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

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### Abstract

**BACKGROUND**—Age-related macular degeneration (AMD) is the most common cause of irreversible visual impairment in the developed world. Advanced AMD is comprised of geographic atrophy (GA) and choroidal neovascularization (CNV). Specific genetic variants that predispose for GA are largely unknown.

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#### Disclosure

Dr. Ambati reports receiving consulting fees from Quark Pharmaceuticals and Allergan; Dr. Zhang reports having an equity interest in Navigen; receiving grant support and lecture fees from Genentech; receiving consulting fees from Acucela and Oxigene; No other potential conflict of interest relevant to this article was reported.

**METHODS**—We tested (i) for association between the functional toll-like receptor-3 (*TLR3*) variant rs3775291 (L412F) and AMD in European Americans and (ii) the effect of TLR3 L and F variants on the viability of human retinal pigment epithelium (RPE) cells *in vitro* and on RPE cell apoptosis in wildtype and *Tlr3*<sup>-/-</sup> mice.

**RESULTS**—The F variant (or T allele at single nucleotide polymorphism at rs3775291) was associated with protection against GA (P=0.005); this association was replicated in two independent GA case-control series (P=5.43×10<sup>-4</sup> and P=0.002, respectively). We observed no association between *TLR3* variants and CNV. The rs377291 variant is probably critical to the function of TLR3, because a prototypic TLR3 ligand induced cell death and apoptosis in human RPE cells with the LL genotype to a greater extent than it did RPE cells with the LF genotype. Moreover, the ligand induced more RPE cell death and apoptosis in wild-type than in *Tlr3*<sup>-/-</sup> mice.

**CONCLUSIONS**—The TLR3 412F variant confers protection against GA, probably by suppressing RPE cell death. Given that double stranded RNA can activate TLR3-mediated apoptosis, our results suggest a possible role for viral dsRNA transcripts in the development of GA and raise awareness of potential toxicity induced by short interfering RNA (siRNA) therapeutics in the eye.

### Keywords

Toll-Like Receptor 3; Age-Related Macular Degeneration; Geographic Atrophy; Single Nucleotide Polymorphism; Apoptosis

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Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the developed world. The disease is broadly classified according to its severity and likelihood of progression. The hallmark of the condition is the presence of drusen, deposits in the macula (central retina). When these are confluent and ‘soft’ in appearance, the affected person is considered to have early/intermediate AMD, even though vision is usually unaffected. The greater the number and size of drusen, the greater the risk of progression to both forms of advanced AMD: (i) geographic atrophy (GA) of the retinal pigment epithelium (RPE) and overlying photoreceptors (also called advanced ‘dry’ AMD) and (ii) choroidal neovascularization (CNV, also called ‘wet’ AMD).

Geographic atrophy is characterized by confluent areas of photoreceptor and RPE cell death, is bilateral in more than half of patients, and is responsible for 10% of the legal blindness from AMD.<sup>1-3</sup> Approximately 900,000 persons in the United States are affected<sup>4</sup>. Despite the prevalence of this disease, its etiology remains largely unknown and there exists no approved treatment.

Loci at the complement factor H gene (*CFH*), *LOC387715/HTRA1*, *C2* and *C3* are associated with all phenotypic variants of AMD, including early AMD, GA, and wet AMD.<sup>5-18</sup> However, the genetic basis of and molecular mechanisms underlying GA that are specific to GA are not known.

There is an emerging consensus that perturbed inflammatory cascades cause susceptibility to AMD.<sup>19, 20</sup> Because of the speculation that microbial and viral entities might provoke the pathological inflammation that drives AMD and given the previously reported potential association of variants in *TLR4* (a bacterial endotoxin receptor)<sup>21</sup> with AMD,<sup>22</sup> we tested for association between polymorphisms in *TLR3*, which encodes a viral sensor that supports innate immunity and host defense,<sup>23</sup> and the AMD phenotypes of soft confluent drusen, GA, and CNV. We then tested for a functional effect of an implicated TLR3 variant in human RPE cells and in the RPE of wildtype and *Tlr3*<sup>-/-</sup> mice.

