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Innate immunity pathways regulate the nephropathy gene *Apolipoprotein L1*

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Abstract

Apolipoprotein L1 (*APOL1*) risk variants greatly elevate the risk of kidney disease in African Americans. Here we report a cohort of patients who developed collapsing focal segmental glomerulosclerosis while receiving therapeutic interferon, all of whom carried the *APOL1* high-risk genotype. This finding raised the possibility that interferons and the molecular pattern recognition receptors that stimulate interferon production may contribute to *APOL1*-associated kidney disease. In cell culture, interferons and toll-like receptor agonists increased *APOL1* expression by up to 200-fold, in some cases with the appearance of transcripts not detected under basal conditions. PolyI:C, a double-stranded RNA TLR3 agonist, increased *APOL1* expression by upregulating interferons directly or through an interferon-independent, IRF-3 dependent pathway. Using pharmacological inhibitors, shRNA knockdown, and chromatin immunoprecipitation, we found that the interferon-independent TLR3 pathway relied on signaling through TBK1, NF-kB, and Jak kinases, and on binding of IRF1, IRF2, and STAT2 at the *APOL1* transcription start site. We also demonstrate that overexpression of the APOL1 risk variants is more injurious to cells than overexpression of the wild-type APOL1 protein. Our study illustrates that anti-viral pathways may be an important inducer of kidney disease in individuals with the *APOL1* high-risk genotype and identifies potential targets for prevention or treatment.

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Conflict of Interest Disclosure:

David Friedman and Martin Pollak are co-inventors on patents filed by Beth Israel Deaconess Medical Center related to APOL1 diagnostics and therapeutics.

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Introduction

Individuals with genetic variants in the ApolipoproteinL1 (*APOL1*; NM_003661.3) gene have greatly increased risk of kidney disease.[1, 2] The high-risk genotypes are associated with elevated risk (7–29 fold) of hypertension-associated end-stage renal disease (H-ESRD), focal segmental glomerulosclerosis (FSGS), and HIV-associated nephropathy.[1, 3, 4] Only individuals with recent African ancestry carry these risk variants, explaining a large part of the 4–5 fold increased rate of kidney disease in African Americans compared with Caucasians.[3] 10–15% of African Americans, or ≈3.5 million individuals, harbor the high-risk genotypes. Two risk variant alleles appear necessary but not sufficient for the increased risk of kidney disease. The factors that determine which carriers of the high-risk genotypes develop disease and which do not remain unknown.

Little is known about *APOL1* or its polypeptide gene product, APOL1. *APOL1* is a member of a 6-gene family on chromosome 22 that evolved by gene duplication.[5] Only the genomes of humans and a few primate species carry the *APOL1* gene and produce APOL1 protein.[5] Human APOL1 is widely expressed, particularly in the vasculature; it also circulates in the densest HDL subfraction, HDL3.[6–9] Notably, circulating APOL1 is the core component of human trypanolytic factor, providing protection against several subspecies of the African trypanosome *T. brucei*.[10]

The two APOL1 variants associated with kidney disease are both located in the C-terminus. Variant G1 is a set of two mutations, S342G and I384M, which nearly always occur together. Variant G2 is a 6 base pair deletion that eliminates amino acids residues 388N-389Y. While all common APOL1 haplotypes protect against T. brucei, the kidney risk variants likely rose to high frequency at least in part because they protect against T. brucei rhodesiense, the parasite that causes acute African Sleeping Sickness.[1, 11]

Several factors suggest that one or both of the APOL1 risk variants may be gain-of-function mutations rather than loss-of-function mutations as a recessive mode of inheritence would suggest. First, only humans, baboons, gorillas, mangabeys, and mandrills are known to possess the intact *APOL1* gene; even our closest living relative, the chimpanzee, does not carry a functional allele.[5] The absence of *APOL1* in nearly all mammalian species suggests that it is not an essential gene in mammalian, or even primate, kidney development or homeostasis. This hypothesis was extended to humans by identification of an Indian individual infected with the opportunistic trypanosomal pathogen, *T. evansi*, who was shown to be APOL1-null.[12] This middle-aged patient had normal renal function, normal blood pressure, and lacked proteinuria, indicating that APOL1 is dispensable for normal kidney development and function.[13] Thus, risk variant APOL1 may have acquired toxic properties rather than lost attributes essential for kidney health.

The strongest risk factor for *APOL1*-associated kidney disease is infection with HIV (odds ratio=29).[4] HIV is a potent inducer of interferons and other anti-viral innate immune programs, while interferons and other inflammatory factors appear to enhance APOL1 expression.[8, 14] We hypothesized that interferon inducers and the *APOL1* risk genotype may

represent an important gene-environment interaction leading to kidney disease in people of recent African ancestry. To explore this hypothesis, we genotyped a cohort of patients who had developed collapsing FSGS (or collapsing glomerulopathy: CG) after treatment with interferons.[15] We subsequently expanded our study to understand how interferons and other innate immunity pathways may impact *APOL1*-associated kidney disease.

Results

We analyzed *APOL1* genotype in archival renal biopsy tissue from eleven individuals (10 African American, 1 Hispanic) with hepatitis C, multiple sclerosis, melanoma, or interstitial pulmonary fibrosis who developed CG during treatment with IFN alpha (α), beta (β), or gamma (γ).[15] Seven of the 11 biopsies yielded adequate preparations of genomic DNA. All 7 were homozygous for high-risk alleles of *APOL1*, whereas individuals with two high-risk *APOL1* alleles constitute about 12% of the African American population.[1, 3] Two of the 7 samples were homozygous for the G2 deletion, while 5 samples were compound G1/G2 heterozygotes.

Early studies indicated that inflammatory factors, including interferon-γ, could upregulate *APOL1* expression.[8, 14] We compared the ability of type 1 (α and β) and type 2 (γ) interferons to stimulate *APOL1* expression in both endothelial cells and podocytes. In both cell types, the rank order of stimulation was γ>β>α, with 200-fold increases in APOL1 mRNA seen for interferon-γ in endothelial cells (figure 1a,b). Increased expression was validated at the protein level using Western blotting (figure 1d) and immunocytochemistry (figure 1e).

A single *APOL1* transcript was detected under basal conditions, but interferon stimulation of cells revealed new bands consistent with additional transcript variants (figure 1c). Amplification and sequencing showed that the dominant band corresponds to transcript variant 1 (RefSeq: NM_003661), encoding a 398 amino acid APOL1 isoform. Tv2 (RefSeq: NM_145343, encoding a 414 amino acid variant) and tv4 (RefSeq: NM_001136541, encoding a 380 amino acid variant) were observed only after interferon stimulation. Tv1 encodes a signal peptide for endoplasmic reticulum targeting and eventual secretion or trafficking to the plasma membrane. In contrast, the signal peptide cleavage site is not present in tv4, likely leading to intracellular targeting. The transcript variants differ only at the N-termini so they all include the C-terminal amino acids mutated in the risk variants. Thus, APOL1 transcript variants likely encode proteins of distinct localization, and potentially different functions.

Only rare cases of *APOL1*-mediated kidney diseases results from administration of exogenous interferons. However, endogenous interferons may have more widespread impact. The innate interferon response is robustly triggered by pathogens, particularly viruses, via pattern recognition receptors. We assayed the ability of the Toll Like Receptor family (TLR1–9) of pattern recognition receptors to activate *APOL1* expression in both endothelial cells and podocytes. The TLR3 ligand polyI:C, a dsRNA mimic, was a potent stimulant of *APOL1* expression in both cell types (mRNA: figure 2a,b; protein: figure 1d).

TLR4 activation was a moderate stimulator in podocytes but only a weak stimulator in endothelial cells (figure 2).

In addition to the cell surface and endosomal TLRs, a wide variety of cytoplasmic pattern recognition receptors (PRR) also recognize viruses and initiate anti-viral signaling programs. Transfection of cells with the Rig-like Receptor (RigL1) agonist 5'-PPP-RNA increased *APOL1* production, as did transfection of single and double-stranded bacterial DNA, revealing multiple microbial stimuli for *APOL1* induction (supplementary figure 1). The primary TLR3 ligands, double-stranded RNAs including polyI:C, are intermediates during the replication cycle of nearly all viruses.[16] This, together with the robust *APOL1* induction by polyI:C in both endothelial cells and podocytes, prompted our subsequent focus on the TLR3 pathway.

