APOL1 risk variants enhance podocyte necrosis through compromising lysosomal membrane permeability

Xiqian Lan,1 Aakash Jhaeri,1 Kang Cheng,1 Hongxu Wen,1 Moin A. Saleem,2 Peter W. Mathieson,2 Joanna Mikulak,3 Sharon Aviram,4 Ashwani Malhotra,1 Karl Skorecki,4 and Pravin C. Singhal1

1Renal Molecular Research Laboratory, Feinstein Institute for Medical Research, Hofstra-North Shore Long Island Jewish School of Medicine School, Hempstead, New York; 2Renal Academic Unit, University of Bristol, Bristol, United Kingdom; 3Laboratory of Clinical and Experimental Immunology, Humanitas Clinical and Research Center, Rozzano, Milan, Italy; and 4Nephrology and Molecular Medicine, Technion Institute of Technology and Rambam Medical Center, Haifa, Israel

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African Americans (AAs) develop higher rates of progressive nephropathy, including focal segmental glomerulosclerosis (FSGS), human immunodeficiency virus (HIV)-associated nephropathy (HIVAN), and hypertension-associated end-stage kidney disease (ESKD) compared with European Americans (EAs) (25, 37, 43). This disparity is the most pronounced in the case of HIVAN, reaching a disparity which is >10-fold (25). This overwhelming population disparity between AAs and EAs and, greater familial clustering among AAs, point to a prominent contribution of underlying genetic factor(s) (14). Recently, increasing evidence has shown that this major health disparity is strongly associated with two coding sequence variants (G1 and G2) in APOL1 (12, 15, 19, 42).

APOL1 is located at chromosome 22q13.1, and it encodes the minor apolipoprotein L1 component of HDL. This apolipoprotein is expressed in the liver, pancreas, kidney, and brain and in macrophages, endothelial, and several other cell types (9, 10). The G1 variant (rs73885319) is a compound missense mutation (S342G:I384M) encoding two nonsynonymous amino acids. The G2 variant is a 6-bp in-frame deletion which has resulted in the loss of two amino acids (N388 and Y389) at the C-terminal helix of APOL1 (18, 48). These two allelic variants arose on separate human phylogenetic lineages, and they have never been observed together on the same parental chromosome. Approximately 34% of AAs possess one of the two risk variants (APOL1Vs), and ~13% have both coding variants (18, 25, 48). In the United States, ~3 million AAs carry both risk alleles. In contrast, APOL1Vs occur infrequently in EAs, ~0.3% carry G1 and 0.1% G2 alleles. These studies are consistent with African admixture (18, 25, 48). Wild-type (WT) APOL1 circulates in plasma and has the ability to kill the trypanosome (Trypanosoma brucei) responsible for causing sleeping sickness in cattle.

The Trypanosoma brucei rhodesiense (Tbr) subspecies is resistant to the trypanolytic effect of APOL1. This is due to the presence of a serum resistance-associated (SRA) factor. SRA allows for Tbr to induce human sleeping sickness (25, 44). The G1 and G2 coding variants confer resistance, in humans, to Tbr. The presence of even a single variant has been shown to confer resistance. Thus these variants have gained prominence in parts of the world with significant rates of sleeping sickness. The risk allele state is now highly associated with increased risk for major forms of nondiabetic kidney disease in descendants, including AAs (18, 19, 25, 42, 48). Recent clinical studies have also implicated the coding variants with renal allograft failure (24, 38). In these studies, kidneys donated from deceased donors with APOL1Vs lasted for shorter periods, independently of recipient ancestry.

Although clinical studies have clearly shown a strong association of APOL1Vs with progressive nondiabetic kidney disease, the cellular function and the role of APOL1Vs in the pathogenesis of kidney injury are still unknown. Localization studies have implicated the podocyte as a target (31). Most proteinuric diseases including glomerular sclerosis and HIVAN are associated with podocytopathy (altered podocyte pheno-
type, reduction in number, and effacement of foot processes) and tubulopathy (32, 33).

In the present study, we examined the effect of APOL1WT (G0) and APOL1Vs (G1 and G2) on human podocytes. Additionally, we have studied the mechanism involving APOL1Vs-induced podocyte injury.

