Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Yang Z, Stratton C, Francis PJ, et al. Toll-like receptor 3 and geographic atrophy in age-related macular degeneration. N Engl J Med 2008;359. DOI: 10.1056/NEJMoa0802437.

Toll-Like Receptor-3 and Geographic Atrophy in Age-Related Macular Degeneration

Supplementary Material and Methods

Patients

All participants underwent a standard examination, which at a minimum included visual acuity measurements, dilated slitlamp biomicroscopy, and stereoscopic color fundus photography. Grading was carried out with the classification established by AREDS.[1] Diagnosis of advanced AMD was based on the presence of GA or CNV (equivalent to AREDS category 4 or 5). Soft drusen were characterized as drusen with indistinct borders and size greater than 125 microns in diameter. Control subjects were > 60 years of age, with no signs of AMD, defined as no drusen or RPE abnormalities in the Utah collection, and controls in the first replication case-control series and AREDS case-control series were defined as being > 60 years of age, having fewer than five small drusen (<63 μm), and no RPE abnormalities. Patient characteristics of the case-control series are listed in Table S1. The ancestral origin of patients was established through self-reporting either by a multiple choice questionnaire (Hopkins, Oregon cohorts; choices were Northern European, Hispanic, African, Asian, Native American, Other) or by open questionnaire (Utah, China: "Please answer the following question: My ancestral origin is …")

In vitro HUMAN RPE APOPTOSIS ASSAY

Cultures were sensitized with IFN-α/β for 24 hours followed by treatment with either PBS or poly (I:C) (5 μg/mL) in high glucose DMEM (Gibco) with 2% FBS (Gibco). After 24 hours treatment, cells were harvested with 0.05% trypsin-EDTA (Gibco), washed in PBS, and resuspended in annexin V staining buffer (BD Biosciences) at 10^6 cells/mL. 100 μL aliquots were then incubated with 5 μL of FITC conjugated annexin-V (BD Biosciences) and 5 μL of propidium iodide (PI; BD Biosciences) for 15 min at 25 °C. Cells were immediately analyzed and annexin V^+/PI^- cells were calculated using Cellquest Pro (BD Biosciences).

In vivo MOUSE RPE ASSAYS

Cell suspensions were isolated from RPE/choroid of C57BL/6J wild-type or *Tlr3*$^{-/-}$mice[2] (The Jackson Laboratory) forty-eight hours after intravitreous injection of poly (I:C) (2 μg) by incubation with collagenase D (20 U/l; Roche Diagnostics). After treatment with Fc block (10 μg/ml; BD Biosciences) for 15 min on ice, 10^6 cells were incubated with FITC-conjugated anti-mouse CD147 antibody (10 μg/ml, eBiosciences, Clone RL73) and APC-conjugated anti-mouse CD31 antibody (20 μg/ml; BD Biosciences, Clone MEC13.3) to identify CD147$^+$CD31$^-$ RPE cells. For cell viability, cells were analyzed with a minimum of 10,000 gated events on a FACSCalibur flow cytometer (BD Biosciences) and CD147$^+$CD31$^-$ fractions were calculated with Cellquest Pro (BD Biosciences). For intracellular activated caspase-3 staining, CD147$^+$CD31$^-$ cells were subjected to fixation and permeabilization (Leucoperm, Serotec), followed by incubation with PE-conjugated rabbit anti-activated caspase-3 antibody (20 μl/10^6 cells, BD Biosciences, Clone C92-605) in the presence of 10% normal rabbit serum. Cells were analyzed with a minimum of 10,000 gated events on a FACSCalibur flow cytometer using Cellquest Pro. Differences in fractions of CD147$^+$CD31$^-$ cells or activated caspase-3$^+$ cells were compared with the Mann Whitney U test.

In situ TUNEL ASSAY in RETINA/RPE

Forty-eight hours after intravitreous injection of poly (I:C) (2 μg), eyes from wild-type and *Tlr3*$^{-/-}$ mice were harvested and immersed in 3.7% paraformaldehyde for 2 hours at 4ºC. Following cryoprotection in 20% sucrose overnight at 4ºC, samples were embedded in OCT (Tissue-Tek) and 10 μm cryosections were prepared. *In situ* TUNEL labeling of apoptotic cells was performed using a commercially available kit (TACS XL Blue, Trevigen). Nuclei were counterstained with Nuclear Fast Red.

