The Role of *Smad4* in Murine T Cells in Terms of IgA Nephropathy and Potential Mechanism of Glomerular IgA Deposition

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A link between glomerular IgA nephropathy (IgAN) and T helper 2 (Th2) response has been suggested; however, the mechanisms are poorly defined because of the lack of an appropriate model. Previously, we reported a novel murine model characterized by lineage-restricted deletion of the gene encoding MAD homologue 4 (*Smad4*) in T cells (*Smad4^{co/co}; Lck-cre*) and found that *Smad4^{co/co}; Lck-cre* mice exhibited massive glomerular IgA deposition, increased albumin creatinine ratio, aberrant glycosylated IgA, IgA complexed with IgG1 and IgG2a, and polymeric IgA, all known features of IgAN in humans. These findings indicate that *Smad4^{co/co}; Lck-cre* mice could be a useful model for studying the mechanisms between IgAN and Th2 response, and furthermore, the disruption of *Smad4*-dependent signaling in T cells may play an important role in the pathogenesis of human IgAN and contribute to a Th2 T-cell phenotype.

Key words: IgA nephropathy, *Smad4* signaling, regulatory T cell, Th2 cytokines

Introduction

IgA nephropathy (IgAN) is the most common form of glomerulonephritis throughout the world. The disease leads to progressive renal failure in a substantial proportion of patients. Despite an expanding international research effort over 40 years, the mechanisms of pathogenesis are still obscure. The hallmarks of this disease are hematuria, variable degrees of proteinuria, low grade proliferative glomerulonephritis and immune deposition of predominantly IgA in the renal mesangium. There is frequently dysregulation of circulating IgA in patients with IgAN; notably, the elevated levels of IgA in serum are aberrantly glycosylated and biased towards higher molecular weight isoforms, and bound in complexes with IgG that are specific for truncated O-glycans¹⁻⁴⁾.

Skewed T helper 2 (Th2) cytokine production has been implicated in the pathogenesis of IgAN. Compared to other forms of glomerulonephritis, IgAN is more common in industrialized nations;

this suggests that less exposure to microorganisms results in decreased T helper 1 (Th1) response and increased Th2 response⁵⁾. Moreover, patients with IgAN exhibit bias toward Th2 cytokine production during disease exacerbation, but heightened secretion of Th1 cytokines during remission⁶⁾. Both human and murine B cells *in vitro* produce higher IgA levels in response to a mixture of recombinant interleukin (IL) -4 and IL-5 (Th2 cytokine) compared to control cultures, and mRNA levels of both core 1 β 1,3-galactosyltransferase and its molecular chaperone Cosmc, as well as β 1,3-galactosyltransferase enzymatic activity are down-regulated by recombinant IL-4 in human B cells⁷⁾. These results suggest that skewed Th2 cytokine production leads to elevated levels of abnormally glycosylated IgA in the serum of patients with IgAN.

Recently, we generated conditional knockout mice in which the gene encoding *Smad4* is deleted selectively in T cells (*Smad4^{co/co}; Lck-cre*) by crossing mice homozygous for a *Smad4* allele flanked with the *lox* sequence with mice expressing a transgene

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encoding a *Cre* recombinase driven by the *lck* promoter⁸). Interestingly, these *Smad4*^{co/co; Lck-cre} mice have higher serum IgA levels than WT mice, and their T cells produce higher levels of Th2 cytokines (e.g. IL-4, IL-5, IL-6 and IL-13) when stimulated *in vitro*. In this article, we demonstrate our published data which shows that the *Smad4*^{co/co; Lck-cre} mice, Th2 prone mice, have IgAN-like phenotype⁹) and propose a possible mechanism with new data in order to answer “how does IgA deposit to glomeruli?”