MATERIALS AND METHODS

Culture of human podocytes. Human podocytes were cultured as previously reported (27, 40). Briefly, immortalized human podocytes proliferated in the growth medium containing RPMI 1640 supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin, 1 mM l-glutamine, and 1× insulin, transferrin, and selenium (ITS; Invitrogen, Grand Island, NY) at permissive temperature (33°C). When the cells attained ~80% confluence, they were transferred at 37°C for differentiation in a medium without ITS for 5–7 days.

Production of pseudotyped retroviral supernatant and transduction of podocytes. Replica-defective viral supernatants were prepared as published previously (21). Briefly, a green fluorescent protein (GFP) reporter gene (from pEGFP-C1; Clontech, Palo Alto, CA) was substituted in place of gag/pol genes in HIV-1 proviral construct pNL4–3. This parental construct (pNL4–3; ΔG/P-GFP) was used to produce VSV.G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by transient transfection of 293T cells using Effectene (Qiagen). The HIV-1 gag/pol and VSV.G envelope genes were provided in trans using pCMV R8.91 and pMD2.G plasmids, respectively (gifts of Dr. Didier Trono, Salk Institute, La Jolla, CA). As a negative control, virus was also produced from pHCMV-IREs2-GFP-ΔB, which contained HIV-1 LTRs and a GFP empty expression vector. The viral stocks were titrated by infecting 293T cells with 10-fold serial dilution. Viral stocks ranging from 10^5 to 10^9 GFP-expressing units (GEU)/ml were obtained. The cells were infected with MOI of 0.5 GEU for 2 h.

Lentivirus preparation. To amplify the DNA fragments corresponding to the whole APOL1 open reading frames, a forward primer GGATCCATGAGGACGCTTTGCTGAGAG (BamHI cleavage site underlined) and reverse primer GTCGACTACATTTGCTTGCCCGCGTCA (SacI cleavage site underlined) were designed based on the sequences around the initial and stop codons, respectively. The PCR-amplified products were double-digested with BamHI and SacI and subcloned into a LG12 lentiviral vector to obtain a transfer plasmid designated LG12-“APOL1” 5. The transcription of targeting genes was driven by a CMV promoter. psPAX2 was the transfer plasmid designated LG12-“APOL1” 5. The transcription of targeting genes was driven by a CMV promoter. psPAX2 was the HIV-1 gag/pol and VSV.G envelope genes were provided in trans using pCMV R8.91 and pMD2.G plasmids, respectively (gifts of Dr. Didier Trono, Salk Institute, La Jolla, CA). As a negative control, virus was also produced from pHCMV-IREs2-GFP-ΔB, which contained HIV-1 LTRs and a GFP empty expression vector. The viral stocks were titrated by infecting 293T cells with 10-fold serial dilution. Viral stocks ranging from 10^5 to 10^9 GFP-expressing units (GEU)/ml were obtained. The cells were infected with MOI of 0.5 GEU for 2 h.

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was quantified using GFP expression. It was observed to be 90%. Subsequently, the overexpression of recombinant APOL1 in podocytes was confirmed. For time course experiments, cell lysates were collected for Western blot assay. Podocytes displayed initial APOL1WT and Vs expression within 24 h of infection (Fig. 1, A and B), with increasing overexpression by 48 h (Fig. 1, C and D).

To determine the effect of APOL1WT and Vs on podocyte injury, we overexpressed these variants in differentiated human podocytes and analyzed podocyte LDH activity. At 24 h, APOL1WT increased podocyte LDH activity, and podocytes expressing G1/G2 variants exhibited even higher levels of LDH activity compared with APOL1WT-expressing cells (Fig. 2A).

We then evaluated the effect of APOL1WT (G0) and APOL1Vs (G1 and G2) on podocyte viability using trypan blue staining. Although both APOL1WT and APOL1Vs displayed an increased number of trypan blue-positive podocytes compared with noninfected podocytes, nonetheless podocytes expressing either G1 or G2 constructs displayed a greater number of trypan blue-positive cells compared with G0 (Fig. 2B).

