Cystinosis Research Foundation Progress Report

Title: ***Elucidating the role of cystinosin-deficient macrophages in nephropathic cystinosis***

Grant number: 414010070101
Investigators: Daryl Okamura M.D. and Allison Eddy, M.D.
Funding Period: July 1, 2008 to June 30, 2011
Progress Report: August 1, 2012 to January 31, 2013

OVERVIEW

The project is progressing well and we are continuing to characterize the macrophage (MΦ) subpopulations within the Ctns-/- kidney. The Ctns null mice are breeding well. For the past 6 months, we have been validating our FACS data with 9 month and 12month old mice which have taken time to generate but have now strengthened our initial observations.

Aim #1: To differentiate the cytokine profile and fibrosis-promoting effects of *Ctns*+/+ and *Ctns*-/- macrophages in mouse models of nephropathic cystinosis and unilateral ureteral obstruction (UUO) induced chronic kidney disease (CKD).

We have been investigating the macrophage subpopulations in Ctns-/- mice to test our overall hypothesis that *Ctns*-/- MΦ are genetically programmed to execute a more aggressive fibrotic response to renal injury than normal *Ctns*+/+ MΦ. Our objective in this past 6 month period was to determine if a specific macrophage subpopulation and/or phenotype was important in the progression of nephropathic cystinosis. In order to decrease the time needed to study these factors, we have chosen to accelerate the timeframe to renal failure by performing a uninephrectomy.

a) *Accelerating nephropathic cystinosis through uninephrectomy*

We performed uninephrectomies on Ctns-/- mice at 6 months of age and analyzed them for interstitial fibrosis by picrosirius red staining at 9 months of age. Our previous progress report validated our model demonstrating a 30% increase in renal fibrosis after uninephrectomy compared to normal Ctns-/- mice (*P<0.01: 9mos normal vs. UniNx*).

As discussed in our previous progress report, we have performed FACS analysis on macrophage subpopulations in the nephropathic cystinotic kidneys at mild (6m) to moderate-severe fibrosis (12m). Macrophages (MΦ) are a heterogenous population and can be divided functionally by their phenotype: M1 – classically activated/pro-inflammatory; and M2 – reparative/wound healing, regulatory, and pro-fibrotic. However, distinct M2 markers to correlate these three phenotypes in the kidney is continuing to evolve. It is fairly well accepted that M1 activation (LPS/IFNγ) is a pro-inflammatory phenotype, but the identification of distinct M2 subsets are still under intense investigation. We have continued to characterize the macrophage subpopulations in the cystinotic kidney with basic macrophage 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. In our early progress reports, we found that the number of M1-type MΦ

(Ly6c$^{med/hi}$) almost doubled between 4 to 6 months and suggests that these cells may initiate an early pro-inflammatory response. Since that time we have analyzed more mice and have focused on the later time points.

Since our last progress report, we have added additional mice and have sufficient statistical power to analyze our findings (Table 1).

	9 month	**12 month**	**9 mos UniNx**
Dendritic	8636 ± 3017	10813 ± 3895	5611 ± 2822
Phagocytic Dendritic	39540 ± 12686	66212± 23092*	12316 ± 1617* ¥¥
M1 Macrophage	41150 ± 11928	90938 ± 30577**	23888 ± 5628¥¥
Regulatory Ly6c lo	27349 ± 9737	12386 ± 6378**	18186 ± 3532

Table 1: Macrophage subpopulations in Ctns-/- mice after uninephrectomy. Events were gated for viable cells and CD45+, normalized to 10^6 cells. The average from three replicates from each mouse was analyzed (5-7 mice/group). (*dendritic – CD11c+ F4/80-; phagocytic dendritic – CD11c+ F4/80+; M1 macrophage – CD11b+ Ly6c$^{med/hi}$; regulatory – CD11b+ Ly6c^{lo}*); * P<0.05: compared to 9m; ** P<0.01: compared to 9m; ¥¥P<0.01: 12m vs 9m uninephrectomy (UniNx).

During the progression from mild to moderate-severe fibrosis (12m) there was a significant increase in the M1 macrophage population by 120% and is the predominant subpopulation within the cystinotic kidney. Phagocytic dendritic cells increased by 67% but regulatory macrophages decreased by 55%. Unfortunately, despite the increase in fibrosis seen in our uninephrectomy model, it has a significantly different pattern in regards to the M1 and phagocytic subpopulation with down-regulation in both groups (P<0.01). However, the M1 and phagocytic subpopulations are the most predominant in the UUO model (*Lin SL et al, J Immunology, 2009*) and will serve as a better model in vivo in which to isolate and elucidate Ctns-/- macrophage function.