AUTHOR CONTRIBUTIONS

Kang Zhang, Nicholas Katsanis, Jayakrishna Ambati, and Zhenglin Yang designed the study; Zhenglin Yang, Peter Francis, Mark E. Kleinman, Perciliz L. Tan, Charity Stratton, Daniel Gibbs, Zongzhong Tong, Haoyu Chen, Xian Yang, Yuhong Chen, Xiang Ma, Lisa Davey, Ryan Constantine, Jiexi Zeng, Jennifer Harmon, Jeanette Buehler, Ling Luo, Beifeng Yu, Andrea Schwager., Erik Pearson, Shrena Patel, Vincent

S. Hau, Yuuki Kaminoh, Norman A. Zabriskie,, Paul S. Bernstein, Wongil Cho., David R Hinton, Michael L Klein, Sara C. Hamon, Emily Simmons, Janet S. Sunness, Betsy Campochiaro, Peter Campochiaro, Donald J. Zack, Nicholas Katsanis, Jayakrishna Ambati, and Kang Zhang gathered the data, Kang Zhang, Nicholas Katsanis, J. Ambati, Haoyu Chen, Chi Wang, Daneil Gibbs, Giovanni Parmigiani, Scott Watkins, Lynn Jorde analyzed the data, vouches for the data and the analysis; Mark E. Kleinman, Wongil Cho, Jayakrishna Ambati performed studies in human RPE cells and mice; Kang Zhang, Nicholas Katsanis, Jayakrishna Ambati, Peter Francis, and Zhenglin Yang wrote the first draft and subsequent revisions of paper and decided to publish the paper and assume responsibility for the overall content and integrity of the article. There were no agreements concerning confidentiality of the data between the sponsor and the authors or the institutions named in the credit lines.

References

1. The Age-Related Eye Disease Study (AREDS): design implications. AREDS report no. 1. Controlled clinical trials 1999;20:573-600.
2. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

Table S1. Disease Status, Gender, and Age of Subjects

Case-control series	Utah case-control series				First replication case-control series			AREDS case-control series		Han Chinese case-control series	
	GA	CNV	Soft Drusen	Controls	GA	CNV	Controls	GA	Controls	CNV	Controls
	232	441	152	359	271	179	421	184	134	140	171
Sex-Number (%)											
Female	139 (60)	221(50)	70 (46)	229 (64)	160 (59)	99 (55)	215 (51)	105 (57)	74 (55)	68(49)	80(47)
Male	93 (40)	220 (50)	82 (54)	130 (36)	111 (41)	80 (45)	206 (49)	79 (43)	60 (45)	72(51)	91(53)
Average Age-years	84.09	83.14	78.01	77.41	83.48	79.51	77.3	78.81	79.57	68.2	67.1

Table S2. List of primers used in SNP genotyping for *TLR3* and *TLR4* variants

Gene	SNP	Forward Primer	Reverse Primer	SNaPshot Primer
TLR4	rs4986790	tgacaaatctgctctagagg	ttataagttttgtcttttac	cttagactactacctcgatg
TLR4	rs4986791	aactcaaatctctcaaaagg	atttcaaattggaatgctgg	gttgctgttctcaaagtgattttgggacaa
TLR3	rs3375291	tcattaaggcccaggtcaag	ttgcgaactttgacaaatgaa	tttattcttggttaggttga
TLR3	rs5743312	tgcattctgcctttaaggtg	tcaggaggatgacaccaatg	cctatctgtgtcacataca
TLR3	rs13126816	cacaatattggataatttccatcaac	tctgggctgtctactctcttcc	aaaaggcaatctagaagaagagcaa
TLR3	rs11730143	ccatgtgccctcacaaaata	tgagagtgaagaaaacgttgc	gagtgagtctctttttaatttttttcatttt
TLR3	rs11732384	aaggccatgagatgggaata	ggtgactaatcctaggaaaacagaa	aactaatgtgagtatatagaattgttctaatatacttaaaaatag
TLR3	rs11721827	atatgggcgtgcctgtgtat	tattttgtgagggcacatgg	tacaaaggttgcaaagtttacaaatcaaact
TLR3	rs10025405	gtaggcaggcttgctttctg	tgcgtagcacattattttatgg	ttcctcccatggtcacagactcaggagatggcgttggc
TLR3	rs5743303	caaccaaagtcgtgggaaat	ttagcaagcatgcaacaagg	gtatgaaggattgttgagatgatgtgtttt

Table S3. Association results of SNPs in *TLR3* and *TLR4*.