Disruption of *Smad4* expression in T cells leads to IgA nephropathy-like manifestations

1. Predominant IgA deposition in glomeruli of *Smad4*^{co/co; Lck-cre} mice

By immunofluorescence, intense IgA deposits were detected in the glomerular mesangium in most T cell-specific *Smad4* deficient (*Smad4*^{co/co; Lck-cre}) mice, whereas trivial IgA deposits were found in control, wild type (*Smad4*^{+/+}; *Lck-cre*, *Smad4*^{co/co}; designated WT) mice (Figure-1A). Weak C3 deposits in mesangial areas and Bowman's capsule were detected in both *Smad4*^{co/co; Lck-cre} and WT mice. By light microscopic

examination of periodic acid-Schiff (PAS) stained sections, the kidneys of *Smad4*^{co/co; Lck-cre} mice exhibited no marked abnormalities (Figure-1A). Specifically, mesangial proliferation, expansion of mesangial matrix, and other glomerular (or extraglomerular) changes were not detected in *Smad4*^{co/co; Lck-cre} mice relative to WT mice.

2. Glomerular function in *Smad4*^{co/co; Lck-cre} mice

Excretion of albumin in the urine, especially when expressed as an albumin to creatinine ratio (ACR), was significantly higher in *Smad4*^{co/co; Lck-cre} mice than in controls (Figure-1B). Although there is no statistical difference between *Smad4*^{co/co; Lck-cre} mice and controls, there was a trend toward an increase in urinary hemoglobin (after freeze-thaw, as a measure of hematuria) in *Smad4*^{co/co; Lck-cre} mice when compared to controls (Figure-1B).

3. Characteristics of circulating IgA in serum of *Smad4*^{co/co; Lck-cre}

As reported previously⁸), the levels of IgA in the serum from *Smad4*^{co/co; Lck-cre} mice at 3 months of age were dramatically increased compared with age-matched WT mice (Figure-2A). We next analyzed

Figure-1 Deletion of *Smad4* in T cell induced predominant IgA deposition in glomeruli and proteinuria

A. Immunofluorescence studies showed glomerular deposition of predominantly IgA, with lesser amounts of IgG and IgM deposition in *Smad4*^{co/co; Lck-cre} mice compared with WT mice at 3 months of age. C3 deposition was occasionally observed in both mesangial areas and Bowman's capsule in *Smad4*^{co/co; Lck-cre} mice, but was restricted to Bowman's capsule in WT mice. PAS staining showed no significant difference between WT and *Smad4*^{co/co; Lck-cre} mice. Original magnification; x400. B. Increased albumin creatinine ratio (ACR) was observed in urines of *Smad4*^{co/co; Lck-cre} mice (n = 9) compared with WT mice (n = 9) (0.043 ± 0.005 vs 0.083 ± 0.007). C. There is no significant difference in the urinary hemoglobin between WT (n = 10) and *Smad4*^{co/co; Lck-cre} mice (n = 10) (14.7 ± 1.94 vs 21.2 ± 2.36). Data are expressed as the mean ± SEM from one of three experiments with similar results; p values were calculated with Student's *t* test. *** p < 0.001

the pattern of glycosylation of IgA; the relative levels of terminal sialic acid (Figure-2B) and terminal or penultimate galactose (Figure-2C) on serum IgA from *Smad4*^{co/co; Lck-cre} mice significantly decreased compared with those on the IgA from WT mice. By sandwich ELISA, the levels of complexes of IgG1-IgA and IgG2-IgA in serum from *Smad4*^{co/co; Lck-cre} mice were significantly higher than those in WT mice (Figure-2, D and E). Finally, Western blotting revealed that the circulating IgA in *Smad4*^{co/co; Lck-cre} mice is predominantly polymeric, whereas most of IgA from WT mice is monomeric (Figure-2F).

4. mRNA levels of β 4GalTs in B-cells from *Smad4*^{co/co; Lck-cre} mice

In mice, β 1, 4-galactosyltransferases (β 4GalT) are involved in the synthesis of IgA glycosylation, and seven β 4GalT genes (β 4GalT1 to 7) have been isolated so far. To determine which β 4GalT is associated with the aberrant glycosylation, β 4GalT (1 to 7) mRNA expressions in purified B cells from *Smad4*^{co/co; Lck-cre} mice and WT mice were analyzed by real-time RT-PCR using housekeeping GAPDH gene as the internal control. Real-time RT-PCR revealed that expression of mRNAs for β 4GalT2 and 4 appeared to be significantly decreased in B-cells from *Smad4*^{co/co; Lck-cre} mice as compared with B cells from WT mice (Figure-2G).