Based on our preliminary data on M1/M2 activation in Ctns-/- bone marrow derived macrophages (BMDM), we hypothesize that the predominance of these two populations, M1 and phagocytic, is related to aberrant macrophage activation in Ctns-/- that has a predisposition toward pro-inflammatory and oxidant gene expression (discussed below). In the next 6 months, we will focus on characterizing the phenotype of the Ctns-/- macrophage in a profibrotic microenvironment (UUO).

AIM #2. To investigate differences in the cytokine production and fibrogenic responses of *Ctns*+/+ and *Ctns*-/- macrophages in response to apoptotic tubular cells using an *in vitro* model system.

As mentioned previously, we have begun to analyze the M1 and M2 response of BMDM. In brief, bone marrow isolated from both Ctns-/- and wild type C57BL6 mice was harvested and differentiated into macrophages with M-CSF for 7 days, and stimulated with M1 (LPS/IFNγ) or M2 (IL4) and cells were harvested after 24h. Total RNA was obtained, cDNA was generated and semi-quantitative real-time qPCR was performed. In our last report, we presented preliminary data from a cytokine qPCR array (SABiosciences) on BMDM that demonstrated vast signaling differences between Ctns-/- and wild type.

M1

Gene	Fold Regulation (Ctns ko:wild-type)
Bmp2	-2.66
Bmp4	-2.26
Bmp6	-2.26
Ccl3	-6.74
Ccl4	-7.18
Ccl5	-2.14
Cntf	1.99
Cxcl16	2.16
Cxcl3	2.51
IL12b	-3.07
IL18	1.93
IL1a	2.20
IL1b	-2.11
IL23a	3.43
IL6	-1.50
Tnfsf10	3.23
Vegfa	3.20

M2

Gene	Fold Regulation (Ctns ko:wild-type)
Bmp2	-6.63
Bmp4	1.99
Bmp6	2.81
Ccl12	2.12
Ccl5	-3.53
Cntf	3.01
Cxcl12	-7.27
Csf1	-1.86
Csf2	2.12
Csf3	5.31
IL12b	3.90
IL1b	-2.09
IL23a	1.99
IL6	4.74
Lif	-4.24
Osm	3.34
Tnfsf11b	-4.99

Table 2 and 3: M1 and M2 gene regulation in Ctns-/- macrophages. Semi-quantitative qPCR array normalized to 5 housekeeping genes. (n=3 Ctns-/- and n=1 wild-type) Relative fold regulation Up Down.

We subsequently have focused our investigation on specific pro-inflammatory and profibrotic genes important in kidney fibrogenesis. In addition, we examined whether the differences in gene expression could be normalized with treatment with cysteamine bitartrate added at the time of M1/M2 activation (Figure 1).

At baseline, Ctns-/- have a 43-fold increase in *iNOS* (*inducible nitric oxide synthase*) expression (P=0.03) and with M1 activation, there is a 6.3-fold increase in *IL6* expression P=0.001) and a 1.5-fold increase in *Nox2* (*NADPH oxidase*, generates superoxide, P=0.03) compared to wild type BMDM. With M2 activation, there was a nearly 6-fold reduction in *IL4 receptor* and a 2.2-fold reduction in *IL10* expression. Both *IL4 receptor* (Lee S et al, JASN, 2011) and *IL10* (Cao Q et al, JASN, 2010) are critical in promoting a reparative M2 phenotype in progressive kidney disease. Interestingly, cysteamine treatment decreased *IL6* expression with M1 activation but remained at significantly elevated levels compared to wild-type and *IL4 receptor* expression remained significantly low. However, both *Nox2* and *iNOS* expression levels worsened, 6.7-fold and 2-fold, respectively, although did not reach significance. However, oxidant generation cannot be correlated with gene expression and further studies have begun to investigate ROS production. *IL10* levels dramatically improved with a nearly 16-fold increase although this did not reach significance (P=0.06) but may be an important factor in the renoprotective mechanism of cysteamine attenuating the fibrotic process in cystinosis. Probably most surprising were the elevated levels of *TGFβ* expression seen with cysteamine treatment and could likely account for the continued progression of CKD in patients well treated with cysteamine.