## METHODS

### PATIENTS

This study was approved by the Institutional Review Boards of the University of Utah, The Johns Hopkins University, and Oregon Health Science University, the Institutional Review Board of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, China, and the AREDS (Age Related Eye Disease Study) Access Committee. All subjects gave informed consent prior to participation. For details of patient ascertainment and case control series information, see Supplementary Materials and Methods.

### GENOTYPING

We genotyped single nucleotide polymorphisms (SNPs) in *TLR3* and *TLR4* SNPs with SNaPshot on an ABI 3100XL genetic analyzer (ABI, Foster City, CA) according to the manufacturer's instructions. The sequences of primers used for each SNP are provided in Table S2; amplification conditions are available upon request.

### STATISTICAL ANALYSIS

All SNP genotyping results were screened for deviations from Hardy-Weinberg equilibrium with no SNPs showing significant deviation ( $p > 0.05$ ). The chi-squared test for allelic trend for an additive model or dominant allele model over alleles was performed with PEPI version 4.0.<sup>24</sup> All SNP results from the same haploblock were adjusted for multiple testing using the False Discovery Rate (FDR) method; adjusted-p value = # of SNPs \* p value/ rank (p value). Odds ratios and 95% confidence intervals were calculated by conditional logistic regression with SPSS version 13.0. Linkage disequilibrium (LD) structure was examined with Haploview (version 4.0).<sup>25</sup> Default settings were used, creating 95% confidence bounds on  $D'$  to define pair-wise SNPs in strong LD.<sup>26</sup> Haploview was also used for allelic association tests.

### *In vitro* HUMAN RPE CELL VIABILITY ASSAY

Primary human RPE cells were isolated from eyes obtained from Advanced Bioscience Resources Inc. (Alameda, CA) and passed through 70  $\mu$ m and 40  $\mu$ m nylon mesh filters (Falcon Plastics, Oxnard, CA). After centrifugation at 1500 rpm for 5 min, the fragments remaining in the filter were gently dissociated and seeded onto laminin-coated 6- well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Irvine Scientific, Santa Ana, CA) with fetal bovine serum (FBS; 25% for primary culture and 10% thereafter) (Omega Scientific, Tarzana, CA), 100  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (Omega Scientific) at 37 °C in 95% air and 5% CO<sub>2</sub>. At confluence, cells were detached using 0.05% trypsin/ 0.02% EDTA (Irvine Scientific, Santa Ana, CA), collected by centrifugation, and expanded. The purity of the RPE cell culture exceeded 95% as confirmed by immunohistological cytokeratin positivity and the absence of contaminating CD11b<sup>+</sup> macrophages or vWF<sup>+</sup> endothelial cells. Homozygote (412LL) and heterozygote (412LF) isolates (P3 or P4) were synchronized for cell cycle state first by cultivating them in high glucose DMEM (Gibco) supplemented with 10% FBS (Gibco) to achieve confluence and then by overnight serum starvation. They were passaged to 96- well plates at a density of 10,000 cells per well (60-70% confluence), followed by stimulation for 24 hours with IFN- $\alpha/\beta$  (1000 U/mL, PBL Interferon Source). Cultures were then treated with poly (I:C) (Invivogen) or poly dI:dC (Sigma-Aldrich). At 48 hours, cell viability was measured with BrdU ELISA (Chemicon) according to manufacturer's instructions. Optical densities of 96-well plates were analyzed on a SpectraMax plate reader (Molecular Devices) at 450 nm using Softmax Pro v4.3. Differences in cell numbers were compared using the Mann Whitney U test (SPSS 15.0 for Windows). For additional *in vivo* and *in vitro* manipulations of these cells, see Supplementary Methods.

## FUNDUS PHOTOGRAPHY

Wild-type and *Tlr3*<sup>-/-</sup> mice were evaluated by dilated fundoscopic examination (1% tropicamide (Alcon)) at baseline and at 2 weeks after intravitreal injection of poly I:C (2 µg). Retinal photographs were acquired on a TRC-50 IX camera (Topcon) with a digital imaging system (Sony) and reviewed by two masked readers.