In endothelial cells, polyI:C had similar effects on *APOL1* expression whether it was added directly to the media or transfected into cells with a lipid transfection reagent (Lyovec) (figure 3a and supplementary figure 2a–c). We could inhibit uncomplexed polyI:C-stimulated *APOL1* induction with the endosomal acidification inhibitor chloroquine (figure 3b), consistent with the inhibition of a TLR3-dependent endosomal pathway. Chloroquine inhibition of *APOL1* induction by transfected polyI:C was greatly attenuated (figure 3c), indicating that transfected polyI:C was also activating cytoplasmic, non-endosomal PRRs.

We suspected that polyI:C stimulation of TLR3 was leading to interferon production, and that the interferons themselves increased expression of *APOL1*. However, when we tested this hypothesis, we found that transcription of interferons was minimal for type 1 interferons (α and β) and absent for interferon-γ when polyI:C was added directly to the cell media (figure 3d,e). Type 3 interferon (lambda-λ) mRNAs were not observed until well after *APOL1* upregulation had occurred (figure 3d,e). This was in contrast to transfection of polyI:C with a lipid transfection reagent, which led to rapid and marked increases of both β- and λ-interferon mRNA expression (figure 3d,e). We confirmed high interferon-β levels after polyI:C transfection by ELISA but could not detect interferon-β protein after cells were stimulated with non-transfected polyI:C (figure 3f). Neither mode of delivery led to measureable interferon-α protein (not shown). These results pointed toward the presence of two pathways leading from dsRNA recognition to *APOL1* expression: one that is TLR3-dependent but interferon-independent, the other at least in part governed by cytoplasmic PRRs operating through interferons.

We sought to determine how this TLR3-mediated signal led to *APOL1* upregulation in the absence of potent interferon induction. TLR3 signaling can proceed through Map kinase, NF-kB, TBK1/IKKε, and Jak-STAT intermediates.[17] We tested chemical inhibitors of this pathway to determine the essential components of the signaling cascade. When we used bx795 to inhibit TBK1/IKKε, a central downstream mediator of TLR3 signaling,[18] we saw markedly decreased *APOL1* expression in both endothelial cells and podocytes (figure 4a,b). The NF-kB inhibitor Bay11–7085 potently suppressed *APOL1* induction in endothelial cells but provided less effective suppression in podocytes (figure 4c,d). None of the MAPK inhibitors was potent in our systems, and the relatively modest effects of Jnk, p38, and Erk inhibitors differed between endothelial cells and podocytes (figure 4c,d).

We also tested inhibitors of the Janus kinases to determine whether this pathway is involved in TLR3-stimulated *APOL1* induction. The Jak1/2 inhibitor INCB018424 also inhibited *APOL1* expression, but only at concentrations where Jak3 inhibition could not be excluded (figure 4e,i).[19] The Jak2 inhibitor TG101348 inhibited *APOL1* production but only at concentrations known to inhibit both Jak1 and Jak3 in addition to Jak2 (figure 4f,i).[19] The Jak3 specific inhibitor WHI-P131[20] potently suppressed *APOL1* expression, with half-maximal inhibition in our system at reported IC50 values (figure 4g,h).[21]

To further delineate this complex signaling pathway, we used shRNA to knock down genes in the TLR3 pathway, focusing on downstream candidate molecules implicated by our pharmacologic experiments. As expected, knock-down of TLR3 or the obligate TLR3 adapter molecule, TICAM1 (TRIF), reduced APOL1 expression almost to baseline, confirming that polyI:C leads to *APOL1* transcription predominantly through TLR3. Knock-down of downstream genes TBK1 (the pharmacologic target of bx795)[22], NF-kB pathway components IKBKG (NEMO) and REL-A (p65), and Janus kinases (Jak1 and Jak3 but not Jak2) all suppressed APOL1 upregulation, consistent with our pharmacologic studies. Knock down IRF3, a TBK1-activate molecule known to play a central role in interferon-independent expression of genes that are typically interferon responsive,[23, 24] also effectively suppressed APOL1 expression (figure 5a).

At the *APOL1* locus we noted the presence of consensus IRF1/2 and Stat2 binding sites just upstream of the *APOL1* transcription start site (TSS), and the functional importance of those sites was supported by evidence of IRF1 and STAT2 binding in Encode project ChIP-on-chip data.[25] Knockdown of these three transcription factors also blunted *APOL1* induction by polyI:C (figure 5). Contrary to all other shRNA that suppressed APOL1 upregulation, knockdown of IRF1 and IRF2 also markedly decreased *APOL1* expression in the basal (unstimulated) state. To validate the shRNA knock-down data for these transcription factors, we performed chromatin immunoprecipiation (ChIP) experiments (figure 6). IRF1 and IRF2 bound the start site both before and after polyI:C treatment, whereas STAT2 binding was not detected under basal conditions but demonstrated abundant binding after polyI:C stimulation. This binding pattern was consistent with shRNA data (figure 5b) showing that IRF1 or IRF2 knockdown decreases *APOL1* expression markedly in both the basal and polyI:C stimulated states, but STAT2 knockdown had altered APOL1 expression only in the setting of polyI:C stimulation.

To address the functional ramifications of upregulating different APOL1 variants, we overexpressed wild-type (human genome reference 19) APOL1 and the APOL1 risk alleles G1 and G2 in HEK293 cells to determine if there was any difference in cell behavior. Using the common TV1 splice isoform, cytotoxicity wild-type *APOL1* transfection was similar to an empty vector control (EV), whereas both G1 and G2 transfection caused enhanced cytotoxicity at expression levels equal to wild-type (figure 7a–c). Our data did not show statistical differences in the toxicity of G1 versus G2 protein. We repeated these experiments using the APOL1 transcript variant lacking a signal peptide and observed only after interferon treatment (TV4) and found similar effects: the G1 and G2 variants were toxic to cells whereas Reference APOL1 was not toxic relative to EV. For the TV4 constructs, the onset of cell death due to G1 and G2 was slower but otherwise similar to TV1.

Discussion

The association between *APOL1* genotype and kidney disease in African Americans is one of the strongest yet reported for a common complex disease. The high-risk variants of *APOL1* have an unusual combination of frequency and effect size, the result of positive selection driven by enhanced innate immunity.[11] Prior to the identification of APOL1 as a major risk factor, the relationship between immune system activation and CG in African Americans had been noted.[26] It will be important to understand the nature of the triggers that lead to overt kidney disease in susceptible individuals. The tight relationship between HIV nephropathy and *APOL1* genotype supports the notion that viruses and perhaps other chronic infections or persistent inflammation may be such triggers. APOL1 has now been associated with kidney disease in another high interferon state, systemic lupus erythematosis, and recently published data highlights the potential for more complex relationships between viruses and APOL1 genotype.[27–29]

It is debatable whether the clinical entity we describe is more accurately termed interferon-induced CG or interferon-associated CG. Each patient had developed new onset CG and nephrotic syndrome while on interferon treatment, albeit with some variation in time to onset of kidney disease. Standard immunosuppressive treatment of CG in these patients had no detectable benefit, but in all cases the renal disease resolved or improved after cessation of interferon therapy, as reflected by decreased serum creatinine concentration and/or improvement in proteinuria. The close temporal relationship between interferon administration and disease may not fulfill Koch's postulates in the strictest sense, but coupled with genotype specificity it strongly suggests a causal relationship. Interferon-induced CG is likely to be quite rare in patients without the high-risk genotype, because no such cases were identified in individuals with non-risk *APOL1* genotypes, despite constituting the vast majority of interferon-treated individuals. The 2:1 ratio of the G2 allele to the G1 allele in interferon-induced CG is in contrast to the reverse ratio in idiopathic FSGS ($p=0.02$).[4] This observation might suggest mechanistic differences between the type of *APOL1* dysfunction conferred by the G1 and G2 alleles, or the nature of the triggers that promote disease in individuals with the different risk genotypes.

We confirmed previous reports that inflammatory factors can induce *APOL1* expression, and extended them to include interferon subclasses, multiple cell types, and the appearance of new *APOL1* transcript variants. The inducible nature of *APOL1* and the appearance of new isoforms in the setting of specific non-genetic factors such as interferons are both potential explanations for the observation that most individuals with the high-risk *APOL1* genotype do not develop kidney disease in the absence of some second hit.