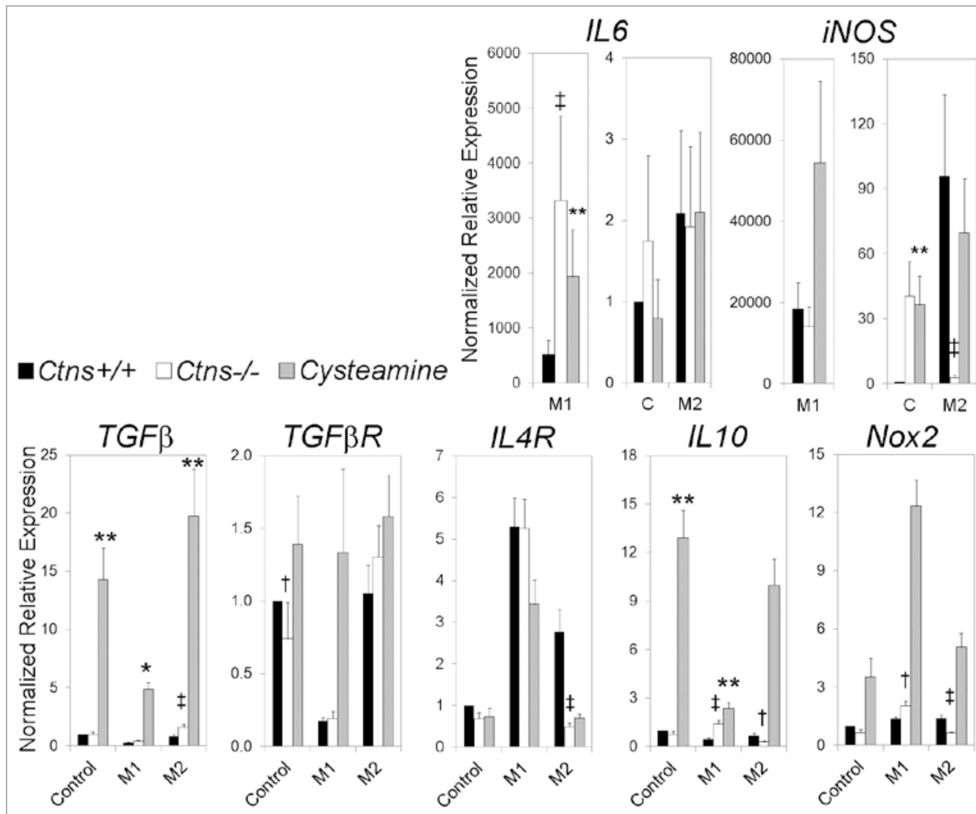

Figure 1. Aberrant macrophage activation. Bone marrow harvested from wild type C57BL6 mice and Ctns-/- mice, 2-3 months of age, and placed in growth media containing M-CSF for 7 days to differentiate into macrophages (BMDM). Cells were seeded at 70-80% confluency and activated with M1 (lipopolysaccharide, LPS, and IFNγ) or M2 (IL4) +/- cysteamine 6nM for 24hours. Cells were lysed and processed for RNA. RNA integrity numbers were determined using the Agilent, only RINs >8 were used to generate cDNA. Semi-quantitative real time RT qPCR was done. Genes were normalized to 18S and GAPDH. Analyzed with REST-MCS software. † $P<0.05$, ‡ $P<0.01$, Ctns-/- vs wildtype; * $P<0.05$, ** $P<0.01$, cysteamine treated vs Ctns-/-.

In the next 6 months, we will perform microarray studies to further understand the range of signaling abnormalities in Ctns-/- macrophages. We will also focus on macrophage functional experiments on migration and phagocytosis, in addition to other assays, to begin to elucidate the mechanisms by which the lysosome transporter cystinosin activates pro-inflammatory, profibrotic signaling pathways.

Grantor Agency:	Cystinosis Research Foundation	Total Award:	$218,966.00
Title of Study: Elucidating the role of cystinosin-deficient macrophages in nephropathic cystinosis			
Principal Investigator: Okamura, Daryl			
		Effective Date of Grant: 8/1/11	
Research Fellow:		Period of this Report: 8/1/12-1/15/13	

Report of Receipts and Expenditures

Receipts:

Payments Received to Date:	164,224.50
Total Available for Expenditure:	**64,731.18**

	Current Expenditures 8/1/12-1/15/13	Cumulative Expenditures	
Salaries and Wages	$22,076.93	$72,991.07	
Supplies and Expenses	$10,075.79	$29,885.15	
Internal Purchase Services	$2,196.62	$7,532.02	
Travel	$0.00	$0.00	
Staff Benefits	$5,876.06	$20,265.54	
Other - Subcontract			
Subtotal - Direct Costs	$40,225.40	$130,673.78	
Indirect Costs - 10%	$4,022.64	$13,067.58	
Total Expenditures	**$44,248.04**	**$143,741.36**	$143,741.36

Unexpended Balance as of : 1/15/13 **$20,483.14**

JoAnn Staheli (signature) 1/30/13

Date

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