 28 after IRI compared to *Ctns wt/wt* chimeric controls (Fig 7D).

In conclusion, our *in vivo* studies strongly suggest that loss of macrophage cystinosin abrogates the fibrotic response in both UUO and IRI. We hypothesize that this is related to an inability of *Ctns*<sup>-/-</sup> macrophages to migrate into injured tissue during kidney injury. Our current ongoing studies on chimeric mice generated with DsRed *Ctns*<sup>-/-</sup> mice are being analyzed to investigate this hypothesis.



**Figure 7: *Ctns* deficient macrophages limit tubular injury after IRI.** (A,B) Representative 400x PAS stained images from day 28 IRI kidneys. Tubular injury scores were assigned in a blinded fashion on 6 images per mouse, n=5-8/group. (C) Graph demonstrates mean tubular injury scores per mouse; Lipocalin2 immunostaining was performed as a marker of tubular injury. Image analysis was performed. (D) Graph represents mean Lcn2 positive area/mouse. Blue—*Ctns wt/wt* sham; Black—*Ctns wt/wt*; Gray—*Ctns ko/wt*. \* P<0.05; \*\* P<0.01. Mean  $\pm$  SEM.