Overexpression of APOL1WT has been reported to induce autophagic cell death in cancer cell lines (47, 49). To determine the type of the cytotoxicity induced by APOL1WT and APOL1Vs, we examined their effect on podocyte morphology under a phase-contrast microscope. Podocytes transduced with G0 or G1- and G2-containing lentiviruses showed a higher percentage of cells expressing a swollen phenotype compared with the vector alone (Fig. 3, A and B). This swollen phenotype precipitated detachment, signifying loss of cell viability. The effect was significantly more pronounced for cells transduced with the G1 and G2 lentivirus.

We then stained podocytes transduced with APOL1G0 and APOL1Vs with PI and Hoechst 33342 and examined these cells under an inverted fluorescence microscope. Interestingly, the swollen cells also contained swollen nuclei (Fig. 3C) and were either necrotic or experiencing necrosis.

Since APOL1WT expression inflicted podocyte injury, we asked whether injury was podocyte APOL1 expression dependent. To determine the effects of lowering expression of APOL1, podocytes were infected with half the dose of lentivirus (titrated as 0.4 pg p24 protein/cell) for 3 h and then incubated in fresh media for 48 h. Live and swollen cells were counted. As shown in Fig. 3, D and E, podocytes expressing the APOL1WT did not display obvious podocyte injury; on the other hand, podocytes expressing G1 or G2 displayed a decreased percentage of viable cells and an increased percentage of swollen cells.

Since higher concentrations of APOL1G0 induced podocyte injury, we asked whether an inflammatory milieu has the potential to enhance podocyte G0 expression. Podocytes were incubated in media containing different concentrations of interferon-γ (0, 1, 2.5, 10, and 20 ng/ml) for 48 h. Subsequently,
Fig. 3. APOL1 risk variants increase podocyte swelling. A–C: human podocytes were transduced with the lentivirus (titrated as 0.8 pg HIV p24 protein/cell) for 3 h and were cultured in the fresh medium for 48 h. Swollen cells (black arrows) were visualized (A) and counted (B) under a contrast-phase microscope. C: swollen cells were stained with propidium iodide (PI) and visualized under a contrast-phase microscope. D and E: human podocytes were infected with half the dose of lentivirus (titrated as 0.4 pg HIV p24 protein/cell), and swollen cells (D) and living cells (E) were counted. Scale bars = 100 and 20 μm for A and C, respectively. *P < 0.05 compared with vector. #P < 0.05 compared with APOL1WT. F: podocytes were incubated in media in the absence or presence of different concentrations of interferon-γ (0, 1, 2.5, 10, and 20 ng/ml) for 48 h. Subsequently, RNA was extracted and cDNA was probed for APOL1 with qPCR. *P < 0.05 compared with 0 interferon-γ.
RNA was extracted and cDNA was probed for APOL1. As shown in Fig. 3F, interferon-γ enhanced podocyte endogenous APOL1 expression in a dose-dependent manner. Thus elevated APOL1 expression in response to an inflammatory milieu could have the potential to contribute to ongoing injury.

To visualize the effect of APOL1Vs on podocyte injury, we performed PI/Hoechst staining after infecting the cells with the lentivirus at 48 hpi. APOL1Vs promoted both primary (PI stained single swollen nuclei) and secondary (PI stained fragmented nuclei) necrosis compared with APOL1G0 (Fig. 4). Taken together, these findings indicate that high levels of APOL1Vs are cytotoxic to human podocytes.

Knockdown of APOL1 doesn’t increase podocyte injury. Gene mutation often leads to the loss of functionality of the encoded protein, in turn causing a loss-of-function disease phenotype with recessive inheritance (e.g., sickle cell anemia). To examine whether podocyte loss of function was involved in APOL1-associated nephropathy, APOL1 expression was silenced in human podocytes [small interfering (si) RNA-APOL1/podocytes], and then podocyte phenotype was examined. The siRNA-APOL1/podocytes displayed a ~60% decrease in protein expression (Fig. 5, A and B), confirming silencing of this gene. However, this was not associated with induction of either LDH activity or cell loss (Fig. 5, C and D). Moreover, siRNA-APOL1/podocytes did not display morphological alterations. These results suggest that APOL1 is not essential for the short-term structural or functional integrity of podocytes. This supports the notion that APOL1Vs-associated nephropathy is not due to the loss of baseline APOL1 functionality in podocytes. It is also important to note that the APOL1 gene exists only in humans and several nonhuman primates and appears to be dispensable in rodents and other mammals (3).

