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Review

The Two Levels of Podocyte Dysfunctions Induced by Apolipoprotein L1 Risk Variants

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Abstract: Apolipoprotein L1 (APOL1) nephropathy results from several podocyte dysfunctions involving morphological and motility changes, mitochondrial perturbations, inflammatory stress, and alterations in cation channel activity. I propose that this phenotype results from increased hydrophobicity of the APOL1 risk variants, which induces two distinct types of podocyte dysfunctions. On one hand, increased hydrophobic interactions with APOL3 cause intracellular variant isoforms to impair both APOL3 control of Golgi PI(4)P kinase-B (PI4KB) activity and APOL3 control of mitochondrial membrane fusion, triggering actomyosin reorganisation together with mitophagy and apoptosis inhibition (hit 1). On the other hand, increased hydrophobic interactions with the podocyte plasma membrane may cause the extracellular variant isoforms to activate toxic Ca^{2+} influx and K^+ efflux by the TRPC6 and BK channels, respectively (hit 2), presumably due to APOL1-mediated cholesterol clustering in microdomains. I propose that hit 2 depends on low HDL-C/high extracellular APOL1 ratio, such as occurs in cell culture in vitro, or during type I-interferon (IFN-I)-mediated inflammation.

Keywords: APOL1 nephropathy; APOL1 risk variants; podocyte dysfunction; APOL1 pore; membrane dynamics; fission; fusion; mitophagy; apoptosis

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1. Introduction: APOL1 Secretion, a Unique Feature in the APOL Family

Among the numerous members of the dynamic mammalian apolipoprotein L (APOL) families, human APOL1 is the only known member to be secreted in the bloodstream, where this protein associates with serum high-density lipoprotein-C (HDL-C) particles, which carry the "good" cholesterol and exhibit anti-inflammatory activity [1]. Therefore, the original function of APOLs must be intracellular [2]. APOL1 is present as both secreted and intracellular isoforms generated by differential transcript processing. In the murine APOL family, mAPOL9 is probably the APOL1 homologue [3], but being devoid of a signal peptide, it cannot be secreted. Thus, the addition of a signal peptide provided a novel function to human APOL1, while keeping original intracellular activities in distinct isoforms. Since secreted APOL1 kills the African bloodstream parasite *Trypanosoma brucei*, the invention of this isoform in primates was presumably linked to the need for resistance to this pathogen, which causes lethal sleeping sickness in Sub-Saharan Africa [4]. Accordingly, APOL1 expression is strongly increased by the Interferon-type I (IFN-I) inflammatory signalling triggered by trypanosome or viral infection [5–7], and APOL1 association with HDL-C particles allows its efficient receptor-mediated uptake in the parasite [4]. Once taken up in the trypanosome digestive system, APOL1 undergoes acidic pH-dependent insertion in endosomal membranes, followed by *T. brucei* kinesin family C1 (TbKIFC1)-mediated transport of these vesicles to the mitochondrion, leading to mitochondrial membrane permeabilisation and transfer of mitochondrial endonuclease G (TbEndoG) to the nucleus, with consecutive DNA fragmentation [8].

In Eastern Africa, the *T. brucei* clone *T. b. rhodesiense* managed to resist APOL1 through expression of the serum resistance-associated (SRA) protein, a truncated form of the main surface antigen of the parasite, the variant surface glycoprotein [9]. Probably due to its

truncation, SRA is trafficked to the parasite endocytic system, where this protein interacts with internalised APOL1, neutralising its trypanosome lytic activity and therefore, allowing *T. b. rhodesiense* to infect humans, causing sleeping sickness [10]. Since SRA interacts with the C-terminal region of APOL1, experimental mutagenesis was undertaken to provide resistance of this region to the neutralising interaction by SRA. This study revealed that some recombinant C-terminal APOL1 variants can indeed restore efficient killing activity on *T. b. rhodesiense* parasites [11]. Remarkably, similar but natural variants were subsequently discovered in human individuals of African origin [12]. The APOL1 variants G1 and G2 (S342G/I384M mutations and 388NY389 deletion, respectively), are widespread in Eastern Africa and exhibit killing activity on *T. b. rhodesiense* [12].

Unfortunately, resistance to African trypanosome infection is strongly correlated with human susceptibility to non-diabetic chronic kidney disease, and such disease was reproduced upon transgenic expression of G1 or G2 in mice, demonstrating the causal effect of the APOL1 variants on the disease [12–14]. G1- or G2-associated kidney disease (APOL1 nephropathy) results from kidney dysfunctions linked to changes in podocyte architecture and motility, triggering the detachment of podocytes from glomeruli following podocyte foot process effacement. IFN-I-mediated inflammation, as occurs upon viral infection, particularly promotes APOL1 nephropathy [14,15]. Accordingly, the effects of APOL1 variants on kidney podocytes in the absence and presence of viral infection were called hit 1 and hit 2, respectively.

Interestingly, in addition to APOL1 G1 and G2, the APOL3 null allele rs11089781 is also linked to chronic kidney disease [16]. Thus, kidney disease results from either the expression of C-terminal APOL1 variants or the absence of APOL3.

In this review, I propose a comprehensive model of podocyte dysfunctions induced by the different isoforms of the APOL1 risk variants. Distinct effects of intracellular and extracellular isoforms contribute to the severity of the disease, depending on the HDL-C/APOL1 ratio in the podocyte environment.

2. APOL1 Structure and Interactions

Based on recent studies [7,17], Figure 1 presents a model of APOL1 structure and interactions, compared with those of APOL3. The N-terminal domain of each protein contains five helices including a four-helix bundle (helices 2 to 5) [18]. To account for their prominent characteristics, helices 2 and 4 are termed here hydrophobic cluster 1 (HC1) and leucine zipper 1 (LZ1). A hydrophobic hairpin helix potentially able to cross a membrane (TM, for transmembrane) separates this domain from the C-terminal region. In the case of APOL1, but not APOL3, negatively charged residues in this hairpin only allow TM insertion under acidic conditions, due to the need for their protonation for helix insertion into lipids. APOL1 trypanosome killing activity absolutely requires a low endosomal pH [8,19], indicating that TM insertion is necessary for this activity. According to its ability for pH-independent TM insertion, recombinant APOL3 can also kill *T. brucei*, but in this case, trypanosome lysis does not require endosomal acidity [19]. Another helix hairpin termed MAD for the membrane-addressing domain (helices MAD1 and 2) is required for APOL1 membrane association [20], likely through interaction with anionic phospholipids [7]. After a long hinge sequence, a second HC-LZ tandem (HC2-LZ2) characterises the C-terminal region of both APOLs. In the case of APOL1 but not APOL3, the HC2-LZ2 tandem strongly interacts with HC1-LZ1, imposing a specific protein folding that underlies the differential ability of these proteins to interact with other proteins [7,17].

