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: February 1, 2012 to July 31, 2012

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. Our studies thus far on the uninephrectomy Ctns-/- mice demonstrate that the rate of fibrosis increases, however, the surgical procedure does alter the macrophage subpopulations within the remaining kidney, the significance of which is still unclear. In addition, some exciting preliminary results suggests that Ctns-/- macrophages have aberrant activation responses to M1 (pro-inflammatory) and M2 (pro-fibrotic/regulatory) cytokine stimulation that may provide some insight into their role in fibrosis progression.

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. We found that there was a 30% increase in renal fibrosis after uninephrectomy compared to normal Ctns-/- mice (Figure 1, *P<0.01: 9mos normal vs. UniNx*).

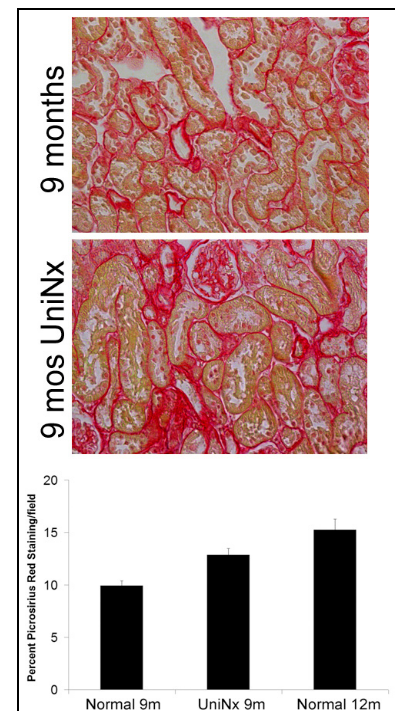

Figure 1: Uninephrectomy accelerates nephropathic cystinosis. Picrosirius red staining was performed on paraffin sections from Ctns-/- mice. (n=3-6/group)

Currently, there are no studies characterizing the macrophage subpopulations within the nephropathic cystinotic kidney as disease progresses. 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 for these three phenotypes are currently lacking and is an active area of investigation by our lab. We have started 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 previous progress report, 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. In addition, the most dramatic changes appear to be occurring in the Ly6c^{lo} and the CD11c+ subpopulations that we speculate may be of the M2 phenotype. Since that time we have analyzed more mice and have focused on the later time points.

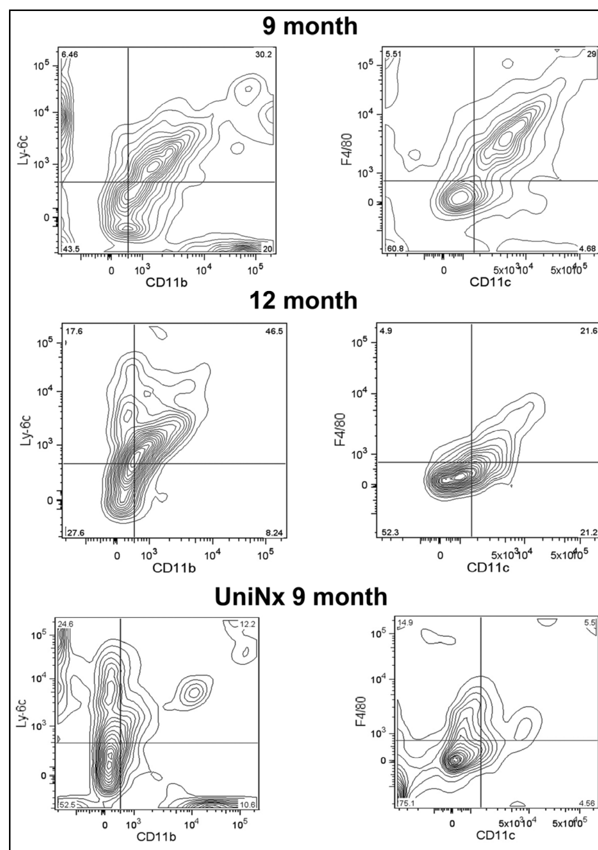

Figure 2: Macrophage subpopulations after uninephrectomy. Uninephrectomy (uniNx) was performed on Ctns-/- mice at 6 months of age and sacrificed after 3 months. UniNx Ctns-/- mice were compared to age matched controls. FACS analysis was performed with representative plots for all time points (n=3-6/group).

