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APOL1 variant-expressing endothelial cells exhibit autophagic dysfunction and
mitochondrial stress

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34 **Abstract**

35 Apolipoprotein L1 (APOL1) gene risk variants (RV) associate with renal and cardiovascular
36 disease particularly in SLE. We hypothesized that in RV-carrying human umbilical vein endothelial cells
37 (HUVECs) cytokine-induced APOL1 expression compromises mitochondrial respiration, lysosome
38 integrity, and autophagic flux. HUVEC cultures of each APOL1 genotype were generated. APOL1 was
39 expressed using IFN γ ; HUVEC mitochondrial function, lysosome integrity, and autophagic flux were
40 measured. IFN γ increased APOL1 expression across all genotypes 20-fold ($p=0.001$). Compared to the
41 homozygous G0 (ancestral) allele (0RV), high risk (2RV) HUVECs showed both depressed baseline and
42 maximum mitochondrial oxygen consumption ($p<0.01$), and impaired mitochondrial networking on
43 MitoTracker assays. These cells also demonstrated a contracted lysosome compartment ($p<0.001$), and an
44 accumulation of autophagosomes suggesting a defect in autophagic flux. Treatment of 0RV HUVECs with
45 a non-selective lysosome inhibitor, hydroxychloroquine, produced autophagosome accumulations similar
46 to the 2RV cells, thus implicating lysosome dysfunction in blocking autophagy. Compared to 0RV and
47 2RV HUVECs, 1 RV cells demonstrated an intermediate autophagy defect which was exacerbated by IFN γ .
48 Our findings implicate dysfunction of mitochondrial respiration, lysosome, and autophagy in APOL1 RV-
49 mediated endothelial cytotoxicity. IFN γ amplified this phenotype even in variant heterozygous cells—a
50 potential underpin of the APOL1/inflammation interaction. This is the first description of APOL1
51 pathobiology in variant heterozygous cell cultures.

52

53 **Introduction**

54 Ancestrally African individuals, particularly those with autoimmunity, suffer from disproportionate
55 rates of cardiovascular, hypertensive, and kidney disease. Two polymorphisms, G1 (SER342GLY;
56 ILE384MET) and G2 (6BP deletion N388/Y389), of the Apolipoprotein L1 (APOL1) gene have been

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57 shown to associate with these adverse phenotypes in individuals of recent African heritage. These mutations
58 have been evolutionarily conserved due to an advantage in resisting *Trypanosoma brucei*, the causal agent
59 of African trypanosomiasis [1], and are therefore largely absent from non-African populations [2, 3]. It has
60 been previously demonstrated that these variants are common in an African American lupus cohort with
61 53% of individuals heterozygous and 13% homozygous for the variants [4]. Despite the variants' high
62 allelic frequencies, the adverse phenotype penetrance varies considerably. It has been reported that
63 endogenous and exogenous interferons, as seen in lupus, can precipitate the adverse phenotype in variant
64 homozygotes [5].

65 Consistent with its innate immune function, APOL1 expression is highly responsive to
66 inflammatory signals including Toll-like receptor (TLR) ligation and inflammatory cytokines such as tumor
67 necrosis factor alpha (TNF α) and interferon gamma (IFN γ) [6]. Immunoprecipitation assays show that
68 interferon regulatory factor 1 and 2 and STAT2 bind the APOL1 promoter heightening expression [5, 7].
69 Therefore, APOL1 variant gene penetrance may be contingent upon environmental second hits [8].
70 Intracellularly accumulated APOL1 contains both a BH3 domain, which participates in initiating
71 autophagy, and a pore-forming domain that can be inserted into phospholipid bilayers, causing tissue injury
72 [9, 10]. This injury is contingent upon APOL1 protein accumulation beyond a toxic threshold. It may be of
73 critical importance to consider that even heterozygous carriers express both ancestral and variant allele
74 copies [11]. Current cell culture models introduce the variants using viral-vector systems hampering the
75 ability to make inferences in the heterozygous state.

76 Several cell types including podocytes, human embryonic kidney cells, and oocytes over-
77 expressing variant APOL1 have demonstrated mitochondrial injury, lysosome compromise, and autophagic
78 flux defects resulting in cell death [12-16]. However, risk variant-mediated toxicity mechanisms have not
79 been studied in vascular disease-relevant cell culture models [17]. Moreover, the G1 and G2 SNPs reside
80 in amino acid coding regions therefore altering protein structural stability and function [18]. Despite this
81 apparent gain of function property [19], the inheritance pattern is thought to be recessive, and the literature
82 has not described variant-associated injury in heterozygous carrying tissues [20].

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83 Endothelial dysfunction has been widely recognized as a risk factor for the development of vascular
84 disease [21]. Accordingly, this study was initiated to address the hypothesis that in HUVECs, variant
85 APOL1 confers mitochondrial stress, autophagy defects and loss of lysosome integrity-- a phenotype
86 heightened by exposure to an inflammatory milieu. Cytokine exposure may additionally drive APOL1
87 expression and amplify injury even in cells heterozygous for the variant. HUVECs were obtained from
88 healthy controls of each genotype – homozygous ancestral allele (0RV), heterozygous (1RV), homozygous
89 (2RV) – to determine the consequences of cytokine exposure across APOL1 genotype.

90

91 **Materials and Methods**

92 **Human subjects**

93 This study abides by the Declaration of Helsinki principles and was approved by the Institutional
94 Review Board of New York University School of Medicine. Healthy pregnant women were recruited from
95 a single center labor and delivery ward. Participants provided written informed consent for fetal umbilical
96 cord collection. Inclusion criteria were: African ancestry (concordant partner), and age >18 years. Umbilical
97 cords that could not be processed within two hours of delivery were excluded. In total, 15 cords were
98 collected between February 2015 and December 2018. For experiments in which human sera were added
99 to the HUVEC cultures (see below), samples were obtained from 5 SLE patients and 3 healthy controls.
100 These subjects were enrolled between February 2015 and December 2018 in the NYU Division of
101 Rheumatology-wide IRB-approved Specimen And Matched Phenotype Linked Evaluation (SAMPLE)
102 biorepository. The SLE patients and healthy controls were African American and >18 years of age. Patients
103 met at least 4 American College of Rheumatology criteria for SLE [22]. Clinical data at the time of sample
104 draw included medications, ACR SLE criteria, autoantibody profile, and SLE disease activity score.

105

106 **HUVEC culture establishment and processing**

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107 Please see the Major Resources Table in the online-only Data Supplement for detailed descriptions
108 of all antibodies and cultured cells used. A cut 5-cm section of umbilical cord was collected in RPMI media
109 (Clonetics Corp.) supplemented with heparin 10 U/mL, penicillin/streptomycin 10 U/mL, and gentamycin
110 10 µg/mL. The umbilical vein was cannulated and perfused three times with HBSS solution to remove
111 clotted blood. The umbilical vein was then perfused with collagenase A (type III) solution and both ends
112 were clamped for 10 minutes to allow separation of umbilical vein cells as described [23]. Subsequently,
113 the vein was re-cannulated and perfused again with HBSS, allowing cells to slowly drip from the vein into
114 EGM-2 BulletKit Medium (Clonetics Corp.). The resulting solution was poured over a cell strainer. Cells
115 were centrifuged and the pellet re-suspended in clean culture media (EGM-2 supplemented with 10% FBS,
116 50 U/ml penicillin, 100 mg/ml gentamicin). The cell isolate contained HUVECs, fibroblasts, and residual
117 blood cells. To yield enriched cultures of HUVECs, the cell suspension was passed through a magnetic
118 bead column to capture CD146+ cells. The residual filtrate was discarded. HUVEC cultures were expanded
119 and passaged for use in these experiments described below. Using FACS analysis, HUVECs exhibited
120 strongly positive staining for both CD31 and CD146. In total, 15 healthy HUVEC cultures were established
121 representing genotypes as follows: 0RV n=8, 1RV n=4, 2RV n=3. There were no differences in donor infant
122 gender distributions across genotype.

123

124 **APOL1 genotyping**

125 To ensure cell cultures representing each genotype in triplicate were available for subsequent
126 analysis, APOL1 genotyping was performed as described previously [4]. Briefly, genomic DNA was
127 isolated from each HUVEC culture using the Qiagen kit (Valencia) according to the manufacturer's
128 instructions. DNA isolates were quantitated using a Nanodrop-1000 spectrophotometer (Nanodrop
129 Products). One hundred ng of genomic DNA was used as a template for conventional polymerase chain
130 reaction (PCR). A single 300 base-pair DNA segment containing the APOL1 polymorphisms, G1
131 (rs73885319 and rs60910145) and G2 (rs71785313), was amplified using AmpliTaq Gold 360 DNA

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132 Polymerase (Applied Biosystems). For quality control, DNA was elongated in both forward and reverse
133 directions using sequences 5'-GCCAATCTCAGCTGAAAGCG-3' and 5'-
134 TGCCAGGCATATCTCTCCTGG-3' respectively. Genotypes were analyzed using the GeneWiz online
135 platform. Successful genotyping was completed on all DNA samples.