In addition to *T. b. rhodesiense* SRA, which binds to the APOL1 HC2-LZ2 tandem following acidic pH-induced disruption of the HC1-LZ1/HC2-LZ2 complex in trypanosome endosomes [3,10,17], another clearly identified APOL1 partner is non-muscular myosin-2A (NM2A), whose three subunits myosin high chain-9 (MYH9), regulatory light chain (RLC) and essential light chain (ELC) are all strongly and stoichiometrically immunoprecipitated from podocyte extracts with anti-APOL1 antibodies [7]. Although the details of this association are still unclear, a direct interaction between APOL1 and the LC subunits can be

envisaged, based on the similarity between these calmodulin-like subunits and neuronal calcium sensor-1 (NCS1), which can strongly bind to APOL3 (see below). Accordingly, APOL1 can directly interact in vitro with the MYL12A RLC (E. Pays, unpublished data). Like its putative mouse homologue, mAPOL9 [3], APOL1 can also bind to the mitophagy receptor prohibitin-2 (PHB2) [21,22], possibly through helix 5 interaction [17].

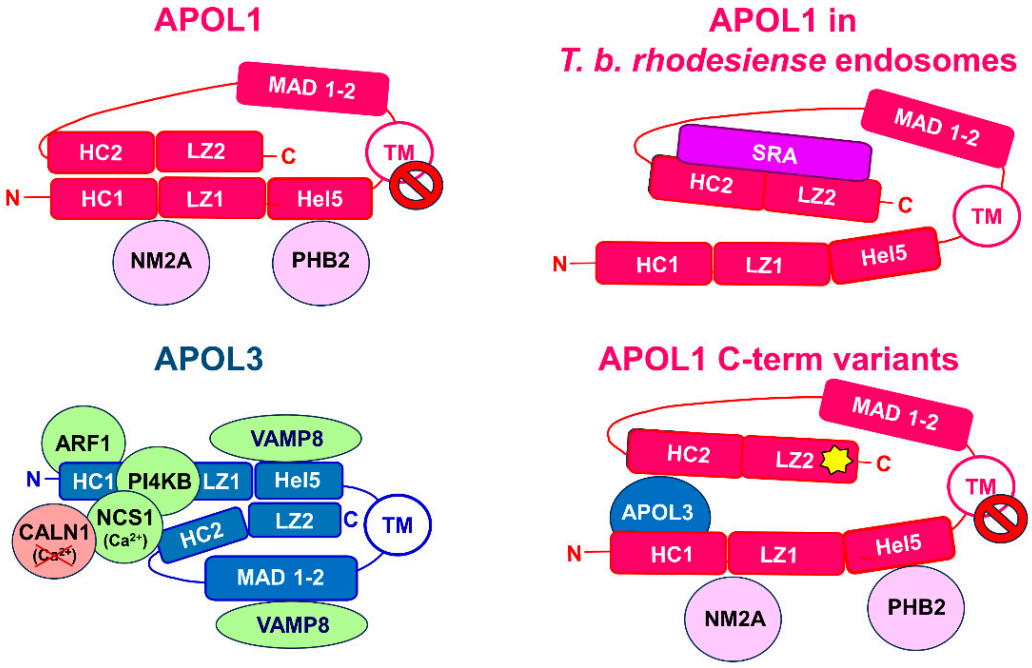

Figure 1. Folding and interactions of APOL1 and APOL3. Models of *cis*- and *trans*-interactions of APOL1, APOL1 C-terminal variants and APOL3 (HC = hydrophobic cluster; Hel = helix; LZ = leucine zipper; MAD = membrane-addressing domain; TM = TM span (⛔: in APOL1, TM insertion strictly requires acidic conditions; ✸: C-terminal G1 and G2 mutations; Ca^{2+}: under calcium-free conditions). For the sake of clarity, each HC-LZ tandem is presented as a linear structure, but these tandems, as well as MAD, are probably folded as double-stranded hairpins. The functions of proteins interacting with APOLs are defined in the text.

APOL1 only weakly interacts with APOL3, but disruption of *cis*-interaction between HC1-LZ1 and HC2-LZ2 by G1 or G2 mutations promotes this interaction, due to increased exposure of HC1 and HC2, which enhances general APOL1 hydrophobicity and favours HC1 interaction between APOL1 and APOL3 [7,17].

In APOL3, no *cis*-interaction between the HC1-LZ1 and HC2-LZ2 tandems was detected, likely due to the lower interaction potential of both helices [17]. In this case, LZ2 could interact with helix 5, preventing APOL3 interaction with PHB2 [17]. The lack of interaction between the two HC-LZ tandems allows higher HC exposure than occurs in APOL1. HC1-mediated hydrophobic interactions promote the binding of different proteins controlling the synthesis of phosphatidylinositol-4-phosphate (PI(4)P) by the PI(4)P kinase-B (PI4KB) at the *trans*-Golgi. In addition to PI4KB itself, APOL3 interacts with the PI4KB activators NCS1 and ADP-Ribosylation-Factor-1 (ARF1), as well as with the PI4KB inhibitor Calneuron-1 (CALN1), depending on Ca^{2+} concentration [7,17]. Finally, APOL3 can also bind to the membrane fusion factor vesicle-associated membrane protein-8 (VAMP8), through combined VAMP8 interactions with the two APOL3 helices flanking the TM hairpin (helix 5 and MAD1) [17].

3. Intracellular APOL1 Function: Vesicular Trafficking Control

APOL1 secretion only occurs in a few African primate species, indicating that this isoform is generally dispensable, being probably only designed to resist bloodstream

African pathogens. Accordingly, individuals naturally lacking APOL1 do not exhibit a detrimental phenotype [23,24], suggesting that APOL1 is dispensable apart from its involvement in pathogen resistance. However, intracellular APOL1 could exert functions only detectable under certain conditions. Accordingly, APOL1 deletion in podocytes (APOL1 KO) appeared to result in a wild-type (WT) phenotype in vitro, but in these cells, both IFN-I-mediated apoptosis and mitophagy were strongly reduced (more than 60 and 90%, respectively) [7,17]. To explain these observations, it is necessary to detail the functions of APOL3 (Figure 2).