Overexpression of APOL1Vs increases podocyte lysosomal membrane permeability. Pérez-Morga et al. (36, 45) reported that APOL1 promotes trypanosomal lysis by forming pores in lysosomal membranes. We considered whether APOL1 could compromise podocyte integrity through a similar mechanism. To test this, we compared the effects of APOL1G0 and Vs on podocyte lysosomal membrane permeability (LMP), with LysoTracker to label lysosomes. Both G1 and G2 variants drastically decreased the number of punctated structures (normal lysosomes) in podocytes compared with vector control (Fig. 6, A and B). Such a decrease may represent a consequence of the dramatic necrotic response evident in Figs. 2–4. To confirm the specificity of APOL1-induced effects on LMP, corresponding podocytes were stained with Lucifer yellow. Vector-treated podocytes displayed containment of dye within lysosomes; on the other hand, G1 or G2 podocytes displayed diffuse lysosomal leak of dye into the cytosolic and nuclear compartments (Fig. 6, A–C).

Since leakage of lysosomal enzymes such as cathepsin L has been reported to cause podocyte injury (39, 41), we examined the leakage of podocyte lysosomal cathepsin L in response to transduction with the APOL1G0 and APOL1Vs encoding lentivirus constructs. In these studies, cathepsin L labeling was carried out using the Magic Red kit as described in MATERIALS AND METHODS. APOL1Vs podocytes displayed diffuse distribution of cathepsin L in the cytosolic compartment; on the contrary, vector-treated podocytes showed containment of cathepsin L within lysosomes (punctate morphology) (Fig. 6, D and E). In turn, a consequence of enhanced LMP and lysosomal cathepsin L leakage is the degradation of the cytoskeletal protein F-actin (41). To compare the effect of APOL1G0 with APOL1Vs on podocyte F-actin, control and experimental podocytes were labeled for F-actin by using Alexa-labeled

![Graph](http://ajprenal.physiology.org.org)
APOL1Vs podocytes displayed scant actin filaments compared with both vector alone and APOL1G0 (Fig. 6, F and G). These findings suggest that APOL1 variants could be compromising the actin cytoskeleton by enhancing podocyte LMP.

A chloride channel blocker inhibits APOL1Vs-induced podocyte swelling. Since APOL1 has been demonstrated to induce lysosomal membrane depolarization and continuous influx of chloride, and subsequent osmotic swelling of the lysosomes in trypanosomes (36), we evaluated the effects of chloride channel blockers on APOL1Vs-induced podocyte swelling. G1 and G2 podocytes were incubated in media containing either buffer or the chloride channel inhibitor DIDS for 48 h. Subsequently, swollen and viable nonswollen podocytes were counted. As shown in Fig. 6, H and I, the chloride channel inhibitor provided protection against G1- and G2-induced podocyte injury.

Blocking membrane traffic attenuates APOL1Vs-induced podocyte injury. Since APOL1 is also a secreted protein and normally present in the circulation, it could reach the lysosomal compartments via an endosomal trafficking pathway (37). Therefore, we studied the effect of chloroquine (CQ), which blocks this pathway. Chloroquine attenuated the number of swollen and dead cells in APOL1Vs podocytes (Fig. 7, A and B). Similarly, changing the media every 12 h resulted in increased cell viability (Fig. 7C); these findings indicated that uptake of secreted APOL1Vs partially contributed to podocyte injury.

To confirm the role of secreted APOL1, podocytes were incubated in media containing conditioned media (CM; collected from vector-, G0-, G1-, and G2-expressing podocytes) for 36 h. Swollen cells and viable cells were quantified. Podocytes treated with CM-G1 and CM-G2 displayed higher numbers of swollen cells and lower numbers of viable cells compared with respective CM-vector and CM-G0 (Fig. 7, D and E). These findings indicate that factor elaborated into the media in the CM-G1 and CM-G2 contributed to podocyte injury.