136

137 **Measurement of serum IFN- α activity**

138 The reporter cell assay for IFN- α has been described in detail previously [24, 25]. In this assay,
139 reporter cells are used to measure the ability of patient sera to cause IFN-induced gene expression. The
140 reporter cells (WISH cells, ATCC #CCL-25) are cultured with 50% patient serum for 6 hours. The cells are
141 lysed, and cDNA is made from total cellular mRNA and then quantified using real-time PCR. Forward and
142 reverse primers for the genes IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), myxovirus
143 resistance 1 (MX1) and dsRNA-activated protein kinase (PKR), which are highly and specifically induced
144 by IFN- α , were used in the reaction [24]. The relative expression of each of these three genes was calculated
145 as a fold increase compared with its expression in WISH cells cultured with media alone and then
146 standardized to healthy donors, and summed to generate a score reflecting the ability of sera to cause IFN-
147 induced gene expression (serum IFN α activity) [24]. This assay has been highly informative in SLE and
148 other autoimmune diseases [26-28].

149

150 **Inflammatory model of APOL1 expression**

151 Single genotype HUVEC cultures grown in EGM-2 (Promocell, Heidelberg, Germany)
152 supplemented with 10% FBS, 50 U/ml penicillin, and 100 mg/ml gentamicin were seeded at 20%
153 confluence in 75 ml culture plates coated with 0.1% gelatin. Once confluent, cells were passaged and
154 harvested once per week using trypsin-EDTA. Only HUVECs between passages 4-8 were used in
155 experiments. For each genotype, untreated controls were compared to cells treated with one of the
156 following: 50% human sera isolated from healthy donors or patients with SLE, IFN γ (50pg/mL), IFN α

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157 (50pg/mL) or TNF α (50pg/mL). Cells were lysed; both protein and mRNA were extracted for immunoblot
158 and qPCR.

159

160 **Immunoblot**

161 Protein concentration was determined using a BCA protein assay kit (ThermoFisher Scientific)
162 following manufacturer's instructions. Appropriate concentrations of cell lysate were diluted with 4X Blot
163 \otimes LDS sample buffer then heated to 70°C for 5 minutes. Samples were resolved on Blot 4-12% Bis-Tris
164 Plus Gels (Life Technologies) and transferred to PVD membranes. Membranes were blocked with Odyssey
165 \otimes Blocking Buffer (TBS) (Li-Cor Biotechnology) for 1 hour at room temperature. After blocking,
166 membranes were incubated with rabbit anti-human APOL1 (1 μ g/mL) (Sigma-Aldrich) and mouse anti-
167 human tubulin (1 μ g/mL) (AbCam) diluted in 5% BSA/TBS-T overnight. Membranes were then incubated
168 with an HCRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:2000) (Santa Cruz
169 Biotechnology) for 1 hour at room temperature. Protein bands were visualized using Li-Cor Image Studio
170 Lite 4.0. Immunoblots were quantified by densitometry of experimental bands relative to loading controls
171 using ImageJ 1.51 Java 1.8 running on Windows 7 or 10.

172

173 **qPCR**

174 Total RNA was extracted from endothelial cells using the RNAeasy Mini kit according to the
175 manufacturer's instructions (Qiagen). RNA was reverse transcribed to prepare cDNA libraries. Both
176 forward and reverse primers for APOL1 were used (sequences above). Levels of expression were
177 normalized by parallel amplification and quantification of GAPDH mRNA levels using forward (5'-
178 ACCACAGTCCATGCCATCAC-3') and reverse (5'-TCCACCACCCTGTTGCTGTA-3') primers.
179 Brilliant SYBR Green RT-PCR (Invitrogen) was used in the qPCR mix to amplify the cDNA product.
180 Results were quantified using the $\Delta\Delta$ CT method.

181

182 **Live cell imaging**

183 HUVECs (1×10^4 cells per 200 μ L media; plate area 34mm²) were seeded on Greiner Bio-One CELL
184 view Cell Culture Slides (Fisher Scientific, Pittsburgh, PA) and allowed to adhere overnight. Cells were
185 either left untreated in EGM-2 media, treated with 50pg/mL of IFN γ , or 50pg/mL of IFN γ plus 25 μ M of
186 non-selective lysosome blocker, hydroxychloroquine, in duplicate for each experiment (n=4). Cells were
187 then stained with LysoTracker red (LTR) probes (ThermoFischer Scientific, Waltham, Ma) and MitoTracker
188 green (MTR) probes (ThermoFischer Scientific, Waltham, Ma) for 30 minutes. Media was replaced with
189 serum free RPMI with glutamine (Mediatech Inc., Manassas, VA). When multiple slides were run, plates
190 were staggered to prevent variation due to time elapsed since staining. Fluorescent microscopy was
191 performed with a Nikon Eclipse Ti with a Plan Apo λ 60x/1.4 Oil Ph3 objective, narrow pass filters, and an
192 Andor Zyla sCMOS 5.5 camera operated by Nikon Elements. For lysosome assessments, the fluorescence
193 intensity as measured by the integrated density was scored using ImageJ 1.51 Java 1.8 running on Windows
194 7 or 10. For mitotracker images, mitochondrial network morphology per cell was assessed using the Mito-
195 Morphology set of macros outfitted for the FIJI distribution of ImageJ as described [29]. The tools and
196 instructions for their usage can be found at <https://github.com/ScienceToolkit/MiNA>.

197

198 **Autophagy assessments**

199 Autophagophore component proteins LC3-II/I were assessed by immunoblot. PVD membranes
200 were treated with rabbit anti-LC3 primary antibodies (1 μ g/mL) (Cell Signaling) diluted in 5% BSA/TBS-T
201 followed by HCRP-conjugated anti-rabbit secondary antibodies (1:20000). Fluorescence units were
202 quantified in the context of APOL1 staining using ImageJ 1.51 Java 1.8 running on Windows 7 or 10.

203 In parallel, single HUVEC cultures of 40,000 cells per 300 μ L of cell media representing each
204 genotype were plated on 0.1% gelatin-coated cover slips (BD Biosciences) housed in 24-well plates (1.9 cm²
205 per well). HUVECs were either left untreated or given 50pg/mL of IFN γ . HUVECs representing each
206 genotype were again given 50pg/mL of IFN γ plus 25 μ M of non-selective lysosome blocker,

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207 hydroxychloroquine, therefore recapitulating the hypothesized lysosomal defect in variant-carrying cells.
208 After treatment for 18 hours overnight, HUVECs were washed with PBS and fixed with 3.7% formaldehyde
209 in PBS for 10 minutes. The cover slips were again washed with PBS and cells permeabilized with 0.5%
210 Triton in PBS for 20 minutes. Following an additional wash step, cover slips were treated with PBS gelatin
211 solution as a blocking step for 1 hour. They were then stained with a DAPI DNA dye (Vector Laboratories)
212 and primary antibodies to anti-human SQSTM1/p62 (Abcam) (both raised in rabbit) diluted in PBS gelatin
213 solution at concentrations of 1:300 each. Cells were again washed and stained with anti-rabbit TRIT-C and
214 Alexa-488 (Fisher Scientific) diluted in PBS gelatin at concentrations of 1:300. The cover slips were
215 mounted on glass slides for visualization.

216 Fluorescent microscopy was performed with a Nikon Eclipse Ti with a 60X N.A. 1.40 Plan APO
217 objective, narrow pass filters, and an Andor Zyla sCMOS 5.5 camera operated by Nikon Elements. Puncta
218 were scored using ImageJ 1.51 Java 1.8 running on Windows 7 or 10. Images of cells were taken to make
219 sure full cells were in the field for measurement. No choices were made based on morphology or intensity.
220 All cells that were fully in each field were traced. A macroinstruction was written to locate discrete bright
221 spots which were identified as puncta. A measurement including a 3-pixel-radius circle centered on each
222 punctum was measured and, for each cell, summed. The integrated density of the total puncta per cell was
223 reported.

224

225 **Mitochondrial Respirometry Assay**

226 Forty-thousand HUVECs representing each genotype (0RV, 1RV, and 2RV) were seeded on V7
227 cell culture plates (Seahorse Bioscience). Cells were either left untreated or treated with IFN γ (50pg/mL)
228 for 18 hours overnight. One hour prior to measurement, cell culture media was replaced with assay media
229 (3mM glucose, 1 mM sodium pyruvate, and 1.5 mM glutamine without FBS at a pH of 7.4). Port injections
230 of oligomycin (1 μ M), FCCP (0.25 μ M) and rotenone/antimycin (1 μ M each) were filled for bioenergetics
231 profiling. Cellular respiration was measured using a Seahorse Bioscience XF 24-3 analyzer.