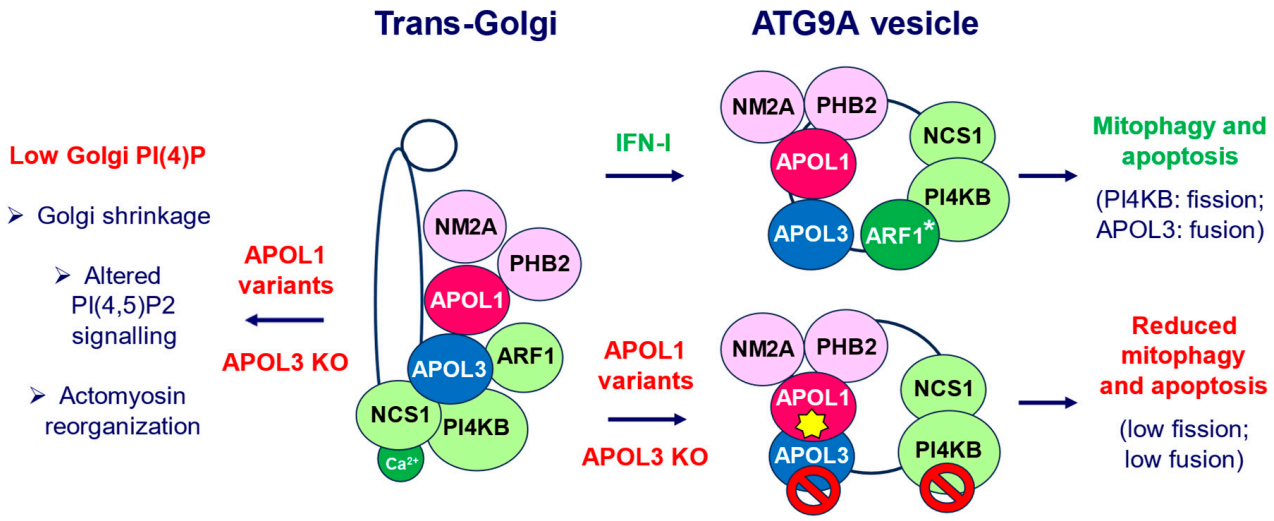

Figure 2. Hypothetical model of the APOL3 and APOL1 activities [7,17]. At the *trans*-Golgi, APOL3 is associated with PI4KB and PI4KB controllers ARF1, NCS1 and CALN1 to regulate membrane fission for secretion. APOL3 also weakly interacts with APOL1, which is associated with the NM2A myosin and mitophagy receptor PHB2. IFN-I signalling affects APOL3 interaction with PI4KB following the binding of activated ARF1 (ARF1*) to PI4KB. This allows PI4KB delocalisation in Golgi-derived ATG9A vesicles. Similarly, APOL1 C-terminal variants can induce APOL3 dissociation from PI4KB, partially mimicking APOL3 KO. PI4KB-carrying ATG9A vesicles are targeted to MERCS, and this may involve association of APOL1 with both NM2A and PHB2. The PI4KB-ARF1 complex of ATG9A vesicles triggers mitochondrial fission and mitophagy, whereas APOL3 promotes mitophagosome fusion with endosomes. In either APOL3-KO cells or APOL1 variant-expressing cells, mitochondrial membrane fission and fusion are reduced, due to PI4KB and APOL3 inactivation, respectively [17] (: see Figure 1).

In striking contrast with APOL1, APOL3 can form a trimeric complex with PI4KB and NCS1 at the *trans*-Golgi, promoting the membrane recruitment of Ca^{2+}-NCS1 to increase PI4KB activity [7,17]. Synthesis of PI(4)P allows the membrane association of PI(4)P-binding factors involved in secretion, particularly Golgi phosphoprotein-3 (GOLPH3), which interacts with the NM2A binder myosin-18 (MYO18) to induce vesicle budding [25]. Disruption of the APOL3-PI4KB-NCS1 complex can be achieved in three ways [17]: (1) IFN-I-mediated increase in ARF1-PI4KB interaction owing to ARF1 activation (GDP to GTP binding) [26], which can interfere with APOL3-PI4KB interaction; (2) G1- or G2-mediated increase in APOL1 interaction with APOL3, which inactivates APOL3 [7]; (3) APOL3 deletion (APOL3 KO). APOL3-PI4KB-NCS1 complex disruption is linked to reduced PI4KB activity at the Golgi, leading to lower Golgi PI(4)P levels. This triggers actomyosin reorganisation, Golgi shrinkage and PI4KB traffic in Golgi-derived vesicles carrying the lipid scramblase autophagy-9A (ATG9A) to mitochondrion–endoplasmic reticulum contact sites (MERCS), where mitophagy is initiated [17,27,28]. Thus, APOL3 interaction with PI4KB and NCS1 ensures the sequestration of these proteins at the Golgi, and interference with this interaction triggers their delocalisation to other membranes such as MERCS. If induced

by IFN-I, the PI4KB/ARF1 transfer to MERCS can induce mitochondrion fission [27], initiating mitophagy [28], and APOL3 completes mitophagy through interaction with VAMP8, promoting membrane fusion between mitophagosomes and endolysosomes [17]. If the PI4KB transfer to MERCS is induced by APOL3 inactivation or deletion, both PI4KB fission and APOL3 fusion activities are reduced, likely due to the lack of ARF1 activation in the first case [17,27].

In APOL1-KO podocytes, mitophagy was inhibited, and in APOL1+APOL3 double KO podocytes, the mitochondrial phenotype due to PI4KB transfer from the Golgi was totally absent, indicating that APOL1 is responsible for PI4KB transfer from the Golgi to MERCS [7,17]. In APOL1+APOL3 double KO cells, the absence of APOL1 allowed restoration of PI4KB activity at the Golgi, albeit at a slightly reduced level. Such PI4KB activity occurring despite APOL3 KO suggests that the most important function of APOL3 is not PI4KB activity control, but PI4KB sequestration at the Golgi membrane, opposing PI4KB delocalisation by APOL1. Supporting the role of APOL1 in such delocalisation, APOL1 was found to tightly associate with both the NM2A myosin, which drives ATG9A vesicle trafficking [29,30], and the mitophagy receptor PHB2, which directs cargoes to auto/mitophagy initiation sites at MERCS [22]. Thus, a clearly defined function of intracellular APOL1 is directing PI4KB traffic to MERCS, such as occurs upon inflammation.

In synthetic membranes in vitro or in trypanosome endosomal membranes in vivo, APOL1 exhibits ionic pore-forming activity [20,31,32]. Under acidic conditions, APOL1 triggers a weak anionic flux, but consecutive shifting to neutral pH induces high cation conductance [31]. Accordingly, in trypanosomes APOL1 permeabilised the lysosomal membrane for the passage of small molecules under 10 kDa, allowing weak anionic fluxes [8,20]. However, trypanosome lysis does not result from such fluxes [8,19]. Lysis rather necessitates the TbKIFC1-mediated traffic of APOL1-containing membranes to the mitochondrion, where apoptotic-like megapore activity (mitochondrial membrane permeabilisation) allows the transfer of the 56 kDa mitochondrial endonuclease TbEndoG into the nucleus, causing DNA fragmentation [8]. Thus, after prior TM insertion in the acidic environment of endosomes, APOL1 activates apoptotic-like mitochondrial membrane megapores, rather than ion pores. Whether APOL1 can form megapores is uncertain, and APOL1 could rather directly or indirectly activate megapore formation by hypothetical trypanosome pro-apoptotic members of the B-cell lymphoma-2 (BCL2) family (Figure 3).