In looking for factors upstream of interferons that induce APOL1, we identified consistently potent stimulation by the dsRNA mimic polyI:C. These data indicate the presence of parallel but distinct pathways, one involving robust interferon induction and the other occuring in the absence of classical interferons. A role for IRF3 in *APOL1* expression may help explain the absence of early interferon induction after TLR3 stimulation. There is substantial evidence for interferon-independent, IRF3-dependent stimulation of antiviral innate immunity genes typically considered interferon-stimulated genes.[23] This IRF3 pathway

precedes the interferon-activated response, with IRF1 and IRF3 acting as the central regulators.[24] In our model system, these parallel pathways correlate with intracellular vs. extracellular dsRNA triggers, but in more complex systems these routes to APOL1 activation may be overlapping. Relating activation of distinct pathways by specific pathogens, in different cell types and perhaps in different subcellular compartments, to specific APOL1 phenotypes is an important direction of future work.

Combining pharmacologic inhibitors, shRNA knock-down experiments, and ChIP assays, we were able to trace out the pathways leading from extracellular dsRNA to induction of *APOL1* without significant induction of interferons. We were able to define several key components of the intermediary signaling pathway between dsRNA/TLR3 and the transcription factors that activate *APOL1*. The most compelling components were those where both pharmacologic and targeted knock-downs blocked APOL1 upregulation. Two components, TBK1 and Jak3, were of particular interest because inhibitors are now entering the clinical domain.[30, 31] In our studies, the NF-κB pathway was also important for transducing the dsRNA signal into *APOL1* expression. Consistent with our current findings, polyI:C stimulation promotes nuclear translocation of p50/65 in podocytes and endothelial cells.[32, 33] We also observed that knockdown of several transcription factors (IRF1, IRF2, IRF3, and STAT2) associated with the cellular response to viral infection diminished *APOL1* expression, indicating that all contribute to induction of *APOL1*.

Despite the recessive mode of inheritance observed in *APOL1* kidney disease, comparative genomics among species and the normal kidney function observed in an APOL1-null human together suggest that *APOL1* renal risk variants act as toxic gain-of-function mutations in at least some settings. Our current cell death data further supports this hypothesis, though extrapolation from our cell-based models to the mechanisms of APOL1 kidney disease in humans should be made cautiously. A previous study began the important work of characterizing APOL1 expression in human kidneys[34], but this approach by itself cannot account for high APOL1 expressing cells that may die and therefore not be visualized. Based on the array of different clinical phenotypes and histopathology associated with the APOL1 high-risk genotype, it is reasonable to expect that there may be more than one means by which the mutant protein may lead to pathology. Explaining the full range of APOL1-mediated kidney disease will therefore likely require exploration in many systems including human cells, animal models, and clinical studies. Our study represents an important springboard for future work that will forge tighter mechanistic links connecting the APOL1 risk genotype and specific clinical entities such as H-ESRD, FSGS, HIV nephropathy, collapsing lupus nephropathy, and likely others.

Our data suggest the possibility of a complex functional triangle comprising viruses, anti-viral defenses, and *APOL1*-mediated kidney disease. Taken more broadly, our data support potential roles not only for TLR3 signaling, but also additional viral recognition pathways (e.g. Rig-I/MDA5) and even bacterial nucleic acid in APOL1 upregulation. Since the risk variants are toxic, enhanced expression of risk variant APOL1 is likely to promote disease, so an understanding of pathways governing APOL1 expression may point toward therapeutic interventions. Several important components of the system have been elucidated

here. Each may be a target for treatment in the future as more is learned about the biology of APOL1 and the mechanisms underlying kidney disease due to high-risk APOL1 variants.

Materials and Methods

Cells, reagents, and additional primer and experimental condition details are included in the supplementary methods section

Patient samples—The interferon-treated FSGS patients for whom good quality genomic DNA could be obtained is summarized in Table 1. A description of the full interferon-treated FSGS cohort is available in Markowitz et al.[15] DNA was extracted from formalin fixed, paraffin embedded tissue using the Biostic FFPE DNA isolation kit according to the manufacturer's instructions. DNA was amplified using primers with the following sequences: forward_GCCAATCTCAGCTGAAAGCG, reverse_TGCCAGGCATATCTCTCCTGG. PCR products were prepared for sequencing using the Qiagen PCR Purification Kit. Sanger sequencing was performed at the Children's Hospital Sequence Core, Boston, MA.

mRNA expression—For the agonist and pharmacologic inhibitor experiments, mRNA was isolated using the RNeasy kit (Qiagen), reverse transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems), and quantified by real-time PCR using an Applied Biosystems 7500 PCR system. In all figures, the expression of the mRNA was normalized to either 18S subunit or B-actin expression. Real-time primer-probe sets were from Applied Biosystems.

Protein expression—Cells were lysed in RIPA buffer supplemented with protease inhibitor tablets (Roche). Equal amounts of protein were run on Biorad Tris-HCl gels followed by semi-dry transfer to PVDF membrane (Millipore). Membranes were incubated in primary antibody overnight at 4°C followed by washing and incubation in secondary antibody for 1 hour at room temperature. Proteins were visualized using the SuperSignal West Pico kit (Pierce/ThermoScientific).

Immunocytochemistry—Cells were plated on collagen-coated round coverslips (BD Biosciences). Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, permeabilized using 0.1% Triton X-100 in PBS for 15 minutes at room temperature, and blocked with 10% goat serum (Life Technologies) in PBS shaking for 1 hour at room temperature. Cells were then incubated with primary antibody (rabbit α-ApoL1, Sigma) diluted in 10% goat serum in PBS overnight at 4°C. After washing, cells were incubated in the dark with fluorescent secondary antibodies diluted 1:250 in 10% goat serum in PBS for 2 hours at room temperature, and washed again. Nuclei were labeled with DAPI stain, coverslipped using Vectashield, and imaged using a Zeiss LSM510 Upright confocal microscope.

ELISA—Quantification of interferons in cell media was performed using VeriKine Human IFN-α Multi-Subtype ELISA Kit and VeriKine Human IFN-β ELISA Kit, both from PBL Interferon Source, according to the manufacturers instructions.

shRNA knockdown experiments—HCAEC were treated with shRNA delivered by lentivirus (5 uL to each well, 105 uL final volume). Media was changed every 24 hrs after virus addition. Four days (96 hrs) post-transfection, cells were treated with polyI:C for 6 hours. Following cell lysis, cDNA was generated and quantified using the Qiagen FastLane Probe Kit and Applied Biosystems 7500 PCR system. Four or five shRNAs were tested for each gene. To reduce the false discovery rate, we set the following parameters. In screening experiments, further exploration of any target gene required that at least 2 different shRNA for any target gene suppressed polyI:C-stimulated APOL1 expression by at least 60%. Effectiveness of target gene knock-down was then confirmed. The most effective shRNA for each gene was tested in 3–7 independent assays for its ability to suppress APOL1 expression.

Chromatin immunoprecipitation (ChIP)—Promoter analysis was performed using TFSearch (Kyoto University), the UCSC genome browser, and the ENCODE project encyclopedia of DNA elements. HCAEC cells (5×10^6 cells per condition) were treated with poly I:C (10 μg/mL) or water for six hours. Cells were fixed in 1% formaldehyde, harvested, and lysed according to manufacturer's protocol (Epitect Chip Kit, SA Biosciences). Fixed lysates were sonicated, divided into aliquots equivalent to 1×10^6 cells, and diluted 10-fold. Diluted chromatin samples were pre-cleared with Dynabeads Protein A (Invitrogen) and immunoprecipitated overnight at 4°C with antibodies against STAT2 (Santa Cruz Biotechnology), IRF1 (Santa Cruz Biotechnology), IRF2 (Santa Cruz Biotechnology), or with rabbit IgG (Cell Signaling). Crosslinks in precipitated immunocomplexes were broken by treatment with Proteinase K (45°C for 2 hours) in a buffered salt solution. DNA was purified using a ChIP purification kit (Active Motif). Primers((5'-AGC TGC TGG GAA GTT GTG AC-3') and (5'-ATC CCA CCT CCA GTT ATG CG-3') spanning the putative IRF1, IRF2, and Stat2 binding site at the APOL1 transcription start site (hg19: 36,649,100-36,649,208) were used to assess transcription factor pull-down of DNA via conventional PCR and real-time PCR. An input control sample (sheared chromatin collected prior to antibody addition, equivalent to 1% of IP sample volume) was processed in parallel to standardize pull-down efficiencies.