FACS analysis of the macrophage subpopulations suggested that there was an increase in all of the subpopulations from 9 months to 12 months except for the regulatory Ly6c^{lo} group but did not reach statistical significance (Figure 2). Interestingly, there was a decrease in all of the subpopulations after uninephrectomy despite an increase in fibrosis (Table 1). However, we only had 3 mice for the 12 month group and more mice are needed to confirm our findings.

	9 month	12 month	9 mos UniNx
Dendritic	8636 ± 3017	13277 ± 3275	5611 ± 2822
Phagocytic Dendritic	39540 ± 12686	44998 ± 17817	12316 ± 1617*
M1 Macrophage	41150 ± 11928	61177 ± 14362	23888 ± 5628
Regulatory Ly6c lo	27349 ± 9737	17378 ± 4592	18186 ± 3532

Table 1: Macrophage subpopulations in Ctns-/- mice after uninephrectomy. Events were gated for viable cells and CD45+. The average from three replicates from each mouse was analyzed (3-5 mice/group). (*dendritic – CD11c+ F4/80-; phagocytic dendritic – CD11c+ F4/80+; M1 macrophage – CD11b+ Ly6c*$^{med/hi}$ *; regulatory – CD11b+ Ly6c*^{lo}; n=3-5/group; * P<0.01: 9m vs UniNx).

In the next 6 months, we will begin to isolate the CD11c and CD11b subpopulations from different time points and analyze their fibrogenic transcriptome signature.

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 in our last progress report, we have begun to analyze the M1 and M2 response of bone marrow derived MΦ from Ctns-/- and Ctns+/+ mice. Bone marrow derived monocytes were isolated from both Ctns-/- and Ctns+/+ mice, differentiated into macrophages with M-CSF, and stimulated with either LPS/IFN-γ (M1) or IL-4 (M2). Total RNA was obtained, cDNA was generated and semi-quantitative real-time qPCR was performed using a qPCR array (SABiosciences). Our preliminary results are based on small numbers (wild-type 1, Ctns-/- 3) but suggest an aberrant response to M1/M2 stimulation.

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.

Although preliminary, these data suggest that macrophage activation/polarization is abnormal in Ctns-/- mice. For example, IL12 and IL6 are M1 cytokines which in Ctns-/- macrophages have completely opposite gene regulation to M1 and M2 stimuli. These data first need to be repeated with increased numbers of replicates, but if confirmed the mechanism and significance will be the subject of future investigation.

Grantor Agency: Cystinosis Research Foundation	Total Award: $218,966.00
Title of Study: Elucidating the role of cystonsin-deficient macrophages in nephropathic cystinosis	
Principal Investigator: Daryl Okmaura, MD	
Co. Principal Investigator: Allison Eddy, MD	Effective Date of Grant: 8/1/11
Research Fellow:	Period of this Report: 2/1/12-7/31/12

Report of Receipts and Expenditures

Receipts:

Payments Received to Date:	109,483.00
Total Available for Expenditure:	**62,323.00**

	Current Expenditures 2/1/12-7/31/12	Cumulative Expenditures	
Salaries and Wages	$27,517.92	$50,914.14	
Supplies and Expenses	$8,845.53	$19,809.36	
Internal Purchase Services	$3,604.64	$5,335.40	
Travel		$0.00	
Staff Benefits	$7,607.56	$14,389.48	
Other - Subcontract			
Subtotal - Direct Costs	$47,575.65	$90,448.38	
Indirect Costs - 10%	$4,757.67	$9,044.94	
Total Expenditures	**$52,333.32**	**$99,493.32**	**$99,493.32**

Unexpended Balance as of : 7/31/2012 **$9,989.68**

8/8/12
Date

Authhorized by: JoAnn Staheli
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Office of Research Finance