## RETINAL MORPHOLOGY

Eyes were enucleated from wild-type and *Tlr3*<sup>-/-</sup> mice and either snap-frozen in OCT (Tissue-Tek) for cryosections followed by staining with hematoxylin and eosin (Richard Allen Scientific) for basic histologic evaluation on an inverted light microscope (Nikon) or fixed in 3.5% glutaraldehyde/4% paraformaldehyde for 2 hours followed by preparation of uranyl acetate- and lead citrate-stained ultrathin sections for transmission electron microscopy studies (Phillips Biotwin 12).

## RESULTS

We tested for association between different AMD phenotypes and two potentially functional variants in *TLR3* (promoter SNP rs5743303 and the coding, non-synonymous SNP rs3775291). Our first sample was a European American AMD case-control series from Utah, with 441 persons with CNV, 232 with GA, and 152 with soft confluent drusen, and 359 unaffected controls (Table S1). We found no significant association between the SNP at 5743303 in *TLR3* ( $P > 0.05$ , Table S3) and any AMD phenotype; However, we observed a significant association between the “T” allele of the non-synonymous coding SNP rs3775291 and protection against GA ( $p = 0.005$ , additive allele-dosage model,  $OR_{het} = 0.712$ , 95% CI 0.503-1.00;  $OR_{hom} = 0.437$ , 95% CI 0.227-0.839; Table 1, Table S3). We did not observe an association between this SNP and CNV ( $p = 0.06$ ) or with soft confluent drusen ( $p = 0.19$ ; Table 1, S4). To test for replication of the association we genotyped an independent sample of European Americans comprising of 271 GA cases, 179 CNV, and 421 normal controls, and observed a significant association between rs3775291 and GA ( $p = 5.43 \times 10^{-04}$ ) but not with CNV ( $p = 0.18$ , Table 1). Nor did we observe a significant association between rs3775291 with CNV ( $p = 0.51$ , Table 1) in a Han Chinese case-control series. A second test for replication yielded a significant association between rs3775291 and GA ( $p = 0.002$ ; Fig. 1 and Table 1, S4) in a case-control sample from the AREDS comprising 184 GA cases and 134 controls (defined as subjects with diagnosis of category 1). Combined analysis of the three GA case-control series of European descent yielded a highly significant association ( $p = 1.24 \times 10^{-07}$ , FDR adjusted) between rs3775291 and GA. All SNPs had a genotyping success rate  $> 98\%$  and accuracy  $> 99\%$  as judged by random re-sequencing of 20% of samples in all case-control series.

We also tested for association between the AMD phenotypes and two SNPs in *TLR4* (rs4986790, rs4986791, which were reported previously to show association with AMD).<sup>22</sup> We found no significant association with rs4986790 or rs4986791 in *TLR4* ( $P > 0.05$ , Table S3).

To refine the association between GA and *TLR3*, we analyzed linkage disequilibrium (LD) block and haplotypes by genotyping six additional SNPs surrounding rs3775291 in the three case-control series of European descent. We found that rs10025405 is in high LD with rs3775291 ( $D' = 0.79$ ) and is also significantly associated with GA ( $P = 0.003$ , Table S3). We observed a disease-associated haplotype, ‘CG,’ made up of a cytosine residue at SNP rs3775291, and a guanine residue at SNP rs10025405 (this haplotype was present in 44.8% of cases and 37.9% of controls,  $P = 0.001$ ). These SNPs were in high LD with each other in both case and control groups (Fig. 1C). The protective haplotype ‘TA’ was present in 21.9% in cases and 30.4% of controls ( $P = 5.92 \times 10^{-6}$ ).