Cytotoxicity Assays—4000 HEK293 cells were plated in each well of a 96-well plate in 100 μl of media. Cells were transfected in serum free Optimem media (Life Technologies) with 100ng of DNA in 0.25 μl Lipofectamine 2000. After 3 hours, media was supplemented with EMEM/FBS. Cytotoxicity and viability were measured using the Multi-Tox-Fluor Multiplex Cytotoxicity assay (Promega). The cytotoxicity component measures a protease released by dead cells using a fluorogenic peptide substrate that cannot cross living cell membranes. The viability component uses a cell-permeant peptide substrate that requires cleavage by a protease active only in living cells. Each condition was run in quadruplicate, with values averaged to obtain a single data point. Staining with anti-APOL1 antibody in parallel experiments showed detectable APOL1 expression in 50–70% of cells.

Human subjects research—Research was conducted under protocols approved by institutional review boards at Beth Israel Deaconess Medical Center and Columbia University.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Genovese G, Friedman DJ, Ross MD, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. Science. 2010; 329:841–845. [PubMed: 20647424]
2. Tzur S, Rosset S, Shemer R, et al. Missense mutations in the APOL1 gene are highly associated with end stage kidney disease risk previously attributed to the MYH9 gene. Hum Genet. 2010; 128:345–350. [PubMed: 20635188]
3. Friedman DJ, Kozlitina J, Genovese G, et al. Population-based risk assessment of APOL1 on renal disease. J Am Soc Nephrol. 2011; 22:2098–2105. [PubMed: 21997396]
4. Kopp JB, Nelson GW, Sampath K, et al. APOL1 genetic variants in focal segmental glomerulosclerosis and HIV-associated nephropathy. J Am Soc Nephrol. 2011; 22:2129–2137. [PubMed: 21997394]
5. Smith EE, Malik HS. The apolipoprotein L family of programmed cell death and immunity genes rapidly evolved in primates at discrete sites of host-pathogen interactions. Genome Res. 2009; 19:850–858. [PubMed: 19299565]
6. Duchateau PN, Pullinger CR, Cho MH, et al. Apolipoprotein L gene family: tissue-specific expression, splicing, promoter regions; discovery of a new gene. J Lipid Res. 2001; 42:620–630. [PubMed: 11290834]
7. Duchateau PN, Pullinger CR, Orellana RE, et al. Apolipoprotein L a new human high density lipoprotein apolipoprotein expressed by the pancreas Identification, cloning, characterization, and plasma distribution of apolipoprotein L. J Biol Chem. 1997; 272:25576–25582. [PubMed: 9325276]
8. Monajemi H, Fontijn RD, Pannekoek H, et al. The apolipoprotein L gene cluster has emerged recently in evolution and is expressed in human vascular tissue. Genomics. 2002; 79:539–546. [PubMed: 11944986]
9. Page NM, Butlin DJ, Lomthaisong K, et al. The human apolipoprotein L gene cluster: identification, classification, and sites of distribution. Genomics. 2001; 74:71–78. [PubMed: 11374903]
10. Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, et al. Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature. 2003; 422:83–87. [PubMed: 12621437]
11. Friedman DJ, Pollak MR. Genetics of kidney failure and the evolving story of APOL1. J Clin Invest. 2011; 121:3367–3374. [PubMed: 21881214]
12. Vanhollebeke B, Truc P, Poelvoorde P, et al. Human Trypanosoma evansi infection linked to a lack of apolipoprotein L-I. N Engl J Med. 2006; 355:2752–2756. [PubMed: 17192540]
13. Johnstone DB, Shegokar V, Nihalani D, et al. APOL1 Null Alleles from a Rural Village in India Do Not Correlate with Glomerulosclerosis. PloS one. 2012; 7:e51546. [PubMed: 23300552]
14. Zhaorigetu S, Wan G, Kaini R, et al. ApoL1, a BH3-only lipid-binding protein, induces autophagic cell death. Autophagy. 2008; 4:1079–1082. [PubMed: 18927493]

15. Markowitz GS, Nasr SH, Stokes MB, et al. Treatment with IFN-{alpha}, -{beta}, or -{gamma} is associated with collapsing focal segmental glomerulosclerosis. Clin J Am Soc Nephrol. 2010; 5:607–615. [PubMed: 20203164]
16. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Molecular cell. 2006; 22:561–569. [PubMed: 16762830]
17. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 2010; 11:373–384. [PubMed: 20404851]
18. O'Neill LA. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. Immunity. 2008; 29:12–20. [PubMed: 18631453]
19. Quintas-Cardama A, Kantarjian H, Cortes J, et al. Janus kinase inhibitors for the treatment of myeloproliferative neoplasias and beyond. Nature reviews Drug discovery. 2011; 10:127–140. [PubMed: 21283107]
20. Goodman PA, Niehoff LB, Uckun FM. Role of tyrosine kinases in induction of the c-jun proto-oncogene in irradiated B-lineage lymphoid cells. The Journal of biological chemistry. 1998; 273:17742–17748. [PubMed: 9651374]
21. Sudbeck EA, Liu XP, Narla RK, et al. Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing antileukemic agents. Clinical cancer research : an official journal of the American Association for Cancer Research. 1999; 5:1569–1582. [PubMed: 10389946]
22. Clark K, Plater L, Peggie M, et al. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. The Journal of biological chemistry. 2009; 284:14136–14146. [PubMed: 19307177]
23. Shapira SD, Gat-Viks I, Shum BO, et al. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell. 2009; 139:1255–1267. [PubMed: 20064372]
24. Dixit E, Boulant S, Zhang Y, et al. Peroxisomes are signaling platforms for antiviral innate immunity. Cell. 2010; 141:668–681. [PubMed: 20451243]
25. Rosenbloom KR, Sloan CA, Malladi VS, et al. ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic acids research. 2013; 41:D56–63. [PubMed: 23193274]
26. Albaqumi M, Soos TJ, Barisoni L, et al. Collapsing glomerulopathy. J Am Soc Nephrol. 2006; 17:2854–2863. [PubMed: 16914539]
27. Divers J, Nunez M, High KP, et al. JC polyoma virus interacts with APOL1 in African Americans with nondiabetic nephropathy. Kidney international. 2013
28. Freedman BI, Langefeld CD, Andringa KK, et al. End-stage kidney disease in African Americans with lupus nephritis associates with APOL1. Arthritis and rheumatism. 2013
29. Larsen CP, Beggs ML, Saeed M, et al. Apolipoprotein L1 risk variants associate with systemic lupus erythematosus-associated collapsing glomerulopathy. Journal of the American Society of Nephrology : JASN. 2013; 24:722–725. [PubMed: 23520206]
30. Reilly SM, Chiang SH, Decker SJ, et al. An inhibitor of the protein kinases TBK1 and IKK-varepsilon improves obesity-related metabolic dysfunctions in mice. Nature medicine. 2013
31. Wojciechowski D, Vincenti F. Targeting JAK3 in kidney transplantation: current status and future options. Current opinion in organ transplantation. 2011; 16:614–619. [PubMed: 21971513]
32. Shimada M, Ishimoto T, Lee PY, et al. Toll-like receptor 3 ligands induce CD80 expression in human podocytes via an NF-kappaB-dependent pathway. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012; 27:81–89.
33. Kaiser WJ, Kaufman JL, Offermann MK. IFN-alpha sensitizes human umbilical vein endothelial cells to apoptosis induced by double-stranded RNA. J Immunol. 2004; 172:1699–1710. [PubMed: 14734752]
34. Madhavan SM, O'Toole JF, Konieczkowski M, et al. APOL1 localization in normal kidney and nondiabetic kidney disease. J Am Soc Nephrol. 2011; 22:2119–2128. [PubMed: 21997392]

Abbreviations

APOL1	ApolipoproteinL1
ChIP	Chromatin immunoprecipitation
CLX	chloroquine
ENCODE	Encyclopedia of DNA elements
FSGS	focal segmental glomerulosclerosis
G1	allele with two amino acid substitutions (S342G, I384M) in APOL1
G2	allele with a two amino acid deletion in APOL1 (del388N389Y)
HCAEC	human coronary artery endothelial cell
HDL	High density lipoprotein
HEK	human embryonic kidney
HUVEC	human umbilical vein endothelial cell
IRF	interferon regulatory factor
Jak	Janus kinase
PRR	pattern recognition receptor
shRNA	short hairpin RNA
siRNA	short interfering RNA
TLR	Toll-like receptor
TSS	Transcription start site
TV	transcript variant