232

233 **Electron Microscopy**

234 Cultured cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium
235 cacodylate buffer (pH 7.2) for 2 hours at 4°C and post-fixed with 1% osmium tetroxide for 1.5 hours at
236 room temperature, then processed in a standard manner and embedded in EMBED 812 (Electron Microscopy
237 Sciences, Hatfield, PA). Ultrathin sections (60 nm) were cut, mounted on copper grids and stained with
238 uranyl acetate and lead citrate by standard methods. Stained grids were examined under a Talos120C
239 electron microscope and photographed with a Gatan OneView camera. Twenty random cells in each sample
240 were imaged for morphological analysis.

241

242 **Statistical Analysis**

243 For each genotype and experimental condition, data were expressed as mean \pm standard deviation.
244 Medians were used when the population cannot be assumed to be normally distributed. Two sample t-tests
245 for two-group comparisons and ANOVA for multiple-group comparisons were used. Data normality was
246 assessed by visual examination of the observed distributions and Kolmogorov-Smirnov tests. Equality of
247 variance was assessed by F-tests. mRNA expressions and cell densities were log-transformed to better
248 satisfy normality. If F-tests failed to reject the hypothesis of unequal variances, two sample t-tests with
249 equal variances for two-group comparisons and ANOVA for multiple-group comparisons were used
250 instead. When ANOVA tests rejected the null hypothesis, post hoc pairwise comparisons were performed.
251 All statistics were carried out using IBM SPSS software. The level of significance was set at 0.05.

252

253 **Results**

254 A unique resource, donor Human Umbilical Vein Endothelial Cells (HUVECS), were isolated from
255 healthy subject umbilical cords representing each of the APOL1 genotypes.

256

257 **Inflammatory stimuli increase HUVEC APOL1 Expression**

258 To investigate the intersection between inflammation and APOL1 expression, healthy HUVEC
259 cultures were established, which were isolated and expanded. All APOL1 genotypes were represented as
260 detailed in the methods section. For each experiment, the number of individual HUVEC donors is outlined
261 in S1 Fig. Previous reports suggest that various inflammatory stimuli can increase APOL1 expression in
262 human podocyte and embryonic kidney cell culture models [5]. The capacity of SLE characteristic
263 cytokines including IFN α , IFN γ , and TNF α to stimulate gene expression in HUVECs was tested. Exposing
264 HUVECs to IFN α , IFN γ , and TNF α resulted in increased APOL1 expression of 8.7 \pm 1.7, 20.8 \pm 13.7, and
265 7.8 \pm 2.6 fold respectively, versus untreated HUVECs (for each cytokine vs untreated, $p < 0.05$) (Fig. 1A).
266 Thus multiple inflammatory cytokines that are integral to autoimmune disease were shown to increase
267 APOL1 expression. On immunoblot, IFN γ -treated HUVECs increased APOL1 protein expression 15.2-
268 fold compared to untreated HUVECs ($p = 0.01$; S1 Fig.). To expose endothelial cells to several circulating
269 cytokines, sera from SLE patients (N=5) and controls were incubated with HUVECs across genotypes.
270 Subsequently, APOL1 expression was assessed. In response to SLE sera, APOL1 expression increased on
271 average 39.8 \pm 9.3-fold compared to 3.6 \pm 0.7-fold in healthy control sera (Fig 1B; note that for the use of
272 sera derivatives, patient donor demographics and clinical data are shown in Table 1). This increased
273 expression was apparent across genotype (S1 Fig). The genotype of the PCR products was concordant with
274 chromosomal DNA with heterozygous HUVECs expressing both variant and ancestral APOL1 alleles. This
275 result empowered a series of interrogations directed at the consequences of cytokine treatment in
276 heterozygous HUVECs.

277

278 **Fig. 1. Endothelial cells treated with inflammatory cytokines induce APOL1 expression.** A. The
279 upregulation of HUVEC APOL1 transcript in untreated HUVECs compared to IFN α (50pg/mL), IFN γ
280 (50pg/mL), and TNF α (10ng) treated for 18 hours (average of 5 experiments, 9 HUVEC donors). Shown
281 on the y-axis are 2- $\Delta\Delta$ CT (transcript normalized to GAPDH) values, and shown on the x-axis are cytokine

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282 treatment. B. Exposure of HUVECs to sera at 1:1 dilution for 18 hours resulted in an upregulation of
283 APOL1 transcription (average of 5 experiments, 9 HUVEC donors). Shown on the y-axis are $2^{-\Delta\Delta CT}$
284 (transcript normalized to GAPDH) values, and shown on the x-axis are treatment conditions. Comparisons
285 are made between the mean fold expression in untreated vs the treatment condition using Kruskal-Wallis
286 test (both 1A and 1B $p < 0.001$) followed by post hoc Dunn test (***) indicates $p < 0.001$, * indicates $p < 0.05$).
287 Abbreviations: Untrt= untreated condition, IFN α = interferon alpha treatment, IFN γ = interferon gamma
288 treatment, TNF α = tumor necrosis factor alpha treatment, HC Sera= healthy control sera, and Systemic
289 Lupus Erythematosus Sera= SLE Sera, each lupus sera (subjects in table 1) was run in triplicate.

290

Table 1. African American (ORV genotype) SLE Sera Donor Demographics, SLE Activity, and IFN score.

Subject					
	SLE 1	SLE 2	SLE 3	SLE 4	SLE 5
Demographics					
Age (years)	62	33	29	47	31
Gender	F	F	F	F	F
SLE Activity					
dsDNA	1	927	181	12	76
C3 (mg/dL)	91	49	68	100	79
C4 (mg/dL)	16	12	7	15	9
IFN Score (units)	28.2	219.7	167.6	1.1	876

291

292 **Table 1.** Demographics and clinical characteristics of SLE serum donor subjects at the time of blood
293 draw. All subjects were African American and APOL1 ancestral allele homozygous.

294

295 **APOL1 variant-carrying HUVECs exhibit defects in mitochondrial respiration**

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296 While HUVEC IFN γ exposure results in pleiotropic responses regardless of APOL1 genotype,
297 we were uniquely positioned to examine the consequences of IFN γ induced expression of APOL1 on a
298 live-cell metabolic assay.

299

300 **Live-cell metabolic assays and the stress response of all APOL1 genotypes**

301 Here, the Seahorse XF24 Analyzer was utilized to measure the APOL1 genotype effect on
302 HUVEC mitochondrial respiration. This assay measures cellular mitochondrial function in real time using
303 well-defined inhibitors, oligomycin, FCCP, and Antimycin A [30, 31]. Baseline oxygen consumption rate
304 (OCR) is first measured, followed by the addition of the ATPase inhibitor, oligomycin, in order to
305 evaluate the non-ATPase-dependent OCR. Next an uncoupling agent, FCCP, is added to allow
306 uninhibited electron crossing at the inner mitochondrial membrane, thus measuring the maximum oxygen
307 consumption. Last, a mixture of complex I and complex III inhibitors, rotenone and antimycin A, is added
308 to inhibit mitochondrial respiration completely. The resultant OCR value reflects non-mitochondrial
309 respiration. These measurements can be used to assess overall bioenergetic health index (BHI), which is
310 proportional to reserve capacity and ATP synthase-dependent OCR and inversely proportional to proton
311 leak and non-mitochondrial OCR [30].

312 We measured OCR in each of the genotypes by treatment condition. As shown in Fig 2 A-C, at
313 baseline, OCR was higher in the 0 risk variant (RV) HUVECs than the 1 or 2 RV HUVECs (89.9 \pm 5.6
314 pmol/min vs 71.7 \pm 4.5 pmol/min vs 66.5 \pm 3.2 pmol/min respectively; p=0.002). Likewise, maximum OCR
315 was higher in 0 RV HUVECs; values dropped with each RV copy, with means of 152.7 \pm 10.7 pmol/min in
316 0 RV carriers, 122.3 \pm 9.6 pmol/min in 1 RV carriers, and 102.6 \pm 4.6 pmol/min in 2 RV carriers (p=0.001).
317 With the addition of IFN γ , maximum OCR fell to 133.2 \pm 11.3 pmol/min, 100.8 \pm 6.2 pmol/min, and 92.9 \pm 6.1
318 pmol/min in the 0, 1, and 2 RV-carrying HUVECs respectively (p=0.002). This reduction trended toward
319 significance in IFN γ -treated 1 RV HUVECs (p=0.06).