In addition, APOL1 trafficking to the trypanosome mitochondrion increases mitochondrial membrane fusion, in a process termed "mitochondrial fenestration" [8]. Such mitochondrial membrane fusion activity is also exerted by APOL3 in podocytes, where it is linked to the protein capacity for TM insertion at neutral pH [8,17,19]. Since APOL1 requires preincubation at low pH to perform membrane fusion [17], trypanosome lysis requires APOL1 TM insertion not for ionic pore activity, but for membrane fusion and megapore activation [8]. Therefore, the weak APOL1 anionic pore-forming activity in endosomes represents a side effect, not directly responsible for trypanosome death. Whether APOL1-containing endosomal membranes can also be trafficked to the trypanosome surface, causing cation fluxes involved in trypanolysis [31], has not been demonstrated yet.

In podocytes, no cation pore-forming activity nor cytotoxicity was attributed to intracellular APOL1 or APOL1 variant isoforms [34]. Similarly, no evidence for APOL3 ion pore-forming activity was detected in podocytes, despite the ability of this protein to insert into membranes for induction of membrane fusion [17]. Instead, either APOL1 or APOL3 deletion strongly reduced inflammation-induced apoptosis, clearly indicating that both APOLs are involved in apoptosis [7]. While this conclusion is in keeping with the capacity of APOL3 for TM insertion, such is not the case for APOL1, not detected in acidic environments and therefore, unlikely to insert into membranes [7]. According to the traffic function of APOL1, inhibition of apoptosis in APOL1-KO cells more likely results from a lack of APOL1-driven APOL3 traffic for apoptosis induction at the mitochondrion. Consistently, apoptosis inhibition was not higher in APOL1+3 double KO podocytes than in single APOL3-KO cells, indicating that APOL1 does not exert apoptotic activity per se [7].

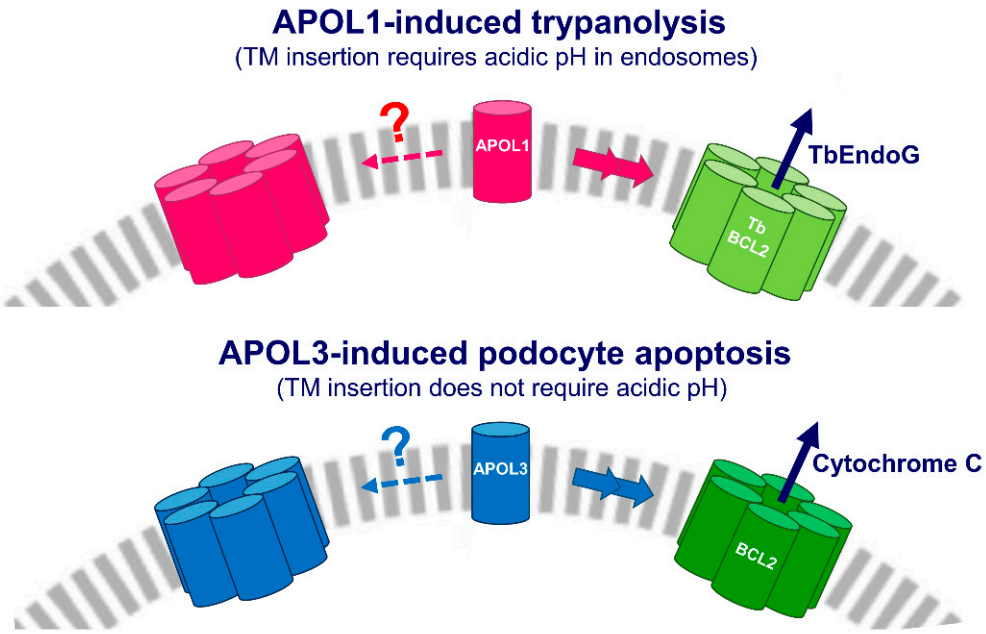

Figure 3. The APOL1 and APOL3 killing activities. In trypanosomes, APOL1 can insert into endosomal membranes owing to the acidic conditions, and APOL1-induced trypanosome lysis results from apoptotic-like megapore activity in the mitochondrial membrane [8]. In podocytes, APOL1 and APOL3 are crucially involved in inflammation-induced apoptosis, through induction of APOL3 trafficking to the mitochondrial membrane and APOL3-induced megapore activity, respectively [7]. For both APOL1 and APOL3, alternative hypotheses can account for death induction: either APOL oligomerisation in megapores or BH3-mediated APOL interaction with BCL2-like proteins (in green), which would trigger activation of pro-apoptotic BCL2 pores for the release of *T. brucei* endonuclease G (TbEndoG) or cytochrome C, in trypanosomes and podocytes, respectively. Whereas no evidence supports the eventual APOL oligomerisation in megapores (question marks), the interaction between murine mAPOL7 and anti-apoptotic BCL-XL in dendritic cells [6] suggests that APOLs could inhibit anti-apoptotic activity (double arrows: induction of apoptosis through inhibition of anti-apoptotic activity). Accordingly, trypanosomatids contain transmembrane BCL-XL-interacting proteins exhibiting a BH3-like motif (termed TbBCL2 here) [33].

Thus, despite the capacity of APOL1 and APOL3 to form ion channels in vitro, no evidence for ionic pore-forming activity was obtained for neither protein within podocytes. Interestingly, this paradox is shared by pro- and anti-apoptotic BCL2 family proteins, whose TM helix hairpin can form monomeric ionic pores in vitro, but rather forms oligomeric megapores in vivo, with a lipidic rather than proteinaceous pore interface [35,36].

While intracellular APOL1 variants do not induce ionic pore-forming activity [34], induction of the G1 or G2 phenotype does not require such activity. As mentioned above, the APOL3 null allele rs11089781 was significantly linked to kidney disease, even in the absence of APOL1 variants [16]. Accordingly, APOL3-KO podocytes exhibited a G1- or G2-like phenotype, albeit with higher severity [7,17]. The difference in phenotype severity between APOL3-KO and G1/G2 cells can be explained by differential APOL3 inhibition: complete APOL3 absence versus partial APOL3 inactivation. Similarly, endogenous replacement of WT APOL1 by a C-terminally truncated version (APOL1Δ: stop at V353), which completely lacks both in vitro pore-forming activity and toxicity in trypanosomes [11,31], induces a severe phenotype very similar to that of APOL3-KO cells [7,17]. Such a severe phenotype could be explained by the much stronger APOL3 interaction with APOL1Δ than with G1 or G2, as expected from the difference between LZ2 alterations in these variants: full LZ2 deletion versus only LZ2 mutations [7,17]. Thus, APOL3 deletion or inactivation, rather than APOL1 variant pore-forming activity, appears to be required for induction of the G1 or G2 phenotype.

Collectively, the only evidence for intracellular APOL1 ionic pore-forming activity in vivo is weak anionic fluxes occurring in trypanosome endosomes [20]. These fluxes occur in conditions quite distinct from the usual APOL environment and are not responsible for trypanosome lysis [8,19,20]. In the trypanosome endosome lumen like under in vitro conditions, APOL1 could be unable to form or activate megapores, resulting in non-physiological ionic pores.