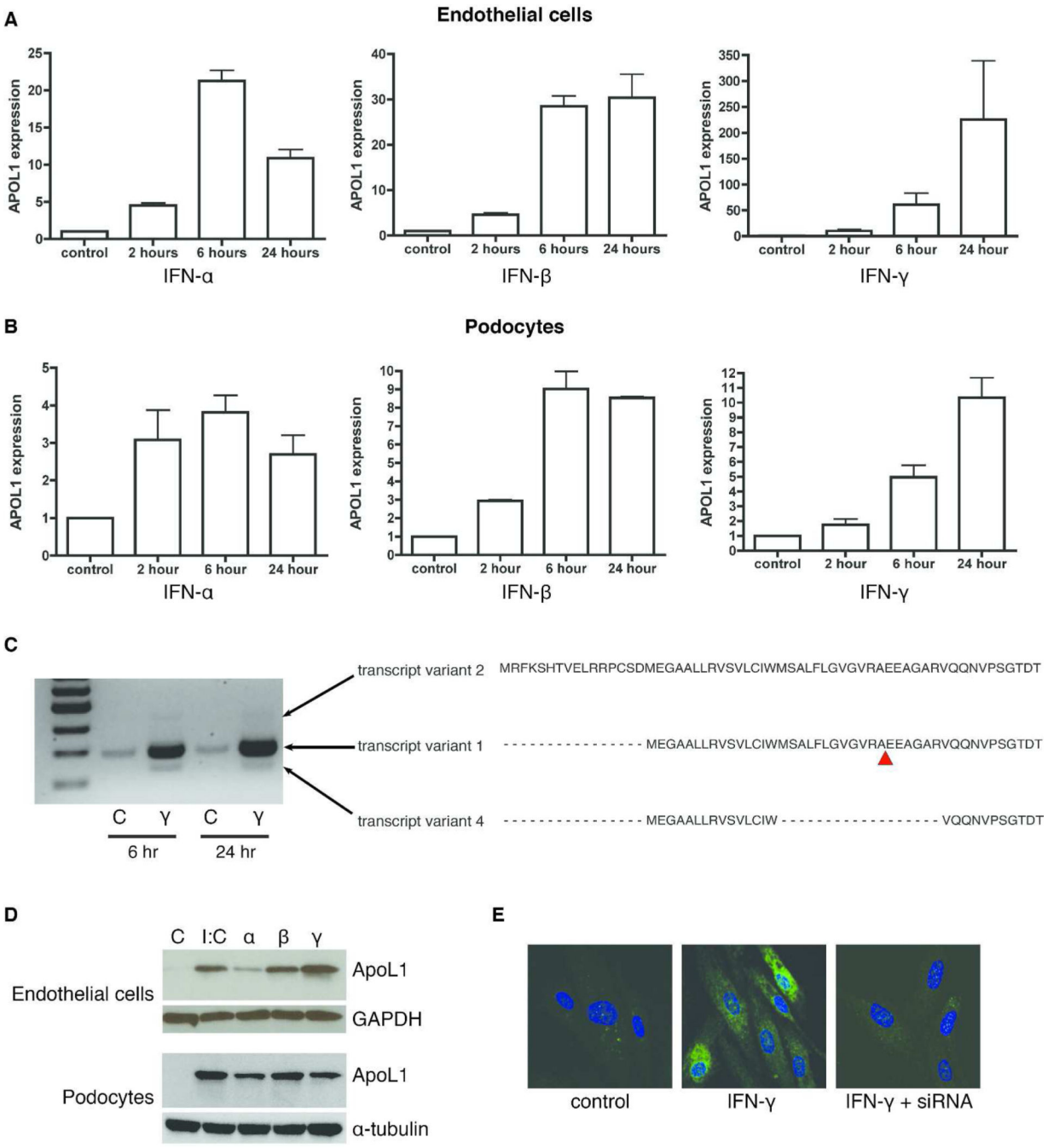

Figure 1.

Interferons induce APOL1 expression and appearance of additional transcript variants. Normalized expression of APOL1 (to 18S subunit) in (A) Human Coronary Artery Endothelial Cells (HCAEC) or (B) podocytes after stimulation with α (100U/ml), β (100U/ml), or γ(10ng/ml) interferon. Values are mean fold increase in APOL1 mRNA +/− s.e.m for a minimum of 3 experiments. (C) Under basal conditions in HCAEC, only APOL1 transcript variant (tv) 1 was detected by PCR. After stimulation with Interferon γ, tv2 and tv4 were also detected at time points indicated. N-terminal amino acid sequences encoded by the

transcript variants are shown at right. Red caret indicates predicted signal sequence cleavage site present in tv1, but absent from tv4. The site in tv2 may be too far from the N-terminus to promote cleavage. (D) Western blot of APOL1 protein in whole cell lysate 24 hours after stimulation with polyI:C (10μg/ml) or interferons (α-100U/ml, β-100U/ml, or γ-10ng/ml) in endothelial cells and podocytes. (E) APOL1 staining was not observed in untreated (control) endothelial cells. APOL1 staining (green) is strong after 24 hours of interferon γ treatment and can be abolished by APOL1 siRNA knockdown. Nuclei are stained by DAPI (blue).

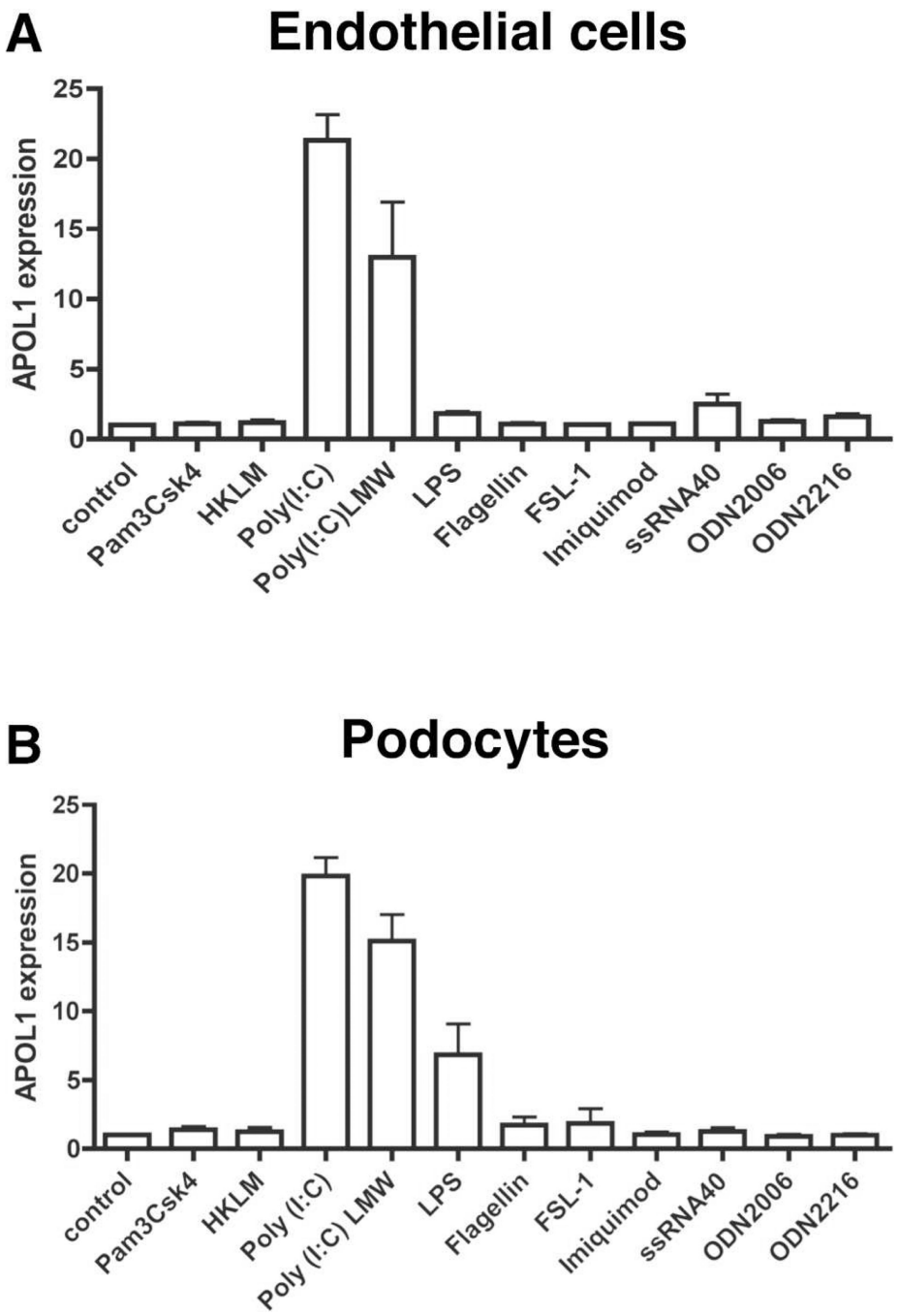

Figure 2.

