Diabetic Complications Consortium

Application Title: Immune Mechanisms in Diabetic Nephropathy

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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) in the US and in many developed countries around the world. Along with hyperglycemia, activation of the renin-angiotensin system (RAS) is arguably the most well established causative factor in DN, as evidenced by the efficacy of RAS antagonists in delaying progression of DN in humans. Nonetheless, understanding of the pathogenesis of DN is limited and there is a significant unmet need for new, specific therapies to prevent or reverse kidney damage in DN. While susceptibility to DN is strongly influenced by inherited factors, specific genetic mechanisms conveying predisposition to DN have not been identified. Elucidation of such factors could lead to novel approaches for treatment.

Our laboratory has developed a mouse model, combining type 1 diabetes with chronic, low-grade activation of the RAS, which recapitulates key features of human DN including: (1) hypertension, (2) robust albuminuria, (3) nodular glomerulosclerosis, (4) accumulation of inflammatory cells in the renal interstitium, (5) dependence on RAS activation, and (6) a powerful influence of genetic background on susceptibility to kidney disease. In microarray studies of glomerular mRNA, we have found that the development of DN is this mouse line is associated with broad up-regulation of gene expression patterns associated with immune activation and inflammation. Moreover, we find discordant mRNA signatures between mouse strains that are susceptible (S) or resistant (R) to DN, with activation of immune and inflammatory pathways in the S strain and broad suppression of these pathways in the R strain. Accordingly, we hypothesized that activation of the immune system is a key determinant of susceptibility to DN. RAS activation, which is critical for the development of DN in humans and in this mouse model, has powerful immunomodulatory effects. Therefore, we suggested that stimulation of adaptive and/or innate immune responses in DN is facilitated by angiotensin II acting via type 1 angiotensin (AT_1) receptors in specific populations of immune and inflammatory cells. Although a role for inflammatory mechanisms in diabetic complications has been suggested previously, the specific contribution of inflammation to DN and the molecular triggers for immune activation in this setting are not clear. We will explored these issues using a combination of cell-specific gene targeting and disease modeling in mice and we determined the activation of immune responses and development of chronic inflammation in DN in order to identify pathways related to renal injury.

1. Project Accomplishments:

To examine the contribution of Ang II signaling in immune cells to the pathogenesis of DN, mice were generated in which AT_{1A} receptors were deleted from T cells (T cell KOs) and macrophages (Mac KOs) using *Cre-Lox* gene targeting. To target T cells, we used transgene in which *Cre* recombinase expression was driven by the CD4 promoter. During maturation of T cells in the thymus, all T cells transiently express CD4, so the *CD4-Cre* transgene will delete floxed target genes in all T cells. Deletion of AT_{1A} receptors from macrophages was accomplished using the *LysM-Cre* transgene, which drives *Cre* expression specifically in macrophages. In both cases, mice on 129 strain background bearing a conditional AT_{1A} receptor allele (*129/SvEv Agtr1a*$^{flox/flox}$) were crossed with the respective Cre transgenic mice, also on inbred 129 genetic background yielding: *129/SvEv CD4-Cre*$^{+}$*Agtr1a*$^{flox/flox}$ *(T cell KO) and 129/SvEv LsyM-Cre*$^{+}$*Agtr1a*$^{flox/flox}$ (*Mac KO*). For all experiments, *Cre*$^{-}$ *Agtr1a*$^{flox/flox}$ *129/SvEv* mice were used as Controls. T cell-specific deletion of AT_{1A} receptors was confirmed by separating and isolating T cells from spleen using flow cytometry. Macrophage-specific deletion is determined using IP injection of BioGel-P 100 beads to stimulate peritoneal macrophages, which are subsequently harvested and double-positive macrophages ($F4/80^{+}$ and $CD11b^{+}$) are isolated by FACS for analysis of AT_{1A} receptor expression by RT-PCR.

In order to thoroughly characterize the role of AT_{1A} receptors on immune cell populationsin DN pathogenesis, we utilized 2 different models of DN. First, we used uni-nephrectomized 129 Akita mice. We have previously found that nephrectomy in 129 Akita mice accelerates the development of albuminuria and renal injury. These animals are generated by intercrossing 129-Akita mice separately with *CD4KOs* and *Mac KOs*. For the

second DN model we utilized the Akita-Renin transgene model described above. Similarly, these animals were generated with a second intercross with the *129-ReninTg* strain.
Accordingly, along with the hypothesis testing studies, we generated the following 6 strains of multi-transgenic mouse lines, all on inbred *129/SvEv* background:

- *Mac KO* (*LysM Cre*$^{+}$ *Agtr1a*$^{flox/flox}$)
- *Mac KO-Akita* (*LysM Cre*$^{+}$ *Agtr1a*$^{flox/flox}$*Ins2*^{C96Y})
- *Mac KO-Akita ReninTg* (*LysM Cre*$^{+}$ *Agtr1a*$^{flox/flox}$*Ins2*^{C96Y}*ReninTg*$^{+}$)
- *T cell KO* (*CD4 Cre*$^{+}$ *Agtr1a*$^{flox/flox}$)
- *T cell KO-Akita* (*CD4 Cre*$^{+}$ *Agtr1a*$^{flox/flox}$*Ins2*^{C96Y})
- *T cell KO-Akita ReninTg* (*CD4 Cre*$^{+}$ *Agtr1a*$^{flox/flox}$*Ins2*^{C96Y}*ReninTg*$^{+}$)

Along with complex and time-consuming breeding involved in generation of the mouse lines described above, we have also made significant progress in experiments specifically addressing the **Specific Aims** of our proposal described below. As it happened, the *Mac KO* lines were generated more quickly than the *T cell KO*s and thus, progress in the studies under Specific Aim 2 are more advanced. Nonetheless, we have all the requisite lines in hand and expect to complete all of the proposed work within the next 8 months.

2. Specific Aims:

Specific Aim 1. ***Define the role of T cell activation by angiotensin II in the development of DN.***

Results: While progress with development of the *T cell KO* lines has been slower than expected, these mice have been generated and preliminary data for these mice are being collected. The uninephrectomy *T cell KO* Akita mice study should conclude in 4 months. Male *T cell KO Akita Renin Tg* mice have been generated and analysis of their phenotypes is in progress and will be carried out over the coming 8 months.

Specific Aim 2. ***Determine whether modulation of macrophage function by the RAS influences the pathogenesis of DN.***

Results: Male *Mac KO Akitas* and *Cre*$^{-}$ *Akita Controls* were generated (n=11 per group), underwent unilateral nephrectomy at 10 wks of age, and have completed phenotyping. After 16 weeks of study, there was no significance difference in body weight (26±0.8 gm vs 26±0.6 gm) or degree of hyperglycemia (594±44 mg/dl vs 596±41 mg/dl). Albumin excretion in the experimental groups is shown in **Figure 1A**. There was no difference in the modest levels of albuminuria between *Control*

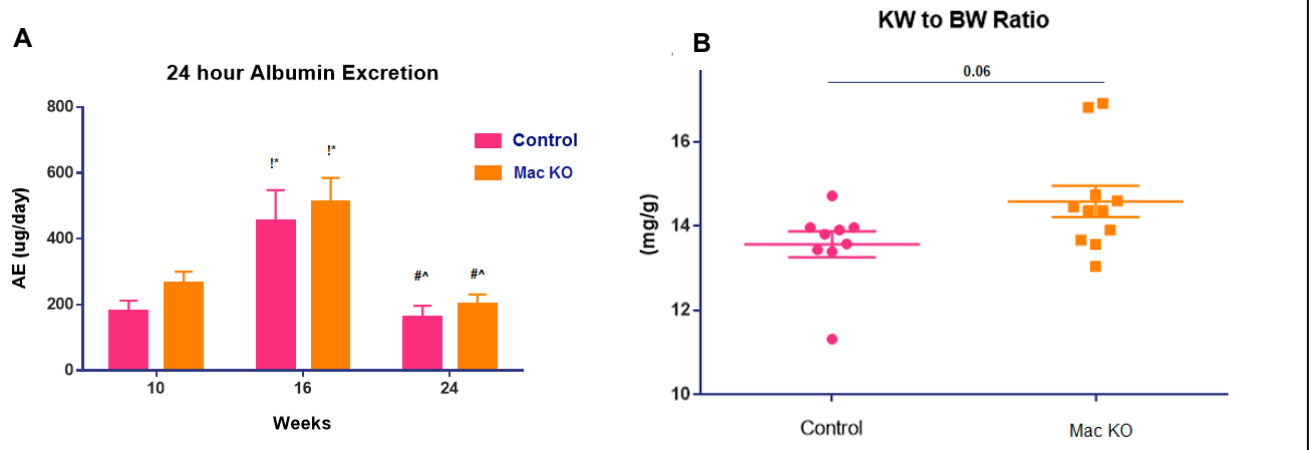

Figure 1. **Albuminuria and Renal Hypertrophy Phenotype**. A) 24 hour albumin excretion rate for male Mac KO and Control Akita mice from baseline (10 weeks of age) to 24 weeks of age (14 weeks after uninephrectomy). !p<0.01 in comparison to 10 wk control, *p<0.01 in comparison to 10 wk Mac KO, #p<0.05 in comparison to 16 wk control, ^p<0.01 in comparison to 16 wk Mac KO. B) Kidney weight to body weight ratio uninephrectomized male Mac KO and Control Akita mice at 26 weeks of age (16 weeks post uninephrectomy).