I conclude that in podocytes, the function of intracellular APOL1 is not ion pore formation, but vesicular trafficking control. Similarly, the intracellular function of APOL3 is not ion pore formation despite the APOL3 capacity for TM insertion at neutral pH, but rather induction of apoptosis and mitophagy-associated membrane fusion.

4. Extracellular APOL1 Function: Specific Innate Immunity

Secreted APOL1 is predominantly carried by HDL-C particles, in a process involving a change in APOL1 folding [1,37]. These particles contain a central core of esterified cholesterol, enveloped by a layer of phospholipids, free cholesterol, and apolipoproteins. The mechanism involved in their association with APOL1 is unknown, but APOL1 contains two cholesterol recognition motifs (cholesterol recognition amino acid consensus, or CRAC) [38], respectively, in the MAD domain (268-LAGNTYQLTR-277) and in HC2 (347-LDVVYLVYESK-357), suggesting a possible APOL1 interaction with free cholesterol at the HDL surface. Interestingly, both CRAC motifs are absent from other human or murine APOLs, linking their presence with the exclusive extracellular function of APOL1. APOL1 binding to cholesterol could be crucially involved in the association of this protein with HDL-C particles because so far, no evidence indicates that HDL-C proteins are involved in this process. In particular, none of the known HDL-C apolipoproteins were found in APOL1 immunoprecipitates or APOL1 interaction screens, despite the abundance of some of them, such as apolipoprotein A1 [7]. In HDL-C particles, the human-specific haptoglobin-related protein (HpR)–haemoglobin (Hb) complex acts as a Trojan horse for receptor-mediated recognition of this complex and consecutive efficient HDL-C uptake by the parasite. Indeed, HpR-Hb can bind to a trypanosome receptor normally meant to internalise the closely similar haptoglobin-Hb (Hp-Hb), which is a parasite growth factor [39].

I conclude that in humans, the major role of HDL-C particles in cholesterol recycling was used to generate a specific immunity system particularly designed to kill bloodstream African trypanosomes, with an APOL1-specific serum carrier binding system (exclusive APOL CRACs), a human-infective trypanosome-specific entry device (exclusive HDL-C HpR-Hb) and a receptor-mediated uptake-dependent intracellular toxin (acidic pH-activated pro-apoptotic APOL1).

5. Intracellular Variants: Kidney Disease Hit 1

As mentioned above, increased hydrophobicity of APOL1 variants inactivates APOL3 due to increased interaction, leading to disruption of the APOL3-PI4KB complex at the *trans*-Golgi and PI4KB trafficking from the Golgi to MERCS, linked to reduced Golgi PI(4)P levels, Golgi shrinkage and reduced mitophagy flux. Such phenotype was not only exhibited in podocytes differentiated in vitro but also in natural urine and glomerular G1 and G2 podocytes [7].

However, PI4KB and NCS1 are transported by Golgi-derived vesicles not only to MERCS but also to the plasma membrane and endosomes [40–42]. Since both Golgi and plasma membrane PI(4)P pools affect the amount of PI(4,5)P2 at the inner plasma membrane leaflet [43], such PI4KB trafficking may influence PI(4,5)P2-mediated signalling [44–46]. In particular, PI(4,5)P2 regulates Ca^{2+} influx through transient receptor potential cation channel-6 (TRPC6), together with K^+ efflux through the Ca^{2+}-dependent big K^+ conductance (BK) channel [47,48] (Figure 4). These channels control the filtration barrier permeability of glomerular podocytes through interaction with the actin cytoskeleton and are involved in various forms of chronic kidney disease [49–58]. Likewise, NCS1 can activate Kv4 K^+ channels [59,60], G-protein coupled receptors (GPCRs) [61–64] and IP3

receptors (IP3Rs), promoting Ca^{2+} release from endoplasmic reticulum stores, particularly at MERCS [65,66]. Thus, PI4KB and NCS1 delocalisation due to APOL3 inactivation by APOL1 variants can strongly affect intracellular signalling. On the other hand, PI4KB trafficking from Golgi to endosomes allows the activation of the antiviral effector stimulator of the interferon gene (STING), initiating the IFN-I inflammatory response [42]. Such observation agrees with the finding that intracellular APOL1 risk variants induce mitochondrial dysfunctions and inflammation upstream from alterations of cation fluxes [67,68], implying that mitochondrial dysfunctions do not result from cellular toxicity, but rather from APOL1 variant effects unrelated to cation pore activity. Accordingly, either APOL3 KO or APOL3 inactivation by APOL1Δ, which cannot affect APOL1 pore-forming activity, induced mitochondrial reactive oxygen species (ROS) production [17].

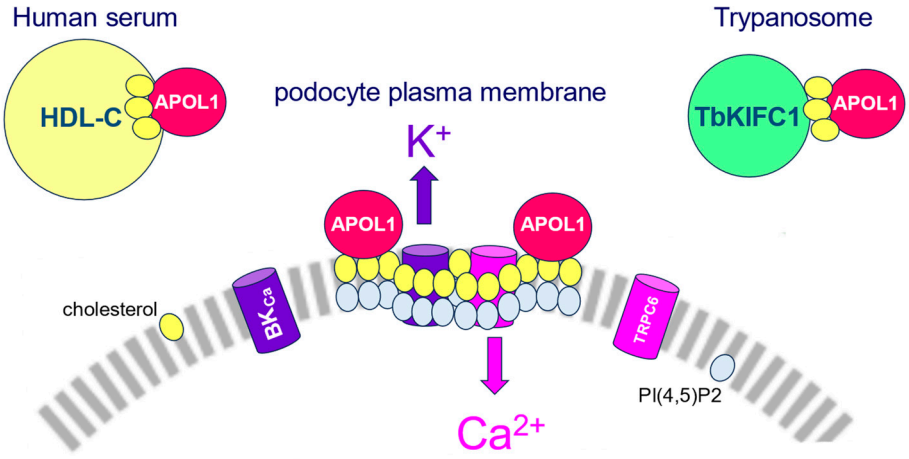

Figure 4. The role of cholesterol in APOL1 trafficking and toxic activity. APOL1 is associated with cholesterol in plasma HDL-C carrier particles or with the *Trypanosoma brucei* cholesterol carrier TbKIFC1 kinesin [69]. I propose a model of podocyte cation channel activation resulting from cholesterol clustering in surface microdomains by HDL-unbound APOL1. Both Ca^{2+} TRPC6 channels and Ca^{2+}-dependent big K^+ conductance BK channels are activated by cholesterol-dependent microdomain formation involving podocin and nephrin.