Figure-2 Characteristics of circulating IgA in mice lacking Smad4 in T-cells are similar to those in patients with IgA nephropathy

A. Markedly increased serum levels of IgA in *Smad4*^{co/co; Lck-cre} mice (n=8) compared with WT mice (n=8) (1773 $\pm$ 294.3 vs 545.2 $\pm$ 71.39). B and C. Aberrant glycosylation of circulating IgA from *Smad4*^{co/co; Lck-cre} mice (n=6, black square) and WT mice (n=6, black triangle) was examined by lectin ELISA. Both sialic acid and galactose on circulating IgA from the mutants were lower than those from controls. Data are expressed as the ratios of specific lectin-binding IgA to total IgA. D and E. *Smad4*^{co/co; Lck-cre} mice (n=6, black square) exhibit an increased in both IgG1-IgA complexes and IgG2a-IgA complexes in the circulation, compared with WT mice (n=6, black triangle). Data are expressed as mean $\pm$ SEM from one of three experiments with similar results; p values were calculated with *t* test or Bonferroni post test. ****p* < 0.001, ***p* < 0.01, and **p* < 0.05. F. The predominance of polymeric IgA was observed in serum from *Smad4*^{co/co; Lck-cre} mice, but not in WT mice, as determined by Western blot. G. mRNA was isolated from purified B-cells (>95% CD19+), and RT-PCR was performed using primers specific for β 4GalT1 to 7, and GAPDH. β 4GalT 2 and 4 mRNA expression in *Smad4*^{co/co; Lck-cre} mice (n=4, black column) were significantly reduced relative to controls (n=4, white column). All samples were normalized to GAPDH levels. Data are expressed as mean $\pm$ SEM; p values were calculated with *t* test. **p* < 0.05

β -actin as a potential target antigen specified by abnormal IgA in *Smad4*^{co/co; Lck-cre} mice

The strong mesangial IgA deposition, albuminuria and presence of structural derangements in circulating IgA in *Smad4*^{co/co; Lck-cre} mice are evocative of human IgAN. One mechanism whereby the abnormal circulating IgA might accumulate in the mesangium and promote proteinuria posits that the IgA has autoimmune specificity for one or more antigens expressed by mesangial cells. Emergence of autoantibodies is more likely in hosts with defective function, such as the *Smad4*^{co/co; Lck-cre} mice. To assess this possibility in *Smad4*^{co/co; Lck-cre} mice, we probed wild type mesangial cell lysates by Western blotting with sera from *Smad4*^{co/co; Lck-cre} and WT mice. A robust protein band of approximately 43 kD, and a weaker 32 kD band, were specifically detected by circulating IgA from *Smad4*^{co/co; Lck-cre} mice, but not by circulating IgA from WT mice (Figure-3A). A band at approximately 82 kD was identified by circulating IgA from

both *Smad4*^{co/co; Lck-cre} and WT mice. The 43 kD band was excised from the gels and subjected to in-gel digestion followed by mass spectroscopy; the most probable mesangial cell protein target is β -actin. Accordingly, wild type mesangial cell lysate was subject to immunoprecipitation with serum from either *Smad4*^{co/co; Lck-cre} or WT mice, using anti-mouse IgA conjugated to Sepharose. Subsequently, the precipitates were separated by electrophoresis and probed by Western blotting using antibodies specific for β -actin and IgA. As shown in Figure-3B, the anti- β -actin antibody stained a band of 43 kD in lanes loaded with immunoprecipitates from *Smad4*^{co/co; Lck-cre} mice, but this band was not found in immunoprecipitates from WT mice. Furthermore, by double immunofluorescence, expression of β -actin was confined to glomeruli of both *Smad4*^{co/co; Lck-cre} and WT mice, whereas co-localization of IgA with β -actin was only observed in glomeruli of *Smad4*^{co/co; Lck-cre} mice, and not in those

Figure-3 Circulating IgA from *Smad4*^{co/co; Lck-cre} mice binds to β -actin in mesangial cells