The TLR3 agonist PolyI:C upregulates APOL1 expression. Normalized APOL1 expression (to 18S subunit) measured by real-time RT-PCR in (A) HCAEC or (B) podocytes after 24 hours of stimulation by the indicated agonists for the specified TLRs. Values are fold increase in APOL1 mRNA/18S mRNA expression for 3 experiments +/− s.e.m. TLR1/2: Pam3cSK4 (triacylated lipoprotein), 1μg/ml; TLR2: HKLM (derived from heat-killed Listeria), 108 cells/ml; TLR3: poly(I:C), low (0.2–1kb) or high (1.5–8kb) molecular weight, 10μg/ml; TLR4: Lipopolysaccharide (LPS), 1μg/ml, TLR5: Flagellin, 1μg/ml; TLR6/2:

FSL1 (diacylated lipoprotein), 1μg/ml; TLR7: Imiquimod, 2μg/ml; TLR8: ssRNA40/Lyovec, 2μg/ml; TLR9: ODN compounds (unmethylated CpG synthetic oligos), 1μM.

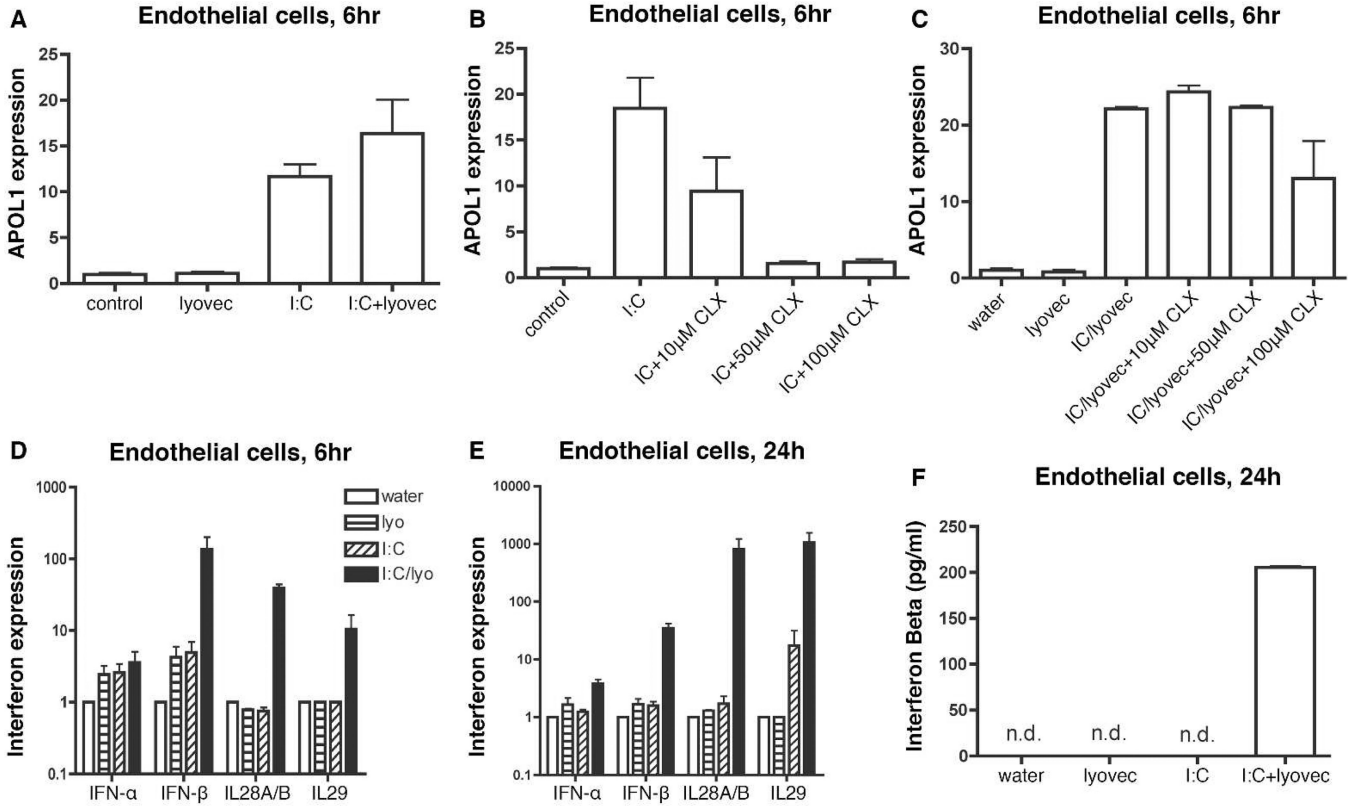

Figure 3.

PolyI:C induces APOL1 expression through parallel pathways. (A) Non-transfected (10μg/ml) and Lyovec-transfected polyI:C (10μg/ml) increase APOL1 mRNA expression in endothelial cells (A) to similar degrees; data for later time points and for podocytes is shown in supplementary figure 2. (B,C) Chloroquine (CLX) treatment blocks APOL1 induction endothelial cells by non-transfected polyI:C (B) but not by transfected polyI:C (C) in endothelial cells. (D–F) Endothelial cells were treated with non-transfected polyI:C (I:C) or transfected polyI:C (I:C/lyo) and fold increases of IFN-α, -β, or –λ (IL28A/B, IL29) mRNA were measured by real time RT-PCR at 6 or 24 hours. IFN-γ was not detected under any conditions. (F) ELISA confirmed mRNA results in (H) for interferon-β (interferon-α was undetectable in all conditions, not shown). In summary, transfected polyI:C is a potent inducer of interferons whereas non-transfected polyI:C has minimal effects on classical interferon production. (n.d.: not detectable).