320

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321 **Fig 2. The bioenergetic profiles of HUVECs across genotype.** APOL1 risk variant associations with
322 attenuated mitochondrial function including maximum respiration, reserve respiration capacity, glycolytic
323 capacity, and bioenergetic health index. Live HUVEC metabolic assays were performed using the Seahorse
324 XF platform. Genotypes are represented as follows: 0RV, 1RV, and 2RV (RV= risk variant). Treatment
325 conditions included: no treatment (Untrt) or stimulation using IFN (50pg/mL, 18h overnight). A-C. In this
326 assay, oxygen consumption rate (OCR) was measured at baseline, upon oligomycin (oligo), FCCP, and
327 antimycin A treatments. Representative oxygen consumption profiles are shown. D-E. Bioenergetic health
328 index (BHI) calculated by APOL1 genotype and treatment condition (5 experiments averaged representing
329 9 HUVEC donors). F. Representative raw tracings of extracellular acidification rate (ECAR) after addition
330 of Oligomycin by genotype and condition. P-values were calculated using one-way analysis of means for
331 cross genotype comparisons. Where the three-comparison ANOVA was significant, post-hoc two
332 comparison analysis was completed. * indicates $P<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

333

334 As shown in Fig 2 D-E, there were no statistically significant differences in bioenergetic health
335 index (BHI) in untreated HUVECs across genotype (0 RV=3.43±0.47, 1 RV=3.20±0.57, 2 RV=3.10±0.37;
336 $p=0.06$); however, treating HUVECs with IFN γ decreased BHI in 1 and 2 RV-carrying cells resulting in
337 significant differences among genotype groups (0 RV=3.41±0.49, 1 RV=3.02±0.48, 2 RV=2.97 ± 0.51;
338 $p=0.025$). Results by experiment and sample are shown in S2 Fig. While this deficiency was apparent in
339 both treated and untreated 2 RV-carrying cells, 1 RV-carrying HUVECs exhibited a difference only after
340 IFN γ treatment, again suggesting that in these cells the adverse phenotype is inducible.

341 To further determine the RV effect on metabolic capacity in HUVECs, the Seahorse XF glycolysis
342 test was used to measure extracellular acidification rate (ECAR). Glycolysis is an oxygen independent ATP
343 production process that converts glucose to lactate. Lactate is the major source of free protons in the culture
344 medium [32]. Therefore, by measuring the rate at which the extracellular medium becomes acidified, the
345 XF24 analyzer measures the glycolytic rate in real time. With the addition of stressor, oligomycin, which
346 blocks mitochondrial respiration, cells are forced to utilize oxygen-independent glycolysis to meet energy

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347 demands [32]. The glycolytic capacity can therefore be measured by the difference between baseline and
348 oligomycin-treated (stressed) glycolytic rate [32]. Overall the glycolytic capacity in untreated endothelial
349 cells was significantly lower in 2 RV carriers than in 1 or 0 RV carriers (0 RV: 5.4 mph/min, 1 RV: 4.8
350 mph/min, 2 RV: 4.2 mph/min, $p=0.02$). This parameter was lowered by IFN γ treatment (0RV: 4.7 mph/min,
351 1RV: 4.5 mph/min, 2RV: 3.9 mph/min, $p = 0.04$, Fig 2F).

352 Consistent with differences in OCR and ECAR, ATP production varied across genotype and
353 treatment condition. At baseline, ATP production was numerically, but not significantly higher –
354 45.6pmol/min – in 0 RV carriers, compared to 33.2pmol/min and 34.2pmol/min in 1 or 2 RV carriers
355 ($p=0.09$). IFN γ treatment increased ATP production in each genotype, however to a lesser extent in variant
356 carriers. The respective values in the 0, 1, and 2 RV HUVECs were 48.7pmol/min, 34.7pmol/min, and
357 37.7pmol/min ($p=0.02$). These results support an association between APOL1 risk variants and impaired
358 mitochondrial function characterized by reduced maximum respiration, reserve respiration capacity,
359 glycolytic capacity, and bioenergetic health index. HUVECs carrying the variants exhibited a more
360 senescent phenotype with overall lower energy production. This observation was more pronounced with
361 each RV copy (i.e. 2 RV > 1 RV > 0 RV), and was exacerbated by IFN γ treatment particularly in the 1RV
362 HUVECs.

363

364 **Mitochondrial structure**

365 To determine genotype-associated differences in mitochondrial structure, mitochondria were
366 stained with fluorescent dye, MitoTracker. HUVECs of each genotype were either left untreated, or given
367 IFN γ overnight and evaluations of mitochondrial ultrastructure included indirect (use of fluorescent dye,
368 MitoTracker), and direct (transmission electron microscopy (TEM)). For the former, sets of 10 HUVEC
369 images across genotype donors and treatment conditions were evaluated for mitochondrial morphology per
370 cell including length and number of branches using an automated protocol [29] (representative images
371 shown in Fig 3 A-B). In untreated HUVECs, 2RV carriers exhibited shorter branch length (sum branch

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372 length: 0RV: 4.3 μ m 1RV: 3.3 μ m 2RV: 3.2 μ m; p= 0.17) and less networking (mean networked branches:
373 0RV: 2.0 1RV: 2.1 2RV: 1.8; p= 0.6) though these differences did not reach statistical significance (Fig 3
374 C-D). Upon treatment with IFN γ , genotype-associated differences became significant (sum branch length:
375 0RV: 4.6 μ m 1RV: 3.1 μ m 2RV: 2.7 μ m; three group comparison p=0.003; Post hoc 2 group comparison
376 0RV vs 1RV p=0.03; 0RV vs 2RV p=0.004. Mean networked branches: 0RV: 2.3 1RV: 1.8 2RV: 1.7; three
377 group comparison p= 0.001. Post hoc 2 group comparison 0RV vs 1RV p=0.01; 0RV vs 2RV p=0.002; Fig
378 3 C-D). TEM images were concordant with this finding. Untreated cells across genotype showed small
379 differences in mitochondrial area (median area for 0, 1, and 2 allele HUVECs=0.09 μ m², 0.08 μ m², 0.07 μ m²
380 respectively) (S3 Fig). Upon IFN γ treatment, these differences became more pronounced (median area +
381 IFN γ 0 RV=0.11 μ m; 1 RV=0.10 μ m; 2 RV=0.07 p <0.001) (S3 Fig).

382

383 **Fig 3. Assessment of mitochondrial structure in resting and stimulated HUVECs using MitoTracker,**
384 **a fluorescent proxy of ultrastructure.** Mitochondrial structure including branch length and networking is
385 attenuated in HUVECs with 1RV or 2RV versus 0RV APOL1 genotype both at baseline and with IFN γ
386 50pg/mL-treatment. Human umbilical vein endothelial cells representing each genotype, 0 risk variant
387 (G0/G0 left columns), 1 risk variant (RV/G0 middle columns), and 2 risk variant (RV/RV right columns)
388 were either left untreated (A) or treated with IFN γ 50pg/mL (B) for 18 hours overnight. Representative
389 immunofluorescence (MitoTracker green stained) images are shown (overall experiment, 10 cells per
390 genotype and condition were measured. In total 12 HUVEC donors and 4 experiments were averaged). C-
391 D Mitochondrial branch length (C) and networked branches (D) measured on confocal microscopy versus
392 genotype and treatment condition (x axis) using the Mitochondrial Network Analysis (MiNA) tools
393 available in the FIJI distribution of ImageJ. Mitochondrial length was measured in microns (μ m). Each
394 additional risk variant associated with a reduced degree of mitochondrial networking--an effect that became
395 statistically significant across the genotypes upon treatment with IFN γ . P-values were calculated using
396 Kruskal-Wallis test for cross genotype comparisons, and Wilcoxon rank sum test for comparing untreated
397 group and IFN group. P<0.01 is indicated by **, and p<0.001 is indicated by ***.

398

399 **APOL1 variant-Carrying HUVECs exhibit lysosomal defects**

400 Lysosomes serve as a cellular “digestive system,” and their function is highly dependent on both
401 an acidic pH and an ATP-dependent pump. To determine genotype-associated differences in lysosome
402 structure, we stained HUVECs representing each APOL1 genotype and treatment condition with
403 fluorescent dye, LysoTracker. At baseline, 2 RV HUVECs exhibited significantly lower lysotracker staining
404 intensity than 0 or 1 RV HUVECs ($p < 0.001$) (Fig 4A). IFN γ exposure significantly decreased lysotracker
405 staining intensity in the 0 and 1 RV carriers ($p = 0.04$ and $p < 0.001$ respectively); in the 2RV carriers
406 lysosome staining intensity did not change with IFN γ treatment but remained lower than that of the other
407 two genotypes (Fig 4B). Next, HUVECs were treated with both IFN γ and HCQ. HCQ is a reagent that
408 blocks lysosome acidification thereby arresting both organelle function and turnover [33, 34]. Treatment of
409 cells with 25 μ M of hydroxychloroquine (HCQ) did not influence HUVEC APOL1 expression (S4 Fig). In
410 all conditions, the co-treatment of IFN γ and HCQ resulted in increased lysosome staining intensity (Fig
411 4C). This effect, however, was significantly less in both 1 and 2 RV-carrying cells. Lysosome staining
412 intensity is quantified in Fig 4D, and results by experiment and sample are shown in S5 Fig. Taken together,
413 each additional RV copy associated with less HUVEC lysosome staining intensity—an observation that
414 was exacerbated by IFN γ treatment.