				Combined		Allelic p-Value		
SNP	Gene	Chromosome	Chromosome Position	Case MAF	Control MAF	Utah	Combined	FDR Adjusted
rs4986790	TLR4	9	119515123	0.053	0.047	0.05	0.60	0.60
rs4986791	TLR4	9	119515423	0.061	0.051	0.05	0.34	0.67
rs5743303	TLR3	4	187225847	0.18	0.179	0.05	0.45	0.51
rs11721827	TLR3	4	187228131	0.16	0.161	0.62	0.95	0.95
rs11730143	TLR3	4	187228296	0.149	0.172	$3.70X10^{-05}$	0.26	0.42
rs11732384	TLR3	4	187229883	0.154	0.169	$9.98X10^{-04}$	0.39	0.52
rs13126816	TLR3	4	187231172	0.224	0.269	0.02	0.06	0.15
rs5743312	TLR3	4	187237250	0.156	0.131	0.03	0.13	0.27
rs3775291	TLR3	4	187241068	0.24	0.332	0.005	$1.55X10^{-08}$	$1.24X10^{-07}$
rs10025405	TLR3	4	187243800	0.482	0.405	0.004	$6.27X10^{-04}$	0.003

Table S4. Genotyping result of *TLR3* variant (rs3775291) in four case-control studies

Case-control series		Utah case-control series				First replicated case-control series			AREDS case-control series		Han Chinese case-control series	
Groups		GA	CNV	Soft Drusen	Control	GA	CNV	Control	GA	Control	CNV	Control
Number		232	441	152	359	271	179	421	184	134	140	171
Genotypic Information	CC [n, (%)]	125(53.88%)	211(47.85%)	70(46.05%)	156(43.45%)	157(57.93%)	86(48.04%)	183(43.47%)	112(60.87%)	61(45.52%)	76(54.29%)	92(53.80%)
	CT [n, (%)]	93(40.09%)	201(45.58%)	74(48.68%)	163(45.40%)	95(35.06%)	81(45.25%)	196(46.56%)	68(36.96%)	62(46.27%)	56(40.00%)	62(36.26%)
	TT [n, (%)]	14(6.03%)	29(6.58%)	8(5.26%)	40(11.14%)	19(7.01%)	12(6.70%)	42(9.98%)	4(2.17%)	11(8.21%)	8(5.71%)	17(9.94%)
HWE		0.83	0.12	0.12	0.97	0.68	0.47	0.61	0.22	0.69	0.22	0.69
Allelic Information	C [n, (%)]	343(73.92%)	623(70.63%)	214(70.39%)	475(66.16%)	409(75.46%)	253(70.67%)	562(66.75%)	292(79.35%)	184(68.66%)	208(74.29%)	246(71.93%)
	T [n, (%)]	121(26.08%)	259(29.37%)	90(29.61%)	243(33.84%)	133(24.54%)	105(29.33%)	280(33.25%)	76(20.65%)	84(31.34%)	72(25.71%)	96(28.07%)