APOL1Vs make podocytes vulnerable to adverse host factors. Only a percentage fraction of the population with the APOL1 risk genotype actually develop chronic kidney disease, suggesting the need for a secondary hit factor (42). Accordingly, we examined the effect of several anticipated adverse host factors (AHFs) on the state of podocyte injury in the setting of the WT and APOL1Vs. To determine the effects of such previously well-characterized AHFs, podocytes expressing vector, G0, G1, and G2 were exposed to H2O2, hypoxia, TNF-α, puromycin aminonucleoside, and HIV for different time periods, as indicated in Fig. 8. Subsequently, the percentage of viable cells was counted. AHFs decreased the number of living cells both in human podocytes infected with vector and with APOL1G0. However, the effects of AHFs were greatly augmented in podocytes expressing the APOL1G1 and G2 risk variants. The most prominent difference was observed in the case of the response to HIV. These findings indicate that podocytes expressing APOL1 are more susceptible to injury in the presence of AHFs. Thus it seems likely that depending on the severity of such secondary hit AHFs, and the degree of APOL1 expression, podocytes may pass a threshold of injury sufficient to cause progressive nephropathy. In this regard, it is noteworthy that some of the AHF’s which cause greater injury in podocytes expressing risk versions of APOL1 might themselves also induce the expression of APOL1. This would be the case especially for HIV and possibly other viral pathogens, which elicit an interferon-γ response (17).

DISCUSSION

There have been many clinical reports suggesting the role of APOL1Vs in the development of APOL1-associated nephropathy. However, to date there is a paucity of information in in vitro studies exploring the mechanism. Accordingly, this study was conducted to test the biological effects of APOL1 and its variants to determine whether there is a pathological counterpart to the differential effect of G0 and its kidney disease risk association variants, in a potentially relevant cell type. We examined several potential mechanisms wherein APOL1Vs could be causative in the greater susceptibility of African Americans to progressive major forms of chronic kidney disease in the category of podocytopathies. Since even APOL1G0 is toxic to cells when markedly overexpressed (47, 49), we titrated lentivirus-expressing APOL1 non-risk and risk variants to achieve levels of expression wherein G0 would have minimal toxicity. At the same doses, G1 and G2 display cell toxicity. These findings provide the first clear-cut biological, functional counterpart to the observed association studies and validate the variants as being causative and not only associated with cellular injury. Podocyte injury was evident as the increased percentages of swollen and necrosed cells. However, higher levels of G0 expression also increased LDH activity, decreased cell viability, and enhanced LMP and necrotic cell death; moreover, comparably high levels of G1 or G2 still further enhanced the manifestation of these phenotypes. These findings suggest that at comparable lower levels of expression,
Fig. 6. APOL1 risk variants increase podocyte lysosomal membrane permeability. A–G: human podocytes were transduced with the lentivirus (titrated as 0.8 pg HIV p24 protein/cell) for 3 h and were cultured in fresh medium for 48 h. LysoTrack red (A and B) and Lucifer yellow (A and C) staining was performed. Cathepsin L activity (D and E) was detected with a Magic Red Cathepsin L kit. F-actin (F and G) was detected with Alexa Fluor 594-conjugated phalloidin. Images were captured with a confocal fluorescence microscope, and representative microphotographs were selected (A, D, and F). Scale bars = 10, 20, and 100 μm for A, D, and F, respectively. The average fluorescence intensity was measured per region of interest (ROI) for each group (B, C, E, and G). H and I, human podocytes were transduced with the lentivirus (titrated as 0.4 pg HIV p24 protein/cell) for 3 h and were cultured in 1% serum medium with or without 100 μM DIDS for 72 h. Then, swollen cells (H) and living cells (I) were counted. *P < 0.05 compared with vector. #P < 0.05 compared with control.
the G1 and G2 variants of APOL1 are more conducive to podocyte cell injury compared with G0 or APOL1WT. This is potentially relevant to the formulation of a threshold effect as an explanation for the recessive or two-risk allele inheritance mode, as has been consistently reported (18).