415

416 **Fig 4. Assessment of lysosomal structure in resting and stimulated HUVECs with or without**
417 **hydroxychloroquine (HCQ) using LysoTracker.** The presence of 1RV or 2RVs associated with less
418 lysosome staining by lysotracker at baseline or with IFN γ treatment. Preventing lysosome turnover with
419 the addition of HCQ treatment increased lysosome staining to a lesser degree in RV carrying HUVECs. A.
420 Immunofluorescent images of untreated HUVECs representing each genotype, 0RV (left column), 1RV,
421 and 2RV (right column). B. Representative images of IFN γ 50pg/mL-treated HUVECs across genotype
422 (parallel layout as per A) for 18 hours overnight. C. Representative images of HCQ (25 μ M) plus IFN γ -
423 treated HUVECs across genotype (layout as per A) LysoTracker staining was performed. Images were

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424 captured by florescent microscopy using a Nikon Eclipse Ti and representative microphotographs were
425 selected. D. The average lysosome intensity per region of interest (Integrated Density) for each genotype
426 and treatment condition group. P-values were calculated using Kruskal-Wallis test for cross genotype
427 comparisons, and Wilcoxon rank sum test for comparing untreated group to the IFN γ treated or HCQ plus
428 IFN γ group. *P<0.05; **p<0.01, ***p<0.001.

429

430 **APOL1 variant-carrying HUVECs display defects in autophagic flux**

431 Autophagosome maturation and degradation (flux) is contingent upon a functioning lysosome [35]
432 which was demonstrated to be compromised in APOL1 variant-carrying HUVECs (above). Therefore,
433 autophagosomes were evaluated using both fluorescent microscopy of SQSTM1 (p62) and transmission
434 electron microscopy (TEM). SQSTM1 (p62) is an autophagophore shuttle protein that is degraded through
435 autophagy and has been utilized in the literature to measure autophagic flux on microscopy. HUVECs were
436 stained for p62 and the number of autophagic puncta (log transformed) per cell was observed. HCQ was
437 again used to arrest the degradation of the autophagosomes by blocking lysosome acidification [36].
438 Therefore, a comparison of APOL1 genotype-dependent differences in autophagy at baseline, upon IFN γ
439 treatment, and IFN γ plus HCQ treatment (autophagic flux inhibition) was established. To assess the degree
440 to which autophagic flux was impaired, the puncta count at baseline or upon IFN γ treatment was compared
441 to the HCQ-treated condition.

442 It was observed that in untreated HUVECs, autophagosome count was the lowest in 0 RV cells
443 and increased with each additional variant allele (log autophagosome count per genotype: 0RV: 1.1 \pm 0.57;
444 1RV: 1.6 \pm 0.48; 2RV: 2.0 \pm 0.70, p<0.001 Fig 5A). Across genotypes, IFN γ exposure increased
445 autophagosome count (0 RV: 1.3 \pm 0.45, 1 RV: 1.8 \pm 0.44, 2 RV: 2.2 \pm 0.31, p<0.001 Fig 5B). In each genotype
446 group, the number of autophagosomes increased in the IFN γ plus HCQ-treated condition; however 2 RV
447 HUVECs exhibited the highest autophagosome count (0 RV: 1.7 \pm 0.38, 1 RV: 2.0 \pm 0.5, 2 RV: 2.5 \pm 0.55,
448 p<0.001 Fig 5C). Autophagosomes were confirmed on TEM by genotype and treatment condition (Fig 5D).
449 Microscopy results are quantified in Fig 5E, and confirmed by p62 immunoblot in Fig 5F. Results by

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450 experiment are shown in S6 Fig. A supporting immunoblot of the LC3 II/I ratio by genotype and treatment
451 condition is shown in S7 Fig. Taken together, these results support an association between HUVEC APOL1
452 genotype and autophagic flux inhibition. Congruent with the lysosome staining results, each additional risk
453 variant had an effect on autophagic flux with interferon exacerbating the phenotype—particularly in the
454 heterozygous condition.

455

456 **Fig 5. APOL1 risk variant-carrying HUVECs display autophagic flux deficiencies.** Assessment of
457 autophagosome accumulation using SQSTM1 (p62) staining, a proxy for autophagic flux inhibition. A.
458 Representative immunofluorescence images of untreated HUVECs stained for SQSTM1 (p62) across
459 APOL1 genotype: 0 risk variants (left column), 1 risk variant (middle column), 2 risk variants (right
460 column). B. Representative immunofluorescence images of IFN γ (50pg/mL)-treated HUVECs stained for
461 SQSTM1 (p62) across APOL1 genotype C. Representative immunofluorescence images of IFN γ plus HCQ
462 (25 μ M)-treated HUVECs stained for SQSTM1 (p62) across APOL1 genotype D. Autophagosomes were
463 confirmed by transmission electron microscopy (Columns: 0RV left, 1RV middle, 2RV right; Rows:
464 Untrt=untreated top, IFN γ -treated middle, IFN γ plus HCQ-treated bottom). E. Log transformed values of
465 p62 positive puncta per cell (y axis) and the treatment condition (x axis) are shown. HUVEC genotype is
466 labeled from left to right. F. Immunoblot of HUVEC lysates showing SQSTM1 (p62) protein concentration
467 compared to tubulin loading control by genotype and treatment condition. Note: HUVECs were treated for
468 18 hours. *P<0.05; ***P<0.001 were calculated from one-way analysis of means for cross genotype
469 comparisons. Where ANOVA rejected the null hypothesis, a post-hoc 2 group analysis was performed.

470

471 Discussion

472 Overall, these data support functionally relevant associations between APOL1 risk genotypes and
473 various endothelial organelle readouts. HUVECs exposed to cytokines characteristically detected in SLE
474 patients, IFN α , IFN γ , and TNF α , strongly up-regulated APOL1 gene expression. Exploiting the use of

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475 HUVECs expressing ancestral and variant APOL1 alleles, it was demonstrated for the first time that variant
476 expression associates with functional consequences such as decreased mitochondrial metabolic potential
477 and mitochondrial fragmentation. Specifically, in 2RV and IFN γ -treated RV heterozygous HUVECs,
478 maximum respiration, reserve respiration capacity, glycolytic capacity, ATP production, and bioenergetic
479 health index were attenuated relative to the ancestral allele homozygous HUVECs. Using a parallel set of
480 conditions, the presence of 1RV or 2RVs also associated with decreased lysosome staining by lysotracker
481 as well as an inhibition of autophagocytic flux, which was based on fluorescent evaluations of SQSTM1
482 and confirmed by transmission electron microscopy. Taken together, these data support that 2 RVs—and
483 under inflammatory stress 1 RV--contribute to a senescent endothelial cell phenotype, which is
484 characterized by overall lower energy production and untoward consequences to autophagosome
485 maturation and degradation (flux), an event that is contingent upon a functioning lysosome. Given the prior
486 reporting of vascular disease in APOL1 variant-carrying SLE patients, these observations in HUVECs may
487 offer mechanistic insights [4, 21, 37].

488 These data add to an overall understanding of APOL1 variant effects in endothelial cells, and the
489 interacting microenvironment which may potentiate cellular injury. Although traditionally this has been
490 characterized by defective nitric oxide synthesis, recent work has demonstrated that autophagy deficiencies
491 contribute to atherogenesis [37, 38]. These results demonstrate a potential mechanistic link between SLE-
492 associated inflammation and APOL1 risk variant status via inducible endothelial injury.

493 APOL1 is a five-domain protein with several intracellular functions [6, 16, 18, 39]. Its expression
494 is up-regulated by cellular stress including inflammatory signals, nutrient deprivation, and hypoxia [9, 10].
495 APOL1's colicin-like pore forming domain may be inserted into cell membrane, lysosome, or
496 mitochondrial phospholipid bilayers in a pH dependent fashion [40]. The G1 and G2 mutations allow for
497 pore formation at lower levels of APOL1 gene expression [40]. Mitochondria may be injured by APOL1
498 variants either directly, or indirectly due to defects in lysosomes and autophagic flux. APOL1 has been
499 shown to cause toxicity by disrupting lysosomes in human podocytes [15]. Since intracellular organelles
500 are not viewed as static and in isolation, there could be a potential consequence to mitochondria. For

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501 example, it has been shown that lysosome membrane permeability allows escape of lysosome hydrolases,
502 including cathepsin-B, which mediate mitochondrial outer membrane permeability and loss of inner-
503 membrane potential [41]. Future studies exploring these mechanisms in APOL1 risk variant-carrying
504 tissues could better explain gene-associated pathobiology. Also mitophagy, an autophagy analogue, is the
505 predominant means by which cells dispose of energy-depleted mitochondria [42]. Finally, others have
506 shown that the APOL1 pore co-localizes with the mitochondria in human embryonic kidney cells, directly
507 causing membrane permeability [43]. The notion that these processes are active in APOL1-expressing
508 endothelial cells is a novel and potentially biologically relevant finding.