A. Blots of mesangial cell lysate incubated with serum samples from *Smad4*^{co/co; Lck-cre} (right) or WT mice (left), followed by anti-mouse IgA antibody. A consistent band at approximately 43 kD (arrow) in mouse mesangial cell lysate was identified by circulating IgA from the mutants, but not controls. B. Mixtures of mesangial cell lysate and serum samples from either *Smad4*^{co/co; Lck-cre} or WT mice were immunoprecipitated with anti-mouse IgA-Sepharose. The immunoprecipitates were then separated by on SDS-PAGE, under reducing conditions and blotted onto nitrocellulose. The membrane was incubated with anti- β -actin (top) or anti-mouse IgA (bottom) antibody. The (~43 kD) bands detected by anti- β -actin antibody were present only in Lanes loaded with immunoprecipitates formed with serum from *Smad4*^{co/co; Lck-cre} mice.

Figure-4 Glomerular IgA deposition was colocalized with β -actin in glomeruli from *Smad4*^{co/co; Lck-cre} mice. Double immunofluorescent staining with (A, D, G, and J, red) β -actin and (B, E, H, and K, green) IgA was performed to evaluate the colocalization in glomeruli from (G-L) *Smad4*^{co/co; Lck-cre} and (A-F) WT mice. Magnified images are D-F and J-L. The staining of β -actin is limited in glomeruli from both (G and J) the mutant and (A and D) controls. Overlayed images show colocalization of IgA deposition with β -Actin in glomeruli from (I and L) the *Smad4*^{co/co; Lck-cre}, but not (C and F) WT mice. Original magnification; $\times 400$

of WT mice (Figure-4).

Discussion

In our studies, *Smad4*^{co/co; Lck-cre} mice develop mesangial IgA deposits associated with increased urinary albumin excretion and dysregulation of IgA synthesis. The latter is manifest by increased serum IgA with aberrant glycosylation that includes an elevated proportion of polymer and that binds to IgG to form mixed isotype complexes. These are features in common with IgA isolated from patients with IgAN. In addition, we investigated a potential mechanism of glomerular IgA deposition in these mutant mice.

Smad4 is a key molecule that influences T cell differentiation; as part of a complex with Smad2 and Smad3, Smad4 serves as a transcription factor for forkhead box P3 (Foxp3), driving the expression of genes important for the genesis of regulatory T cells (Tregs). Although the *Smad4*^{co/co; Lck-cre} mice represent a useful model for the investigation of the balance between Th2 and Tregs, the means by which deletion of Smad4 promotes bias towards Th2 function remains unclear. A reciprocal relationship between Th2 cells and Tregs is recognized. The transcriptional activator STAT6 enhances the expression of the master regulator of Th2 differentiation, GATA3. STAT6 and GATA3 synergize with STAT5 activation to induce the secretion of Th2 cytokines by activated Th2 cells. Apparently, Foxp3 can bind directly to GATA3; engagement in this heterodimeric complex diminishes the activity of each binding partner. The loss of *Smad4* would likely diminish Foxp3, freeing GATA3 from inhibition while simultaneously impairing differentiation of Tregs. Indeed, a progressive decline in mucosal Treg has been described in the *Smad4*^{co/co; Lck-cre} mice and in *Smad3*^{-/-} mice¹⁰. Upon stimulation of T cells, overproduction of Th2 cytokines, unopposed by Tregs, might lead to exaggerated production of IgA that bears aberrant glycans. Several reports indicate that, *in vitro* and *in vivo*, Th2 cytokines lead to increased production and defective glycosylation of IgA. This interpretation is also supported by a recent report showing that upon OVA challenge, glomerular IgA deposition was detected in only double-transgenic mice that overexpress GATA3 and the ovalbumin (OVA)

-specific T cell receptor¹¹.