The absence of a cellular phenotype after knockdown of endogenous APOL1 in human podocytes is also consistent with the observation that APOL1 is dispensable for kidney integrity (22). Interestingly, frequent changes of incubation media decreased APOLVs-induced podocyte injury, while direct exposure of conditioned media of G1 or G2 promoted podocyte injury; these findings indicate that a secretory component of APOL1Vs contributes to podocyte injury. Thus it is premature to dismiss the potential contributory roles of both intracellular and secretory APOL1 gene products in the pathophysiology of kidney injury in different clinical states and perhaps reflecting isoforms of APOL1 with differences in the signal peptide domain (8). Since chloroquine partially attenuated APOL1-induced podocyte injury, a role of endosomal traffic in transport of APOL1 may be inferred. Interestingly, the chloride channel inhibitor DIDS attenuated APOL1-induced podocyte swelling; these findings indicate that secretory effector molecule(s) transported via membrane trafficking may be targeted to lysosomes, in turn contributing to abnormal chloride influx and lysosomal swelling. As a cellular organelle, the lysosome is a membrane-bound vesicle loaded with low pH-dependent hydrolytic enzymes. The integral double-layer membrane around lysosome limits these enzymes within the lumen and protects the cytotoxic proteins from degradation. Increasing LMP will lead to the leakage of lysosomal enzymes into the cytosol, resulting in a series of severe cell injuries, including cytoskeleton degradation, apoptosis, and necrosis (1, 4, 5, 20). In the present study, overexpression of APOL1G1 and G2 in podocytes dramatically increased both LMP and the leakage of lysosomal components into the cytosol, which was also accompanied by APOL1-induced F-actin degradation and necrotic cell death in podocytes.

In this study, chloroquine, a known membrane traffic blocker, partially attenuated APOL1Vs-induced podocyte injury; moreover, the change of media every 12 h also provided partial protection against APOL1Vs-induced podocyte injury. Direct exposure of the conditioned media of G1 and G2 to podocytes inflicted podocyte injury. All of these findings indicate that uptake of secreted APOL1Vs through endocytosis might have also contributed to podocyte injury and that abnormal chloride flux could be contributing to osmotic lysosomal swelling, overwhelming the capacity to remove damaged lysosomes. Lysosome-mediated cell injury is also consistent with the observations of Perez-Morga et al. (36) displaying the effect of APOL1 on the depolarization of lysosomal membranes and continuous influx of chloride. Since it appears from the current findings that endosomal pathways may be required for APOL1 to target lysosomes, as also suggested by other investigators studying APOL1-induced lysosomal injury (36, 45), modulation of such membrane traffic, in addition to chloride channel blockade, could be used as a potential therapeutic means to ameliorate APOL1Vs-induced podocyte injury. Nonetheless, it will be important in future studies to delineate the details of the membrane traffic pathways and their role in the induction of podocyte lysosomal injury associated with APOL1Vs.

Genetic epidemiological studies have demonstrated that, while the odds ratios for kidney disease risk are high in association with APOL1Vs, only a small percentage of the population with harboring of APOL1Vs actually develops kidney disease. This suggests that genetic susceptibility is combined with a nongenetic second, which transforms risk to disease causation. This is especially prominent in the case of...
HIVAN, wherein in the absence of APOL1 genetic susceptibility, poor control of HIV viral load leads to HIVAN with a very high likelihood (3, 11). To examine the pathobiological underpinnings of this second-hit formulation, we evaluated the effect of G0, G1, and G2 versions of APOL1 in the presence of AHFs such as H2O2, hypoxia, TNF-α, puromycin aminonucleoside, and HIV. AHFs induced only mild podocyte injury in vector- and G0-expressing cells; however, the injury’s effect was more pronounced on top of a higher level of baseline injury in G1 and G2 podocytes. Thus it appears that both the expression levels of APOL1 and severity of one or more second hits may determine the sustainability of podocyte injury, past a threshold which may not be altered by compensatory mechanisms utilized for the maintenance of integrity of the glomerular filtration barrier (16). Consistent with the clinical and epidemiological observations, the second-hit counterpart was the most pronounced in the case of HIV. Among individuals with APOL1Vs genotypic susceptibility, >50% will develop HIVAN, which is about 10 times higher than those without HIV infection. This type of injury can be mitigated by successful viral control (2, 25, 30, 32). These studies indicate that APOL1Vs may cause particularly severe renal injury in the setting of HIV infection. In a previous study, we noted that HIV increased lysosomal enzyme cathepsin L activity, which further compromised the integrity of the podocyte cytoskeleton (7). Taken together, we speculate that APOL1Vs and HIV may act synergistically on such lysosomal damage, leading to enhanced leakage of lysosomal components, exceeding the capacity to deal with damaged lysosomes by normally available pathways, and stronger lysosomal cathepsin L activity than in the case of either APOL1Vs or HIV alone. This could be a possible mechanism for the prominence of the association of APOL1Vs with HIVAN. Thus it appears that both the expression levels of APOL1 and the severity of a second hit act in concert to determine the extent of podocyte injury. In patients, there may also be compensatory repair mechanisms utilized for the maintenance of integrity of the glomerular filtration barrier.