509 While there are current APOL1 primary cell culture models in the literature, most have employed
510 viral vector systems to deliver the gene [5, 12, 44]. Importantly, these models showed that APOL1 variant
511 expression coincides with mitochondrial fragmentation, lysosome compromise, and an abundance of
512 autophagosomes [13, 15]. However, exaggerated APOL1 expression beyond that expected in native cells
513 poses a limitation on the clinical interpretation of these results. Moreover, viruses themselves can not only
514 induce APOL1 expression but also engage other autophagy pathways, making it difficult to assign risk variant-
515 mediated effects [45]. Therefore, HUVECs naturally expressing the variants were utilized acknowledging
516 shortcomings inherent in this approach as well.

517 Though experiments were repeated across multiple donors of each genotype, the possibility that non-
518 APOL1-related genetic variation contributed to our findings cannot be excluded. Both male and female
519 samples were utilized, and despite even distribution across genotype, differences in autophagy due to sex
520 cannot be excluded. Also, HUVEC cells may not recapitulate endothelial cell behavior in other vascular beds
521 more relevant to atherosclerotic disease. Finally, the threshold at which APOL1 expression influences
522 lysosome function is not clear based on this study. Further work determining the intracellular concentration
523 of APOL1 and timing of lysosome injury is warranted given the above findings.

524 In sum, variant APOL1 expression, particularly in the presence of inflammatory stimuli typical of
525 autoimmune diseases such as SLE, confers endothelial dysfunction mirrored in mitochondrial stress,
526 lysosomal dysfunction and impaired autophagic flux. Treatments aimed at compensating for these

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527 metabolically compromised cellular states may improve the vascular consequences facing APOL1 variant
528 carriers.

529

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534

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APOL1, interferon, and endothelial cell autophagy

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704 **Supporting Information**

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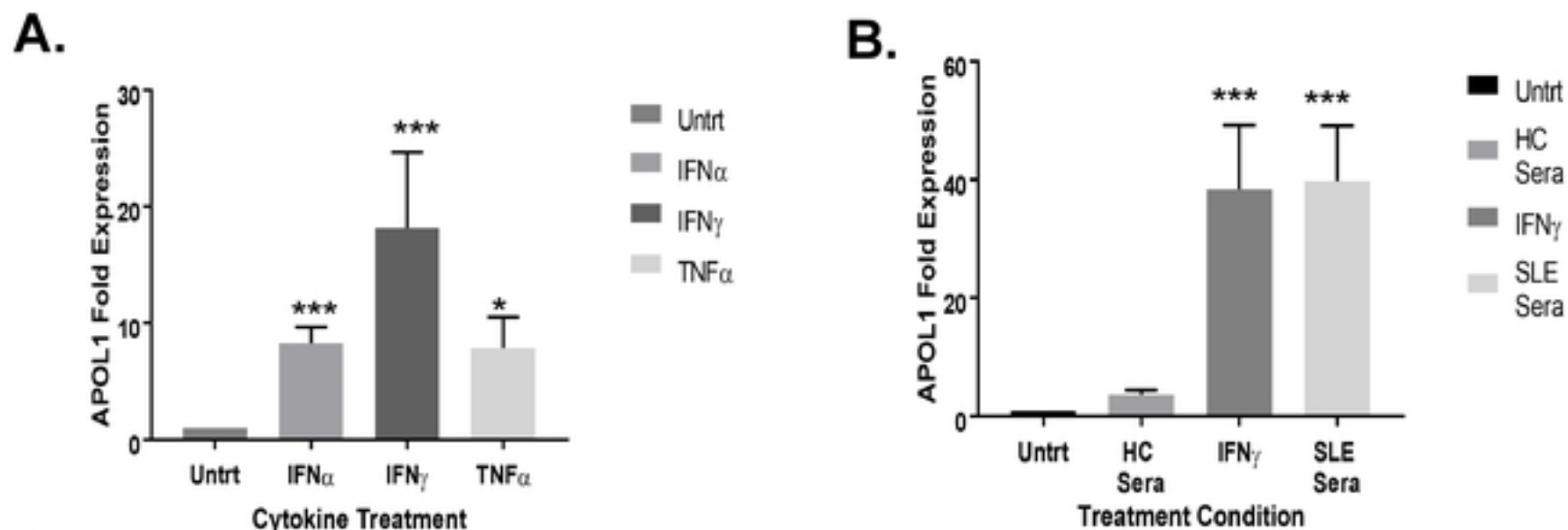


Figure 1. Endothelial cells treated with inflammatory cytokines induce APOL1 expression. **A.** The upregulation of HUVEC APOL1 transcript in untreated HUVECs compared to IFN α (50pg/mL), IFN γ (50pg/mL), and TNF α (10ng) treated for 18 hours (average of 5 experiments, 9 HUVEC donors). Shown on the y-axis are $2^{-\Delta\Delta CT}$ (transcript normalized to GAPDH) values, and shown on the x-axis are cytokine treatment. **B.** Exposure of HUVECs to sera at 1:1 dilution for 18 hours resulted in an upregulation of APOL1 transcription (average of 5 experiments, 9 HUVEC donors). Shown on the y-axis are $2^{-\Delta\Delta CT}$ (transcript normalized to GAPDH) values, and shown on the x-axis are treatment conditions. Comparisons are made between the mean fold expression in untreated vs the treatment condition using Kruskal-Wallis test (both 1A and 1B $p < 0.001$) followed by post hoc Dunn test (*** indicates $p < 0.001$, * indicates $p < 0.05$). Abbreviations: Untrt= untreated condition, IFN α = interferon alpha treatment, IFN γ = interferon gamma treatment, TNF α = tumor necrosis factor alpha treatment, HC Sera= healthy control sera, and Systemic Lupus Erythematosus Sera= SLE Sera, each lupus sera (subjects in table 1) was run in triplicate.