Akita mice and *Mac KO Akitas* at 10 weeks and before nephrectomy (179 ±33 µg/day and 265±35 µg/day). By 16 weeks of age (6 weeks after uninephrectomy), albumin excretion had increased significantly in both *Control Akita* and *Mac KO Akita* mice, but there was no difference in the absolute levels of albuminuria between the groups (455±93 µg/day vs. 512±73 µg/day, respectively). At 24 weeks of age (14 weeks after uninephrectomy), albumin excretion was significantly reduced from 16 weeks in both *Mac KO* and *Control Akita* mice, but there was no difference in absolute albuminuria levels between the groups (199±32 µg/day and 162±35 µg/day, respectively). There was also not a significant difference in kidney weight to body weight ratio between *Mac KO* and *Control Akita* mice (14±0.3 mg/g and 15±0.4 mg/g, $p<0.06$, respectively) as shown in Figure 1B. We have also carried out preliminary histopathological analysis of kidneys from *Control* and *Mac KO* animals. Kidney sections were stained for fibrosis (Masson's Trichrome), mesangial expansion (PAS), T cell infiltration (anti-CD3), and macrophage infiltration (anti-F4/80). Representative immunostained sections are shown in **Figure 2A**. There were no apparent differences in T cell of macrophage infiltration between the groups. We also graded the extent of glomerular injury using a sem-quantitative scale. As shown in Figure 2B, there was no significant difference in the extent of glomerular injury between *Mac KO* and *Control Akita* mice

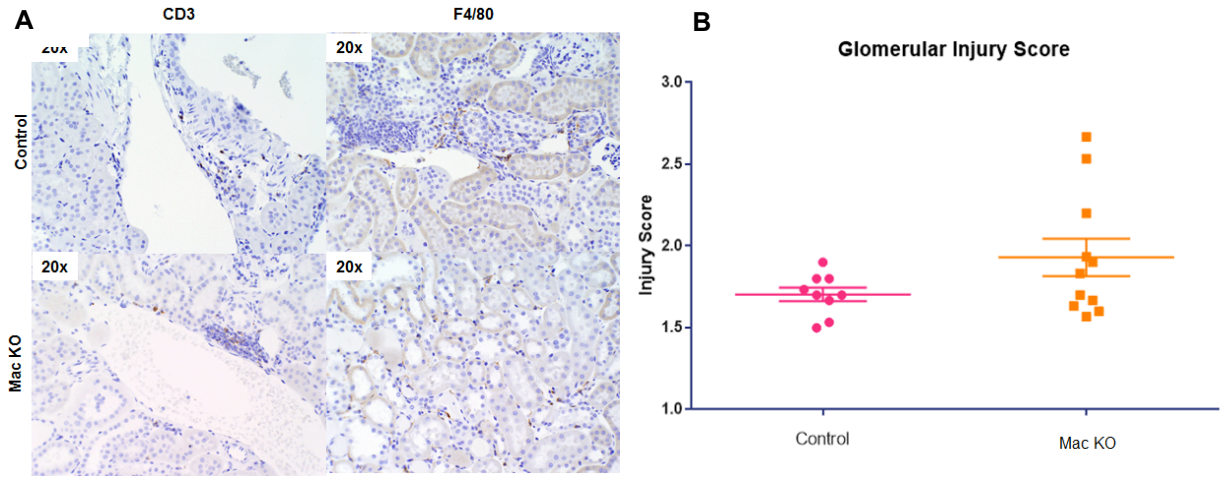

Figure 2. **Histology analysis**. A) 20X histology kidney cortex stained for CD3 (T Cell marker) and F4/80 (macrophage marker) in Mac KO and Control Akita mice at 26 weeks of age. B) Glomerular injury score for Mac KO and Control Akita mice at 26 weeks of age (the scoring scale used was 1 to 4).

From our initial results we conclude that elimination of AT_{1A} receptors from macrophages did not clearly affect the severity of kidney disease in the Akita mice with uninephrectomy. However, we hypothesize that superimposing RAS activation in combination with hyperglycemia in the *Akita RaninTg* model may uncover a more substantial contribution of AT_{1A} receptors on macrophages in the progression of diabetic nephropathy. Male *Mac KO Akita Renin Tg* mice have been generated and analysis of their phenotypes is in progress and will be carried out over the upcoming 8 months.

3. Publications:

None.