Although we observed that APOL1 variants would lead to podocyte loss, the underlying molecular mechanism is still unknown. The T. brucei subspecies rhodesiense and gambiense resist the lytic activity of Apol1WT and can infect humans, causing sleeping sickness (29). In the case of Tbr, resistance, their lysis involves interaction of the SRA protein with the C-terminal helix of APOL1 (14, 19, 25). This observation led us to believe that there may be functional SRA analogs in human serum or on the cell vesicle membranes. We propose that it would be important to examine whether the APOL1 protein may have the capacity to cause lysosomal membrane damage, while this toxic function is blocked in the WT through binding to such a functional SRA homolog at the C terminus.

To examine this possibility, we constructed an artificial G1/G2 APOL1 variant containing both mutations of G1 and G2 on the same sequence. We observed that expression of G1/G2 showed far greater toxicity to podocytes compared with G1 or G2 alone. For example, trypan blue staining showed a ratio of dead cells at 48 hpi was close to 50% for either G1 or G2, but 70%
for the combined G1/G2 construct (data not shown). These findings are consistent with the gain-of-injury formulation noted and provide a potentially important tool to screen for protective molecules. While the current study focused on the effects of APOL1 on podocytes in culture, this does not exclude additional sites of expression, cellular targets, or mechanisms. We also recognize that cells in culture can amplify and accelerate cellular injury processes, causing them to appear in relatively shorter time periods, compared with the corresponding clinical situations. This may be attributed to lack of compensatory defense and repair mechanisms, which apply in the whole organism. Cell culture systems amplify effects, which take longer or are milder in vivo, and this can be attributed to a hostile cell growth environment with an absence of biological matrices, reduced serum factors, and induction of increased cellular expression of protein. Nevertheless, this platform has enabled the clear-cut discernment of differential biological effects of APOL1 and its variants in a relevant experimental system that has been used extensively in the past to study mechanisms involved in podocytopathies. By titrating expression of APOL1 G0 to levels which did not induce podocyte injury, but in which corresponding levels of G1 and G2 expression did inflict such injury, together with the differences in degrees of injury at higher concentrations using multiple assays, and the augmented adverse host factor effects, this study provides compelling preliminary evidence that extends beyond genetic association and should enable causative mechanisms to be examined.

In summary, overexpression of APOL1G0, G1, and G2 are toxic to podocytes. However, importantly, the APOL1 G1 and G2 variants induce a greater degree of podocyte injury, which manifests differently from one of cellular swelling and necrosis. This effect is accompanied by evidence of enhanced LMP, F-actin disruption, and can be blocked partially by chloroquine and a chloride channel inhibitor. It is very likely that many other mechanisms may be involved, as these cells other than podocytes may be targeted as well. Nevertheless, as far as current literature is concerned, this is the first report of a differential effect of APOL1 risk variants on cellular function in kidney cells and therefore provides a platform for unraveling the molecular interaction that is responsible. On that present, this study provides insight into the mechanisms involved in APOL1-associated nephropathy and would help to set up high-throughput compound screening and potential prevention and therapy for APOL1-associated nephropathy.

REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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APOL1 AND PODOCYTE NECROSIS