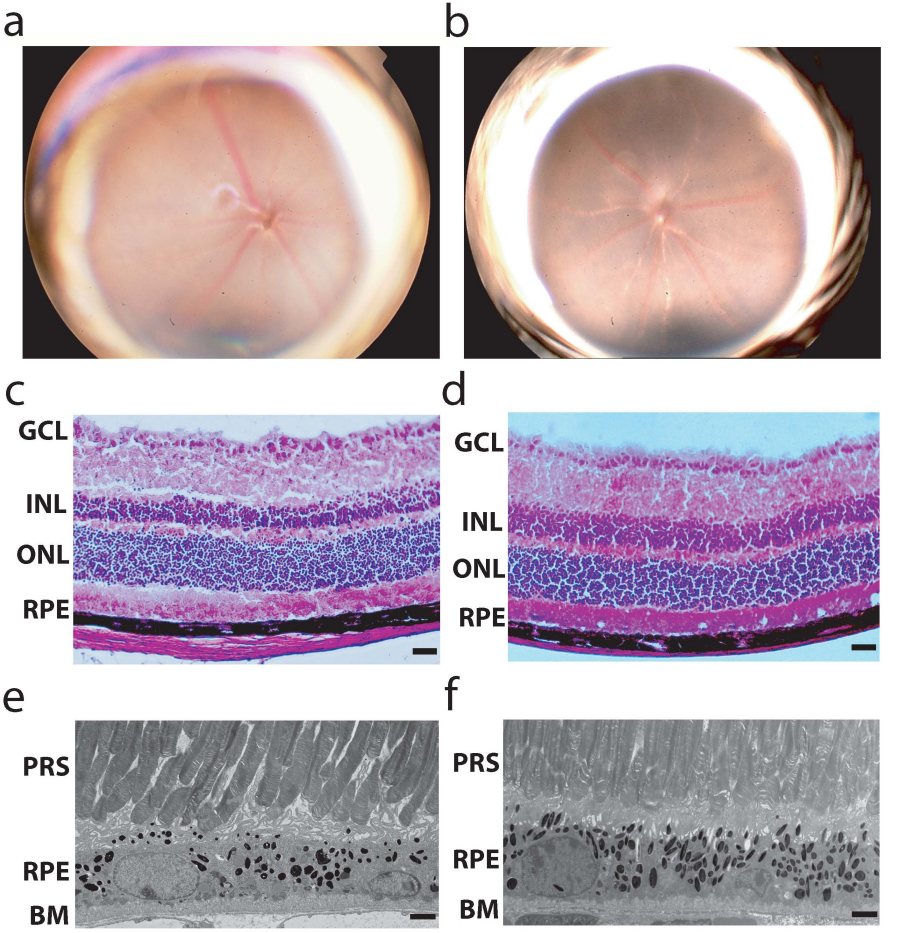
Supplemental Figure Legends

Figure S. 1

Color photograph of the fundus of *Tlr3*$^{-/-}$ mice (a) revealed a normal retinal appearance resembling that of wild-type mice (b). The retinal morphology of *Tlr3*$^{-/-}$ mice (c) resembled that of wild-type controls (d). The ultrastructure of the outer retina, RPE, and Bruch's membrane of *Tlr3*$^{-/-}$ mice (e) resembled that of wild-type mice (f) as evaluated by transmission electron microscopy. GCL=ganglion cell layer; INL=inner nuclear layer; ONL=outer nuclear layer; PRS=photoreceptor segments; BM=Bruch's membrane. Images representative of n=3. Scale bar, 10 μm (c,d), 2 μm (e,f).

Figure S. 2

Color photographs of the fundus of uninjected wild-type (a) and Tlr3-/- (b) mice showing grossly normal retinal features. Color fundus photograph taken two weeks after intravitreous injection of poly (I:C) (2 μg) shows areas of subretinal atrophy (black-arrows) consistent with geographic loss of RPE cells in wild-type (c) but not in Tlr3-/- mouse (d) retinas. In situ TUNEL assays at forty-eight hours after poly (I:C) (2 μg) injection demonstrated far more numerous positive (blue) cells overlapping with nuclei (stained red by Nuclear Fast Red) in the inner and outer nuclear layers (INL and ONL; black arrow-heads) of the retina of wild-type mice (e) than in the retina of Tlr3$^{-/-}$ mice (f). Scale bar, 20 μm. Images representative of n=4.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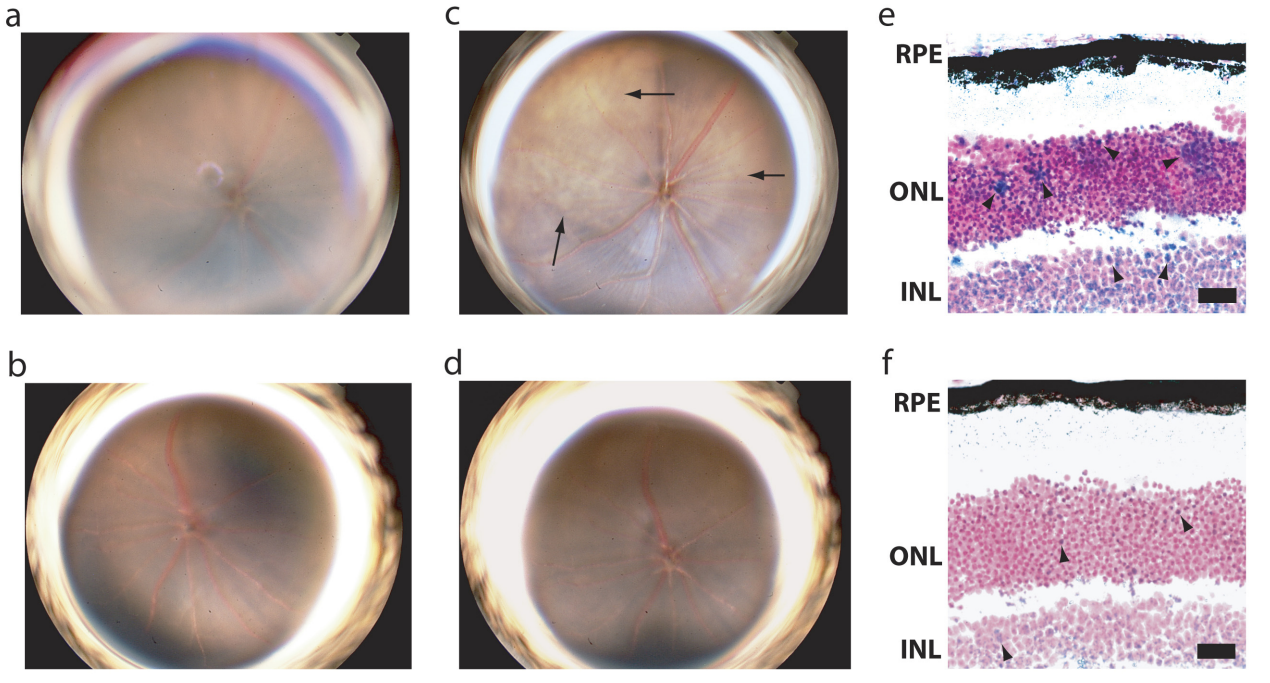
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