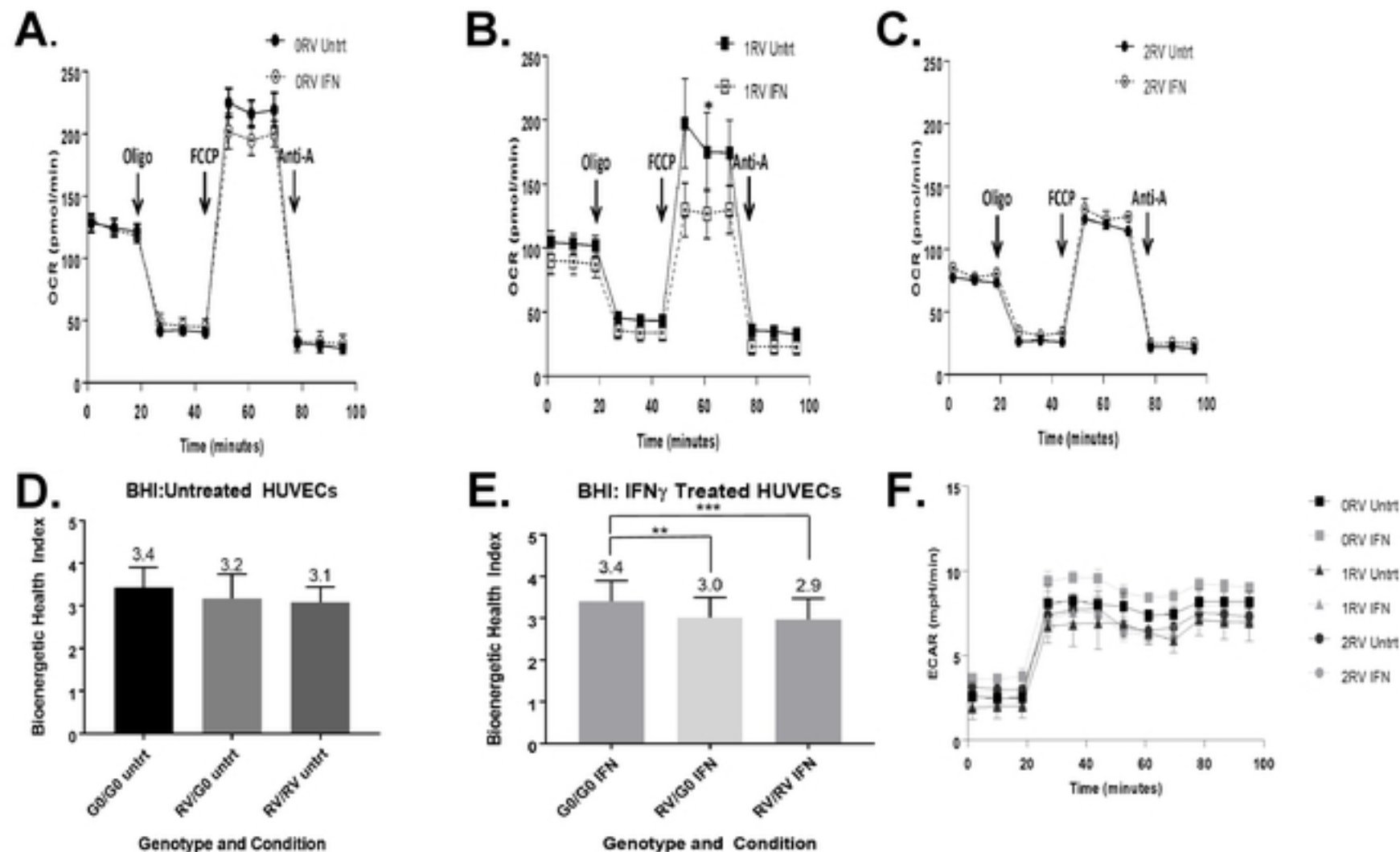


Figure 2. The bioenergetic profiles of HUVECs across genotype. APOL1 risk variant associations with attenuated mitochondrial function including maximum respiration, reserve respiration capacity, glycolytic capacity, and bioenergetic health index. Live HUVEC metabolic assays were performed using the Seahorse XF platform. Genotypes are represented as follows: 0RV, 1RV, and 2RV (RV= risk variant). Treatment conditions included: no treatment (Untreated) or stimulation using IFN (50pg/mL, 18h overnight). A-C. In this assay, oxygen consumption rate (OCR) was measured at baseline, upon oligomycin (oligo), FCCP, and antimycin A treatments. Representative oxygen consumption profiles are shown. D-E. Bioenergetic health index (BHI) calculated by APOL1 genotype and treatment condition (5 experiments averaged representing 9 HUVEC donors). F. Representative raw tracings of extracellular acidification rate (ECAR) after addition of Oligomycin by genotype and condition. P-values were calculated using one-way analysis of means for cross genotype comparisons. Where the three-comparison ANOVA was significant, post-hoc two comparison analysis was completed. * indicates $P < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

Figure 2

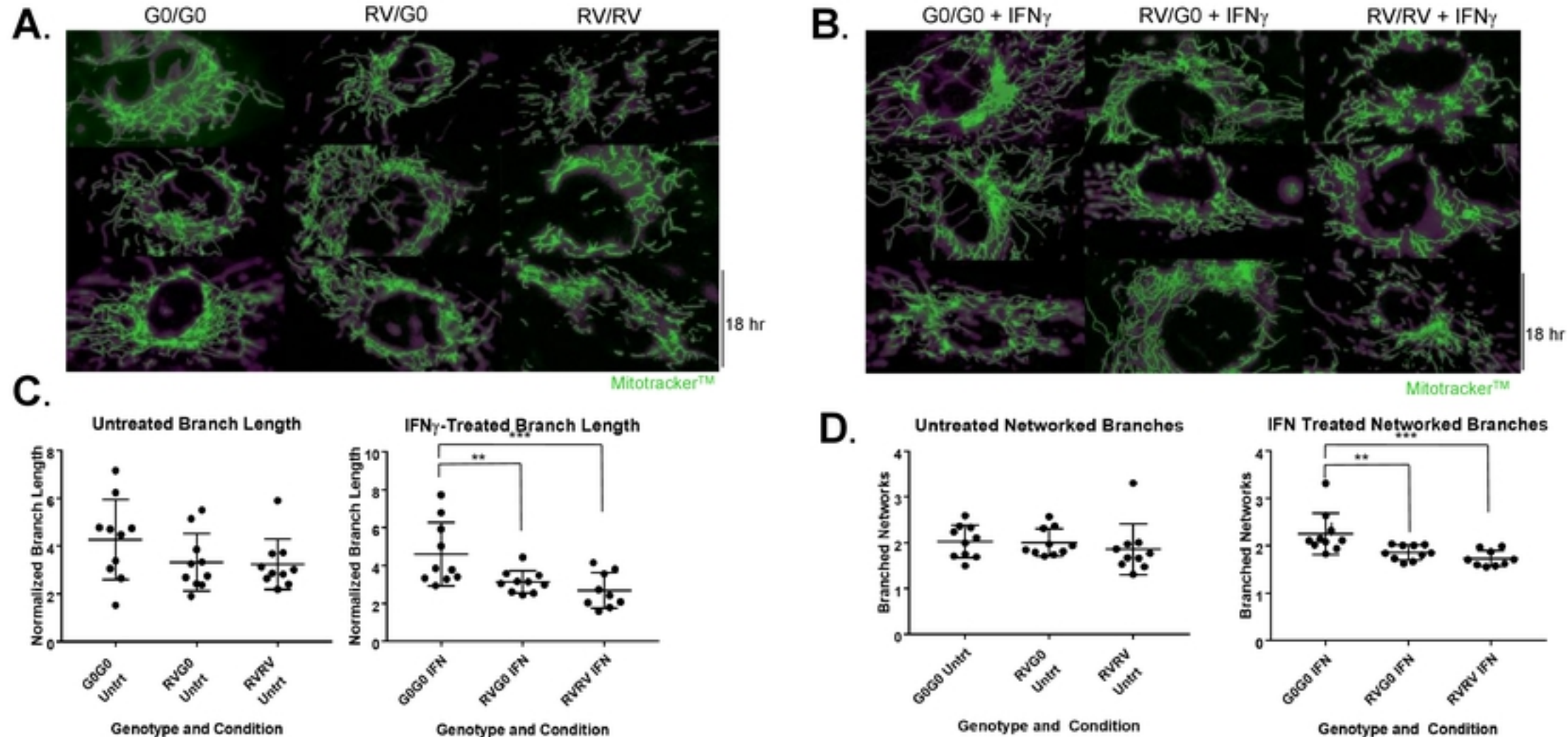


Figure 3. Assessment of mitochondrial structure in resting and stimulated HUVECs using MitoTracker, a fluorescent proxy of ultrastructure. Mitochondrial structure including branch length and networking is attenuated in HUVECs with 1RV or 2RV versus 0RV APOL1 genotype both at baseline and with IFN γ 50pg/mL-treatment. Human umbilical vein endothelial cells representing each genotype, 0 risk variant (G0/G0 left columns), 1 risk variant (RV/G0 middle columns), and 2 risk variant (RV/RV right columns) were either left untreated (A) or treated with IFN γ 50pg/mL (B) for 18 hours overnight. Representative immunofluorescence (MitoTracker green stained) images are shown (overall experiment, 10 cells per genotype and condition were measured. In total 12 HUVEC donors and 4 experiments were averaged). C-D Mitochondrial branch length (C) and networked branches (D) measured on confocal microscopy versus genotype and treatment condition (x axis) using the Mitochondrial Network Analysis (MiNA) tools available in the FIJI distribution of ImageJ. Mitochondrial length was measured in microns (μ m). Each additional risk variant associated with a reduced degree of mitochondrial networking; and effect that became statistically significant across the genotypes upon treatment with IFN γ . P-values were calculated using Kruskal-Wallis test for cross genotype comparisons, and Wilcoxon rank sum test for comparing untreated group and IFN group. P<0.01 is indicated by **, and p<0.001 is indicated by ***.