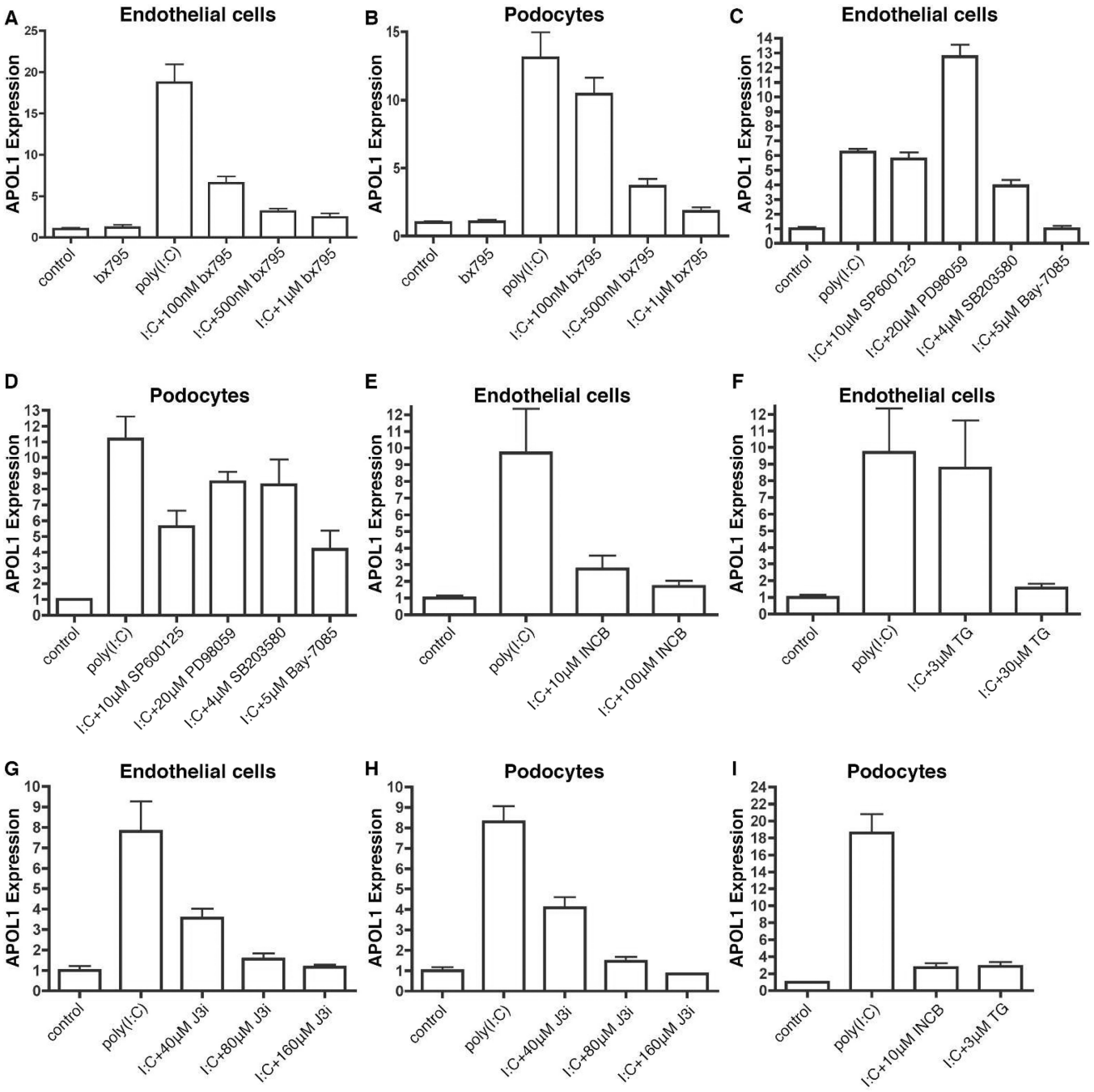

Figure 4.

Induction of APOL1 in endothelial cells and podocytes by polyI:C is blocked by TBK1/IKKε, Jak, NF-kB, and MAP kinase inhibitors. In all subfigures, cells were pretreated with drug and stimulated with 10μg/ml of polyI:C with normalized APOL1 expression measured by real-time RT-PCR. (A,B) In endothelial cells and podocytes, the TBK1/IKKε inhibitor bx795 blocks APOL1 upregulation. (C,D) Blockade of APOL1 upregulation by inhibitors of NF-kB (Bay-7085), JNK (SP600125), p38 (SB203580), and ERK (PD98059) in endothelial cells (C) and podocytes (D). (E–I) In endothelial cells and podocytes, APOL1 stimulation

was blocked to varying degrees by (E, I) the Jak1/2 inhibitor INCB018424, (F,I) the Jak2 inhibitor TG101348, (G,H) the Jak3 specific inhibitor WHI-P131 (J3i).

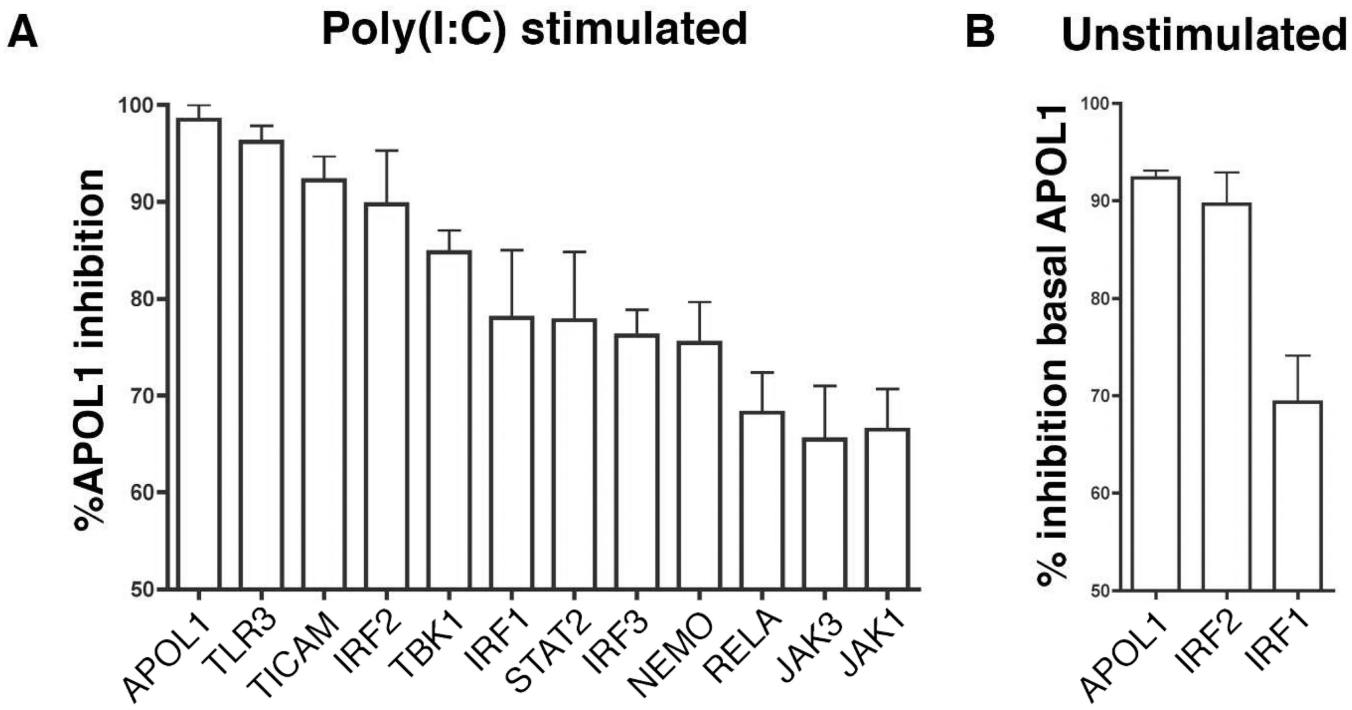

Figure 5.

shRNA knockdown of several downstream components of the TLR3 pathway block APOL1 stimulation by polyI:C. (A) APOL1 upregulation in HCAEC after stimulation with polyI:C (10μg/ml) was inhibited by pretreatment with shRNA against TLR signaling pathway components. (B) Of the shRNA that blunted APOL1 upregulation by polyI:C, only shRNAs against IRF1 and IRF2 reduced the amount of basal APOL1 expression. $p<0.01$ for all shRNA shown vs. control shRNA. N = 3–7.