Collectively, the reduction of Golgi PI(4)P level, induction of PI4KB trafficking to other intracellular membranes such as MERCS, and resulting reduction of mitophagy, account for the phenotype of G1 or G2 podocytes under non-inflammatory conditions [7,17]. Reproduction of this phenotype upon transgenic expression of G1 or G2 in mice [13] probably results from APOL1 variant effects on a murine APOL3 homologue, likely mAPOL8 [3].

I conclude that intracellular G1 and G2 are responsible for hit 1 of the disease.

6. Extracellular Variants: Kidney Disease Hit 2

Several observations linked the severity of APOL1 nephropathy with the level of circulating APOL1 [14]. Accordingly, APOL1-linked cytopathology was found to strictly depend on APOL1 variant secretion [70]. Because virus-induced IFN-I signalling triggers increased APOL1 expression, and because this signalling is crucially involved in disease severity [71], inflammation is clearly responsible for disease hit 2. This effect did not appear to result from higher levels of circulating APOL1 variants as compared with WT APOL1 [72], but the variants were associated with enlarged HDL particles [73].

In many studies reporting the results of ectopic APOL1 expression in various cell types cultivated in vitro, G1 or G2 induced more important cytotoxicity than WT APOL1, and this was attributed to the extracellular association of secreted/circulating APOL1 with the plasma membrane, triggering increased surface Ca^{2+} influx and K^+ efflux, linked to stress signalling involving IP3-induced Ca^{2+} release [34,37,74–78]. As discussed above, intracellular APOL1 or APOL1 variants do not form ion pores in vivo, and without prior

acidification, APOL1 cannot form TM pores in synthetic membranes in vitro or in trypanosomes in vivo [11,19,20,31]. The secretory pathway used by extracellular APOL1 or APOL1 variants isoforms does not involve transit through endosomal vesicles, and if this occurred, APOL1 would be prevented from being secreted due to TM insertion in endosomal membranes like occurs within trypanosome endosomes, therefore reducing the levels of circulating APOL1. Likewise, hypothetical surface trafficking of the secreted APOL1 isoform after eventual TM insertion into Golgi membranes, owing to low Golgi pH, would obviously inhibit APOL1 secretion, while also causing intracellular ionic fluxes before reaching the plasma membrane. Finally, in either the bloodstream or cell culture medium, TM insertion of secreted APOL1 in the plasma membrane is also very unlikely, due to insufficiently acidic conditions. Thus, membrane pore formation by secreted APOL1 is quite improbable.

The correlation between APOL1 presence at the cell surface and increased Ca^{2+} and K^+ fluxes [34,37,74] does not imply that these distinct fluxes occur in APOL1 pores, and the physical evidence of TM APOL1 insertion in the plasma membrane has not been provided yet. Inhibition of both K^+ flux and cytotoxicity by anti-APOL1 antibodies, SRA or the anti-APOL1 VX-147 compound [77–79] does not demonstrate the existence of APOL1 pores, because these inhibitors are likely to inactivate surface-exposed APOL1 without specifically targeting the pore. It is at least equally plausible that secreted APOL1 triggers Ca^{2+} influx and K^+ efflux following activation of existing podocyte cation channels, for instance through membrane disturbance due to hydrophobic interactions.

The activity of several cation channels, like Ca^{2+} TRPC6 channels and Ca^{2+}-dependent K^+ BK channels, which are both crucially involved in podocyte function [49–58], is regulated in lipid microdomains containing cholesterol at the outer membrane leaflet and PI(4,5)P2 at the inner leaflet [45–48,80–85] (Figure 4). Cholesterol- and sphingomyelin-rich regions in the outer leaflet align with clusters of PI(4,5)P2 in the inner leaflet by a mechanism termed *trans*-bilayer coupling [46,86]. Cholesterol controls the activity of the TRPC6 Ca^{2+} channels, which mainly occurs at lipid rafts and is very sensitive to mechanical tension on these microdomains [87–89]. In particular, podocin, which oligomerises in pedicels lipid rafts together with nephrin to form the filtration slits, binds and recruits cholesterol to the TRPC6 ion channel complex at the slit diaphragm, regulating the activity of TRPC6 in a cholesterol-dependent manner [82,90–92]. Moreover, cholesterol also affects podocyte BK K^+ channel activity, which is connected to TRPC6 [49,51,82–85]. Thus, interfering with cholesterol at the plasma membrane outer layer is likely to affect podocyte cation fluxes, possibly also enhancing PI(4,5)P2-mediated activation at the inner membrane layer, depending on changes in intracellular PI(4)P levels. In the serum, APOL1 associates with cholesterol-rich HDL-C particles, and in trypanosomes, APOL1 associates with the TbKIFC1 kinesin, involved in cholesterol transport [8,69] (Figure 4). Therefore, if not sequestered in HDL-C particles, secreted APOL1 is likely to influence surface cholesterol organisation, possibly through cholesterol clustering in microdomains, affecting both K^+ and Ca^{2+} fluxes. Secreted G1 and G2 may exacerbate such effects because the hydrophobicity of these variants is significantly higher than that of WT APOL1 [7], which also explains the variant-specific higher K^+ permeability after insertion in synthetic membranes in vitro [32]. Accordingly, the APOL1 variants appear to increase the HDL size, suggesting enhanced cholesterol recruitment [73].

Whereas the unitary conductance of podocyte BK channels is ∼105 pS under physiological conditions [82], the unitary conductance of either WT or variant APOL1-induced K^+ pores was estimated to be 25–28 pS [77], which is clearly below either BK or APOL1 conductance [31]. Because cholesterol can affect BK channel activity [83,84,92], APOL1-mediated recruitment of cholesterol at the TRPC6-BK ion channel complex may limit BK conductance despite channel activation. This could occur through competition for cholesterol binding between APOL1 and the BK activating accessory β1 subunit [84] or involve cholesterol propensity to increase lateral stress and stiffness of the phospholipid bilayer, constraining the physical dimensions of the BK hydrophobic internal vestibule [93].

The APOL1 domain involved in membrane interaction is MAD (Figure 5). Given the affinity of APOL1 for anionic phospholipids such as phosphoinositides and cardiolipin [7], this domain may be responsible for interaction with these phospholipids. Accordingly, both MAD1 and MAD2 helices contain stretches of positively charged amino acids resembling phosphoinositide-binding motifs (Figure 5). When APOL1 is incubated in vitro with mitochondrial membranes, the MAD domain is largely protected against proteinase K digestion, suggesting MAD burying in the outer membrane layer [8]. Accordingly, MAD is the only domain inaccessible to recognition by monoclonal antibodies in cell surface-bound APOL1 [37], confirming that it is likely to be involved in the effects of secreted APOL1 on the plasma membrane. Interestingly, APOL1 MAD contains a cholesterol binding motif (CRAC) absent in all other APOLs, which are intracellular. Thus, the MAD CRAC may be linked to the extracellular function of APOL1. Like occurs for TbKIFC1 [69], MAD interaction with cholesterol may involve additional binding to phospholipids next to the CRAC (Figure 5).