The phenotype of *Smad4*^{co/co; Lck-cre} mice is reminiscent of human IgAN and suggests that loss of Smad4-signaling in T cells associates with IgA deposition in the mesangial area, dysregulation of IgA, and proteinuria. However, there are some discrepancies between *Smad4*^{co/co; Lck-cre} mice and spontaneous IgAN in humans. Notably, strong C3 deposition associated with mesangial IgA, morphological changes in glomeruli and hematuria are generally observed in human IgAN, but were not observed in *Smad4*^{co/co; Lck-cre} mice. These discrepancies may represent requirements beyond glomerular IgA deposition, such as complement activation and/or inflammatory mediators, for evolution of hematuria and/or morphologic expression of glomerular injury. Recently, a genome-wide association study (GWAS) of human IgAN identified a major IgAN susceptibility locus within the complement factor H gene (CFH) cluster at chromosome 1q32. As CFH regulates complement activation through inhibition of the C3 convertase, the GWAS suggests that dysregulation of complement activation could be one of the mechanisms underlying progressive glomerular dysfunction. In addition to relatively minor glomerular complement deposition in spite of severe IgA deposition, the lack of hematuria and morphologically evident glomerular injury in *Smad4*^{co/co; Lck-cre} mice may be explained by the strain of these knockout mice. According to reports in different murine models, glomerular dysfunction caused by IgA deposition is more pronounced in the BALB/c strain compared to other backgrounds despite similar mesangial deposition of IgA and C3¹². Although the basis for these strain differences is unclear, we expect that deletion of *Smad4* in BALB/c mice would lead to more morphologic and functional evidence of glomerular injury than observed in the current (C57BL/6 × SvEv129 × FVB) strain.

Understandings of mechanisms of immune deposit formation are very important to elucidate processes of glomerular injury in glomerular diseases because most of glomerular diseases manifest various immune depositions such as IgG, IgA, IgM, C1q, C3, C4, C5, and mannose binding lectin in the glomeruli. Mechanism (s) of glomerular immune deposits formation are generally divided by three categories that (1) Circulating

immune complex trapping; (2) *In situ* immune deposit formation exogenous antigens; (3) *In situ* immune deposit formation endogenous antigens. To examine the immune deposit formation, we focused on the third mechanism. Recent progress in proteomics technology allows us to discover new target antigens readily. For example, the M-type phospholipase A₂ receptor (PLA₂R) in podocytes was identified by proteomics as a target antigen in 70%–80% of patients with idiopathic membranous nephropathy¹³, 20 years after “megalin” was identified as the target antigen in podocytes in a membranous nephropathy rat model (Heymann's nephritis). Although no single pathogenic antigen is recognized by circulating IgA in human IgAN, actin has been implicated in patients with IgAN. In the present study, we showed reactivity of serum IgA from *Smad4*^{co/co; Lck-cre} mice with a dominant 43 kD protein from an extract of mesangial cells; proteomic analysis indicated that β -actin is the likely target antigen. Immunoprecipitation and double immunofluorescence experiments support the conclusion that circulating IgA against β -actin exists in serum from *Smad4*^{co/co; Lck-cre} mice. β -actin is a moderate-sized protein consisting of $\approx$ 375 amino acids and is very highly conserved in most of cells. Cytoskeletal actins, such as β -actin, are involved in the structural integrity and motility of cells; actin interacts and associates with a number of other proteins, including actin-binding proteins/calcium associated proteins, are essential for controlling and regulating actin filament assembly. The binding of specific IgA to β -actin might alter cytoskeletal signal transduction and thereby interfere with cell functions. Immunofluorescence findings for *Smad4*^{co/co; Lck-cre} mice show dominant mesangial IgA deposition. β -actin is ubiquitous, the specific IgA to β -actin in *Smad4*^{co/co; Lck-cre} mice may deposit to mesangial cells as well as podocytes and endothelial cells. It is tough question that why the specific IgA is tended to deposit to mesangial cells, balance between deposition and clearance of IgA in each cell may be attributed.

We conclude that *Smad4*^{co/co; Lck-cre} mice produce heightened amounts of IgA that is mostly polymeric, aberrantly glycosylated, and complexed with IgG. These mice develop mesangial IgA deposits and proteinuria, reminiscent of human IgAN. In addition, we recognized that some of the circulating

IgA in *Smad4*^{co/co; Lck-cre} mice has specificity for β -actin expressed in mesangial cells. *Smad4*^{co/co; Lck-cre} mice could therefore provide us with new insights into the role that skewed Th2 cytokine production plays in altering circulating IgA and/or formation of mesangial immune deposits. Ultimately, these insights might promote the development of a new therapeutic strategy for IgAN.

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