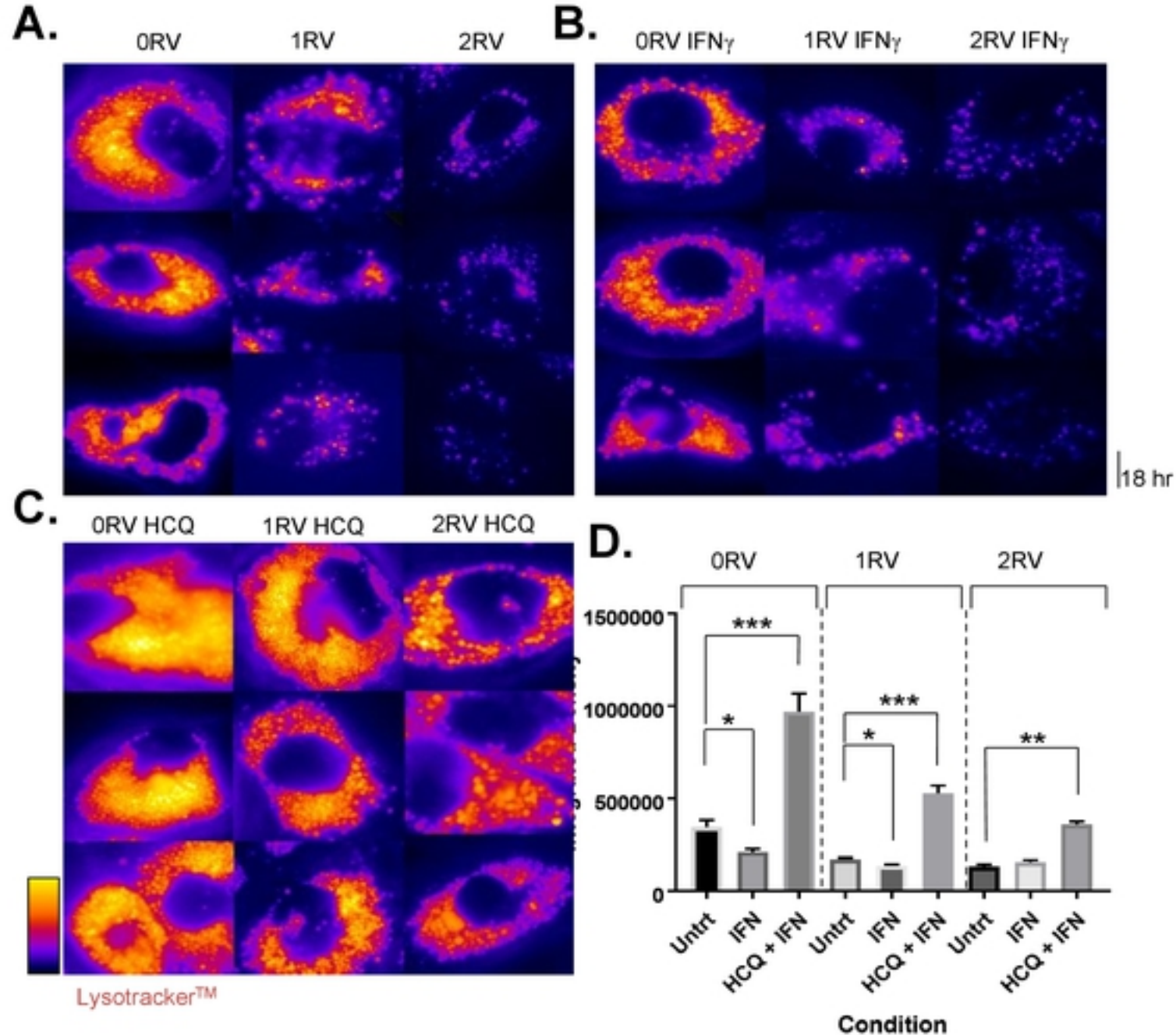


Figure 4. Assessment of lysosomal structure in resting and stimulated HUVECs with or without hydroxychloroquine (HCQ) using LysoTracker, a fluorescent proxy of lysosome. The presence of 1RV or 2RVs associated with less lysosome staining by lysotracker at baseline or with IFN γ treatment. Preventing lysosome turnover with the addition of HCQ treatment increased lysosome staining to a lesser degree in RV carrying HUVECs. A. Immunofluorescent images of untreated HUVECs representing each genotype, 0RV (left column), 1RV, and 2RV (right column). B. Representative images of IFN γ 50pg/mL-treated HUVECs across genotype (parallel layout as per A) for 18 hours overnight. C. Representative images of HCQ (25 μ M) plus IFN γ -treated HUVECs across genotype (layout as per A) LysoTracker staining was performed. Images were captured by florescent microscopy using a Nikon Eclipse Ti and representative microphotographs were selected. D. The average lysosome intensity per region of interest (Integrated Density) for each genotype and treatment condition group. P-values were calculated using Kruskal-Wallis test for cross genotype comparisons, and Wilcoxon rank sum test for comparing untreated group to the IFN γ treated or HCQ plus IFN γ group. *P<0.05; **p<0.01, ***p<0.001.

Figure 4

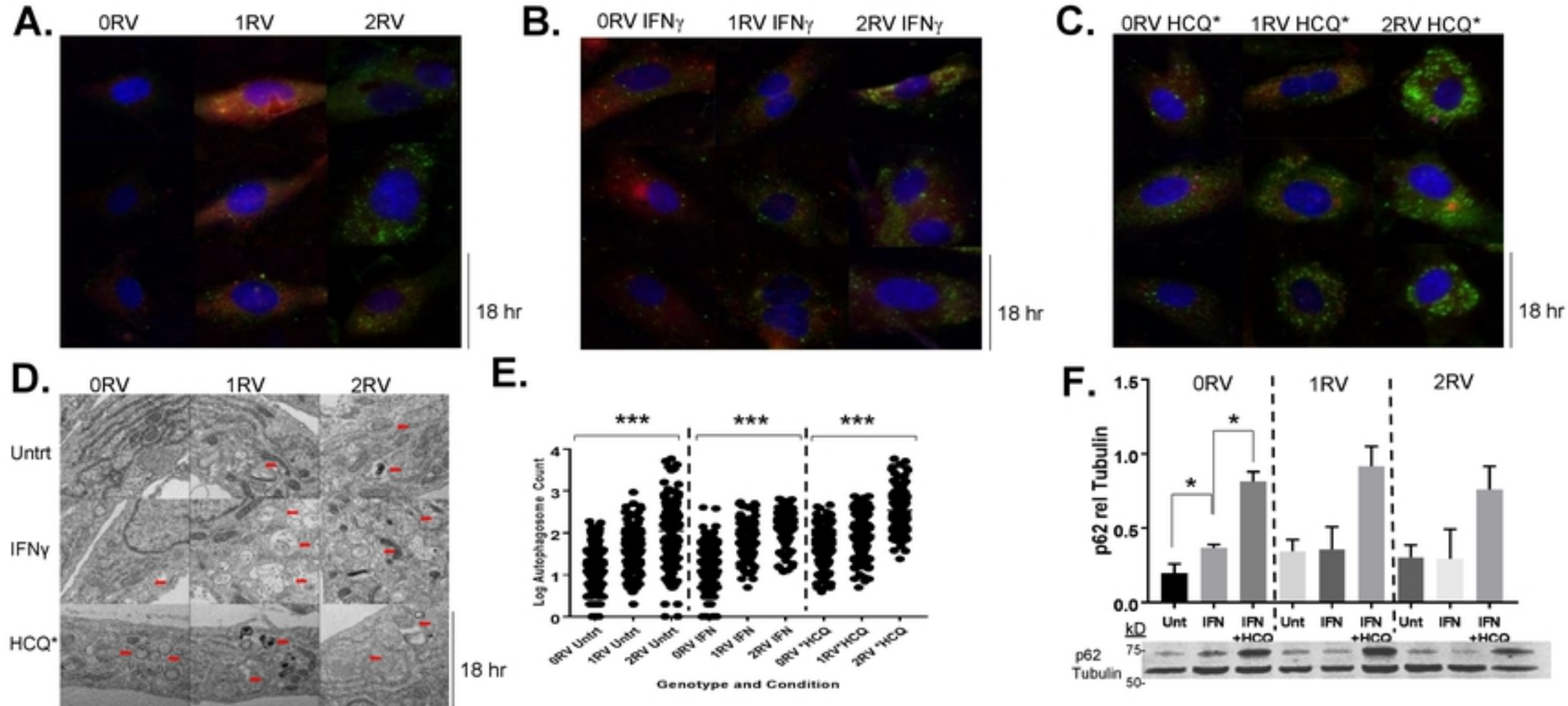


Figure 5. APOL1 risk variant-carrying HUVECs display autophagic flux deficiencies. Assessment of autophagosome accumulation using SQSTM1 (p62) staining, a proxy for autophagic flux inhibition. A. Representative immunofluorescence images of untreated HUVECs stained for SQSTM1 (p62) across APOL1 genotype: 0 risk variants (left column), 1 risk variant (middle column), 2 risk variants (right column). B. Representative immunofluorescence images of IFN γ (50pg/mL)-treated HUVECs stained for SQSTM1 (p62) across APOL1 genotype C. Representative immunofluorescence images of IFN γ plus HCQ (25 μ M)-treated HUVECs stained for SQSTM1 (p62) across APOL1 genotype D. Autophagosomes were confirmed by transmission electron microscopy (Columns: 0RV left, 1RV middle, 2RV right; Rows: Unt=untreated top, IFN γ -treated middle, IFN γ plus HCQ-treated bottom). E. Log transformed values of p62 positive puncta per cell (y axis) and the treatment condition (x axis) are shown. HUVEC genotype is labeled from left to right. F. Immunoblot of HUVEC lysates showing SQSTM1 (p62) protein concentration compared to tubulin loading control by genotype and treatment condition. Note: HUVECs were treated for 18 hours. *P<0.05; ***P<0.001 were calculated from one-way analysis of means for cross genotype comparisons. Where ANOVA rejected the null hypothesis, a post-hoc 2 group analysis was performed.