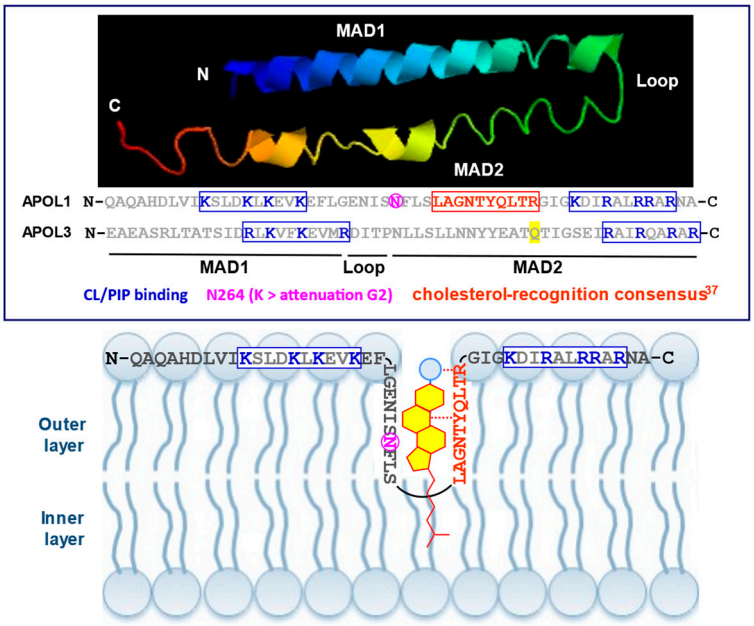

Figure 5. The APOL1 membrane-addressing domain (MAD). The MAD domain promotes APOL1 membrane association [20], probably through the binding of positively charged residues (blue) to anionic phospholipids (CL = cardiolipin, PIP = phosphoinositides). This domain is also present in APOL3. The sequence highlighted in red is a putative APOL1-specific cholesterol-binding motif [CRAC, for cholesterol recognition amino acid consensus: (L/V)-X1–5-(Y)-X1–5-(K/R)] [38]. In APOL3, the CRAC motif is disrupted by the residue highlighted in yellow. Conversion of N264 (pink, encircled) to K is strongly correlated with reduced G2 toxicity [94,95]. In silico modelling (https://zhanggroup.org/I-TASSER/, accessed on 13 March 2024) predicts a hairpin-like double helix structure, but interactions with membrane lipids in the outer plasma membrane layer could result in deep MAD insertion as shown in the model at the bottom, with N264 facing the CRAC. Thus, the N264K mutation may impair cholesterol recognition, linking APOL1-induced cytotoxicity to cholesterol binding.

A mutation close to this motif (N264K) correlates with a reduction in G2 toxicity [94,95], despite its location clearly outside the segments conferring in vitro ionic pore-forming activity [8,19,31]. As shown in the model presented in Figure 5, N264 may face the cholesterol-binding motif in the membrane's outer layer, and therefore, the N264K mutation could impair cholesterol binding. Moreover, attenuation of APOL1 variant toxicity can also result from the recruitment of these variants to lipid droplets [96], which like HDL-C particles contain free cholesterol and phospholipids, further confirming that APOL1 variant toxic effects involve hydrophobic interactions, particularly with cholesterol. Consistently,

the higher hydrophobicity of the G1 and G2 variants [7] may account for their greater clustering than WT APOL1 in microdomains at the podocyte surface [34], as well as for their higher interference with cholesterol transport [97]. More precisely, disruption of the interaction between HC1-LZ1 and HC2-LZ2, as occurs in APOL1 variants, is expected to increase the accessibility of a second CRAC motif present in HC2 (Figure 6). As the MAD CRAC, this motif is not found in other APOLs. Because the HC2 peptide exhibits in vitro membrane-destabilising properties at neutral pH [10], hydrophobic interactions by HC2 may particularly affect the plasma membrane organisation. In support of the crucial role of the HC2 CRAC in podocyte cytotoxicity, disruption of this CRAC following sequence truncation at V353 in APOL1Δ (Figure 6) could explain the loss of APOL1-induced cytotoxicity upon APOL1Δ ectopic expression [74]. Even in the absence of APOL1 variants, cholesterol is clearly involved in kidney disease [98], and cholesterol depletion efficiently protects from this disease [99]. Thus, different conditions promoting cholesterol-mediated signalling may result in a common dysfunctional podocyte phenotype.

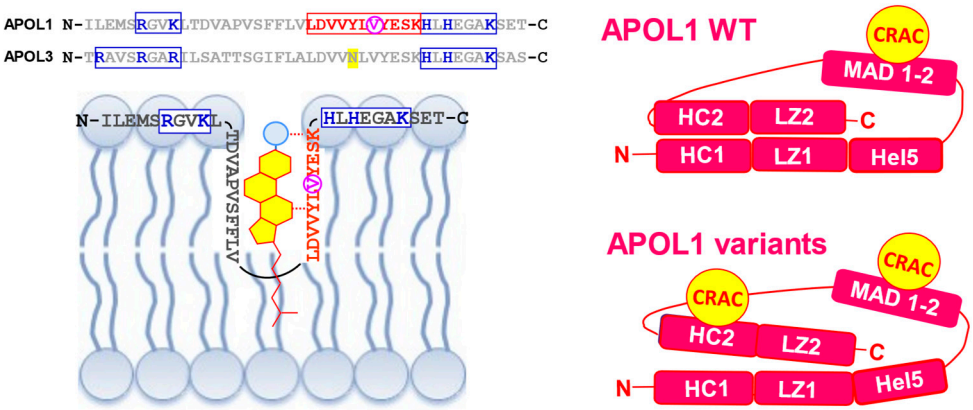

Figure 6. The HC2 cholesterol-binding site. The APOL1 and APOL3 HC2 regions share putative phospholipid-binding sequences (blue boxes), but only APOL1 contains a putative CRAC motif (red box), which is the second of the two APOL1 CRAC motifs (highlighted in yellow in APOL3 sequence: the CRAC-disrupting residue). Encircled in violet, V353 is the C-terminal amino acid of APOL1Δ, which lacks the ability to induce cytotoxicity at the surface of podocytes [74]. The APOL1 HC2 CRAC is expected to be only accessible in C-terminal APOL1 variants, owing to disruption of the interaction between the two HC-LZ tandems (**right panel**).

Intracellular signalling triggered by surface APOL1-induced K^+ efflux involves both GPCR and IP3R activation [78]. As mentioned above, activation of these receptors, as well as STING-mediated inflammation, may already occur at the hit 1 stage of the disease. The functional connection between the surface cation fluxes and GPCR activation further suggests that these fluxes do not occur through APOL1 pores, because the evolutionary recent and unique conversion of an intracellular APOL into a secreted version, supposedly inventing the novel capacity of an APOL to form surface cation channels, appears difficult to reconcile with such a functional connection. It seems much more plausible that activation of existing surface pores involves signalling by already connected receptors, as occurs between BK channels and IP3Rs in lipid rafts [100]. Therefore, hit 2 appears to result mostly from a quantitative increase in the first response to APOL1 variants.