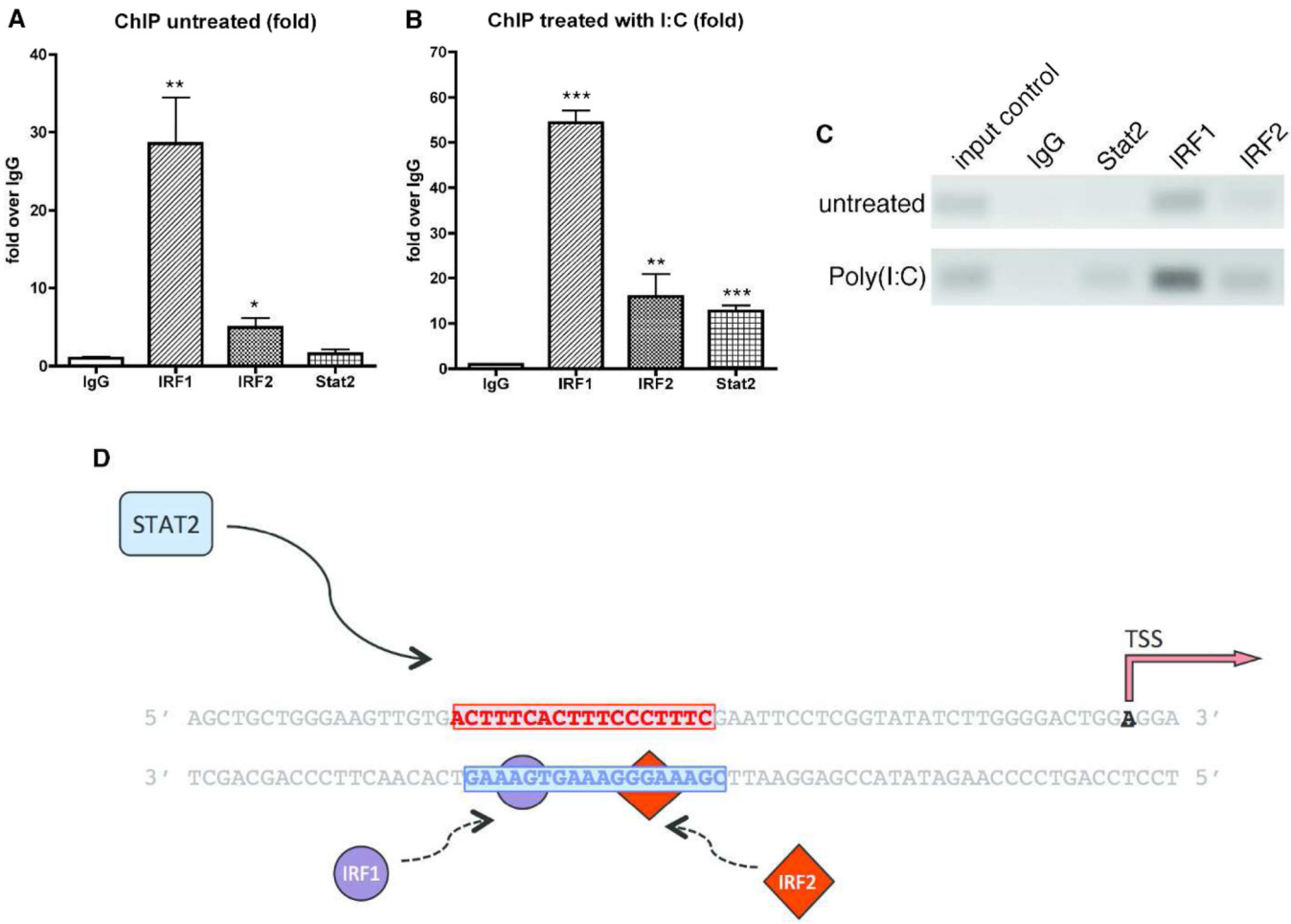

Figure 6.

Chromatin Immunoprecipitation (ChIP) validates transcription factor binding at the APOL1 locus. (A) Transcription factor binding of IRF1, IRF2, and STAT2 to the APOL1 promoter in lysates from endothelial cells under basal conditions, as measured by real time PCR at the APOL1 promoter (N=5). (B) Transcription factor binding of IRF1, IRF2, and STAT2 to the APOL1 promoter in lysates from endothelial cells 6 hours after stimulation with 10μg/ml polyI:C, as measured by real time PCR (N=5). (C) Results of representative ChIP experiment using standard PCR. (D) IRF1 and IRF2 bind to a canonical IRF binding site (blue box) near the APOL1 transcription start site (TSS) under basal conditions and their binding increases after polyI:C stimulation. Sequence amplified in ChIP experiments is shown. STAT2 does not bind detectably under basal conditions, but is recruited to the promoter after stimulation with polyI:C. STAT2 may bind the interferon stimulated response element (red box) as part of a STAT1/STAT2/IRF9 complex in the absence of direct interferon stimulation. *p<0.01, **p<0.001, or ***p<0.0001 vs IgG.

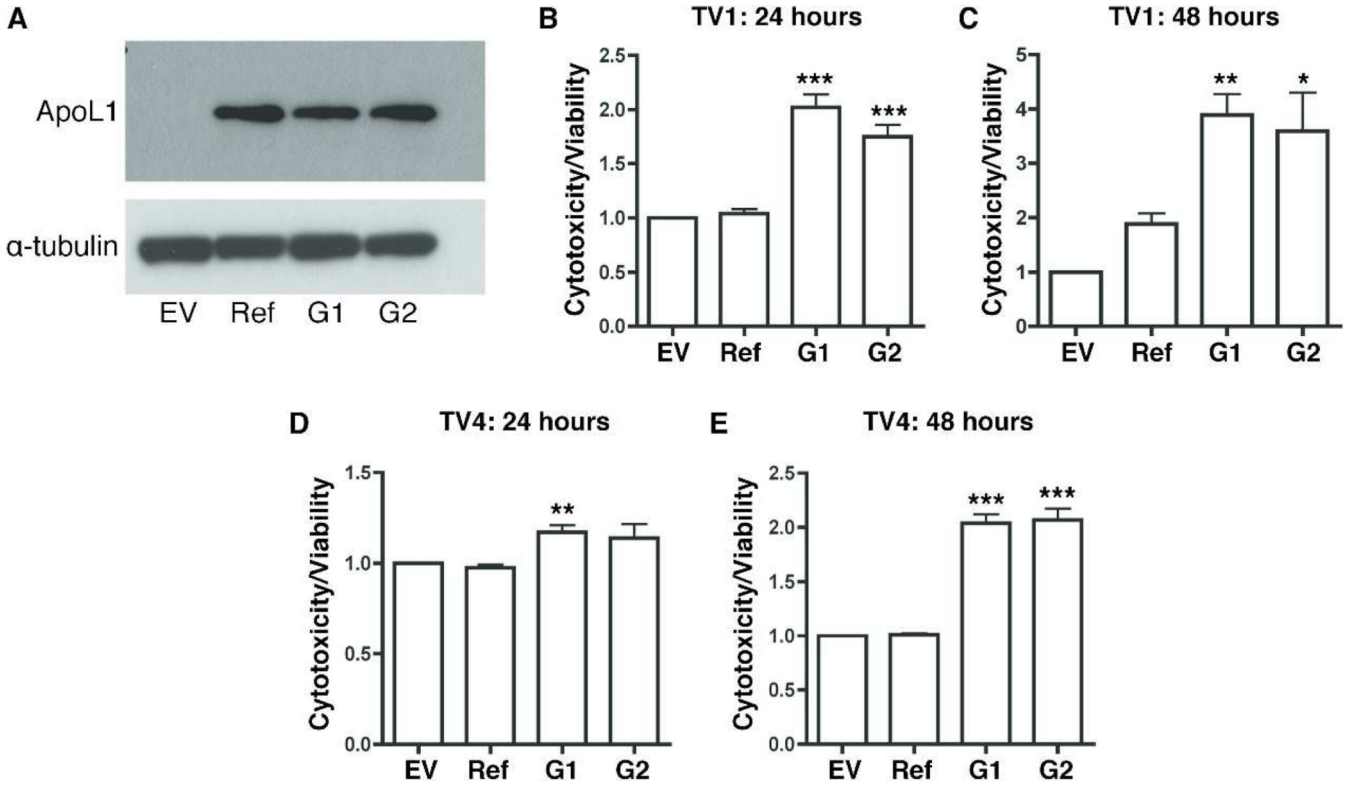

Figure 7.

Kidney risk variants of APOL1 are more toxic to HEK293 cells than "wild-type" APOL1. (A) Plasmids encoding Reference hg19 (Ref) APOL1, the G1 risk variant (with S342G and I384M), the G2 risk variant (del388N389Y), or empty vector (EV) were transfected into HEK293 cells. Equal expression was noted by immunoblot at 24 hours (shown for transcript variant 1). (B–E) The ratio of cytotoxicity:viability was measured 24 (B,D) or 48 (C,E) hours after transfection with APOL1 transcript variant 1 (B,C) or transcript variant 4 (D,E). Data from different experiments were normalized using EV values. *$p<0.05$, **$p<0.005$, or ***$p<0.0001$ versus Reference APOL1. N=7 for panel B, 5 for panel C, 4 for panels D,E.

Table 1

APOL1 genotypes of individuals with interferon-associated FSGS.

	Race/ Ethnicity	Gender/ Age	Indication	Interferon type	Scr (mg/dL)	Upr (g/day)	APOL1 Genotype
1	Black	46F	HCV	Alpha	3.2/1.2	10.0/3.6	G1/G2
2	Hispanic	64M	HCV	Alpha	1.5/1.0	4.5/neg	G2/G2
3	Black	69M	HCV	Alpha	6.9/5.6	3.3/NA	G2/G2
4	Black	36F	Melanoma	Alpha	1.4/1.0	27.0/4.5	G1/G2
5	Black	33F	MS	Beta	2.6/1.7	1.9/0.5	G1/G2
6	Black	37F	MS	Beta	2.3/1.2	2.4/1.4	G1/G2
7	Black	67M	Pulm. Fibr.	Gamma	3.5/2.1	4.0/0.65	G1/G2

APOL1 genotyping for a case series originally reported in Markowitz et al. (reference 16). In addition to *APOL1* genotype, the serum creatinine and urine protein levels are shown while receiving and after discontinuing interferon. Abbreviations: hepatitis C virus (HCV), multiple sclerosis (MS), pulmonary fibrosis (pulm. fibr.), serum creatinine (Scr), milligrams per deciliter (mg/dL), urine protein in grams per day (Upr). The G1 allele is defined by two amino acid substitutions: S342G and I384M. The G2 allele is defined by in-frame deletion of amino acids 388N and 389Y.