I conclude that surface hydrophobic interactions by APOL1 variants are responsible for hit 2 of the disease, through increased activation of cation fluxes occurring not through hypothetical APOL1 channels, but through cholesterol-sensitive TRPC6 and BK channels.

7. Hit 2 Results from Low HDL-C/High APOL1 Ratio

Under in vitro culture conditions, secreted APOL1 variants accumulate on the podocyte surface and induce podocyte cytotoxicity, while intracellular variant isoforms appear to be harmless [34,70]. Whereas bloodstream HDL-C particles efficiently sequester secreted

APOL1, the cell culture medium only contains very low HDL levels, and APOL1 is presumably largely HDL-unbound. This difference is accompanied by a differential level of APOL1 expression, which is generally higher upon ectopic expression in vitro. Consequently, the HDL-C/circulating APOL1 ratio is much lower in vitro than in vivo. Following viral infection, the in vivo HDL-C level is lowered [101–103], and APOL1 expression is strongly increased [5–7]. For instance, COVID-19 infection is linked to an HDL reduction of approximately 25% [103], and the viral mimetic poly(I:C) may increase APOL1 expression more than 10-fold [6]. Moreover, low serum HDL-C level is a significant predictor of chronic kidney disease progression [104–107]. Therefore, under inflammatory conditions or kidney disease, the ratio between HDL-C particles and circulating APOL1 is low because of the simultaneous lowering of HDL-C levels and increase in APOL1 levels, strongly increasing the bloodstream level of HDL-unbound APOL1 and mimicking the in vitro cell culture conditions.

I propose that a low HDL-C/high APOL1 ratio promotes podocyte cytotoxicity caused by HDL-unbound APOL1, worsening kidney disease.

8. Evolutionary Considerations: From Apoptosis Control to Apoptotic Toxin Building

From the control of intracellular membrane dynamics (membrane fission, fusion and trafficking) [3], APOLs evolved to provide resistance to pathogens [108]. APOL3 kills intracellular bacteria through detergent-like fusion activity on bacterial membranes [109], possibly involving the fusion of APOL3-targeted bacterial membranes with VAMP8-containing endosomal membranes like occurs for mitophagy completion in podocytes [17], thus triggering mitophagy-like degradation of bacteria. The secreted APOL1 isoform appears to have been specifically invented to kill African trypanosomes through induction of apoptotic-like mitochondrial megapore formation. In both APOL1/3 and apoptotic BCL2 proteins cases, the TM hairpin can exhibit monomeric ionic pore-forming activity in vitro, but this activity does not appear to be exerted under physiological conditions in vivo. In the BCL2 family, oligomerisation is linked to megapore activity [35], and this process is inhibited by cholesterol [110,111]. Likewise, the monomer-to-oligomer conversion could also be required for APOL-induced membrane permeabilisation and apoptosis [6–8]. However, the almost complete inhibition of podocyte apoptosis occurring upon expression of C-terminally truncated APOL1Δ, which strongly binds to APOL3 [7], rather suggests that APOL3 inactivation inhibits pro-apoptotic BCL2 proteins (Figure 3). Based on the interaction between murine mAPOL7 and the anti-apoptotic BCL2-like B-cell lymphoma-extra-large (BCL-XL) in mouse dendritic cells [6], it is tempting to propose that APOL3 interacts with anti-apoptotic BCL2 family members, possibly controlling pro-apoptotic BCL2 pore activity (Figure 3). Such interaction could be mediated by the BCL-homology-3 (BH3)-like motif present in LZ1 of APOLs [6]. In trypanosomes, membrane-inserted APOL1 could also activate mitochondrial megapore formation by BH3-bearing membrane proteins [2,33].

To confer resistance to lethal parasites, intracellular APOL1 of some primates was converted into a secreted toxin while keeping intracellular isoforms for the conservation of initial APOL1 functions. The safety of this newly formed bloodstream toxin was ensured by the combination of strict acidic pH-dependence of pore formation and tight APOL1 sequestration by serum HDL-C particles. However, under conditions of low HDL-C/high APOL1 ratio such as that occurring in in vitro cell cultivation or during inflammation, HDL-unbound APOL1 can accumulate in microdomains at the cell surface, possibly inducing cholesterol clustering and consecutive activation of TRPC6 and BK surface cation channels. Higher cholesterol clustering by more hydrophobic APOL1 variants could increase the activation of these channels, causing cytotoxicity responsible for the inflammation-linked worsening of kidney disease. Therefore, this system may still need evolutionary adaptation to fully prevent APOL1 toxicity under inflammatory conditions. Such could be the case with the N264K mutation, which attenuates G2 toxicity [94,95], possibly owing to interference with cholesterol interaction (Figure 5). To verify this hypothesis, CRAC mutations expected to prevent cholesterol interaction, such as R277Q, could be tested for their effect on APOL1

toxicity. However, according to this hypothesis, N264K APOL1 would exhibit reduced association with HDL-C particles, precluding APOL1 trypanosome lytic function. Such loss of function could be innocuous outside of Africa, except in some regions where African trypanosomes have disseminated, like the Indian continent [23]. Another approach to reduce APOL1 toxicity could involve adenosine-to-inosine RNA editing, carried out by adenosine deaminase acting on RNA (ADAR), which can suppress inflammation-induced APOL1 expression [112]. Thus, ADAR could counter APOL1 production during acute inflammation, increasing the protective HDL-C/APOL1 ratio.

9. Conclusions

In some primates including humans, the conversion of APOL1 into an efficient trypanosome lytic factor triggered an evolutionary arms race resulting in the generation of potentially toxic variants, particularly under low HDL-C/high circulating APOL1 ratio such as occurs during inflammation. I propose that this toxicity results from increased hydrophobicity of both intracellular and extracellular APOL1 variants, affecting APOL3 and surface cation channel activities, respectively. The kidney seems to be the principal victim of such toxicity, presumably because actomyosin and surface cation channels critically control the key blood filtration function of the kidney, but APOL1 variants could also participate in other pathologies like neurotransmission disorders or cancer metastasis, which both involve APOL3 control of PI4KB and NCS1 [2]. Treatment of kidney disease could be achieved by disruption of APOL1 variant hydrophobic interactions with APOL3 and/or with the plasma membrane, using either synthetic peptides of the APOL3 interacting helices, lipid droplets [96] or over-expressed VAMP8 [113]. Alternatively, given the relative absence of dysfunctional phenotype in APOL1-KO cells or individuals [7,23], APOL1 inactivation by specific drugs or antibodies could treat the disease [79]. However, as APOL1 is involved in the induction of mitophagy, APOL1 inactivation could result in defective mitophagy, reducing the ability to resist viral infection.

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