Hidden subdivision (stratification) can generate false-positive associations in case-control series.<sup>27</sup> We have tried to keep this to a minimum by choosing European-American case-control series from limited, distinct/localized geographic areas in the United States (Utah case-control series: Salt Lake City; first replication case-control series: Baltimore, Salt Lake City, Eugene, OR). In particular, those in our first series are all Utahns of European descent.<sup>40,41</sup> The slight degree of subdivision therein<sup>42</sup> is unlikely to cause the strong association that we observed. Furthermore, it is improbable that stratification would underlie association in both tests of replication. Finally, the AREDS case-control series was investigated previously for substructure, and no evidence of significant stratification was found.<sup>14, 18</sup>

We next turned to the question of allelic effect of the *TLR3* variant rs3775291. The prototypic TLR3 ligand polyinosinic:polycytidylic acid (poly (I:C)), a synthetic long dsRNA molecule that activates TLR3,<sup>28</sup> induced cell death in primary human RPE cells homozygous for the 412L variant (which is encoded by the C allele at rs3775291) in a dose-dependent fashion (Figure 2a), consistent with the known cytotoxic effect of TLR3 activation. In contrast, polydeoxyinosinic:polydeoxycytidylic acid (poly dI:dC), which does not activate TLR3,<sup>28</sup> did not reduce RPE cell viability (Figure 2a). Poly (I:C) induced cell death was reduced by 50 ±12% (p=0.02) in 412LF human RPE cells when compared to 412LL cells (Figure 2b). In addition, we found that induction of apoptosis (as indicated by Annexin V<sup>+</sup>PI<sup>-</sup> expression) was reduced by 50±9% in 412LF cells compared to 412LL cells (p=0.03; Figure 2c).

We then tested the effect of TLR3 activation of administering poly (I:C) into the vitreous humor of wild-type or *Tlr3*<sup>-/-</sup> mice. The retinas of uninjected wild-type and *Tlr3*<sup>-/-</sup> mice retinas appeared normal on dilated fundus examination (Supplementary Figure 1a and 1b). Histologic evaluation revealed intact neural retinal layers, RPE, and choroid (Supplementary Figure 1c and 1d). Ultrastructural examination revealed orderly photoreceptor arrays and confluent RPE (Supplementary Figure 1e and 1f) in both mouse strains.

Fundus examination two weeks after poly (I:C) injection revealed that wild-type mice developed features consistent with geographic loss of retinal and RPE cells; such features were not evident in *Tlr3*<sup>-/-</sup> mice (Supplemental Figure 2a-d). In support of these observations, flow cytometric analyses demonstrated that 48 h after poly (I:C) administration, there was a 61±4% (P=0.03) greater loss of RPE cell numbers in wild-type mice compared to *Tlr3*<sup>-/-</sup> mice (Figure 2d). Similarly, there was a 60±18% (P=0.03) greater induction of RPE cell apoptosis (as indicated by caspase-3 activation) in wild-type mice compared to *Tlr3*<sup>-/-</sup> mice following poly (I:C) administration (Figure 2e). Although activated caspase-3 is specific for apoptotic-related cell death,<sup>29-31</sup> we also identified late apoptotic/necrotic cells by *in situ* TUNEL labeling. In correlation with the caspase-3 activation data, there were 75±1% (P=0.05) fewer TUNEL-positive cells in the retina and RPE at 48 h after poly (I:C) administration in *Tlr3*<sup>-/-</sup> mice compared to wild-type mice (Figure 2f, Supplementary Figure 2e,f).

## DISCUSSION

Our data indicate that the T allele of SNP rs3775291 is associated with protection against GA in patients with AMD and that this protective effect is likely mediated by a reduction of dsRNA-induced cell death in RPE cells *in vitro* and *in vivo*. We did not observe an association between SNPs in *TLR3* and CNV or early AMD. *TLR3* therefore would seem to have an effect specifically on the GA subphenotype. Indeed, it is important to underscore that this association is evident only when controls are limited to no drusen (Utah case-controls series) or less than five small drusen (AREDS category 1) in the two replication series. Persons with more than five small drusen or RPE changes (sometimes considered to be in the normal range) were not included in the study, suggesting that the *TLR3* genotype is potentially implicated in early events in the pathogenesis of AMD. We speculate that while *HTRA1* and *CFH* predispose



individuals to early and late AMD, and that TLR3 activation (which is enhanced with the 412L variant) might promote progression to the GA phenotype. Once definite GA appears, it generally progresses contiguously from preexisting areas of involvement. Such a consumptive course is consistent with previously healthy areas of RPE being affected by adjacent diseased tissue. If activation of the viral dsRNA sensor TLR3 contributes to this progressive process, it is conceivable that intercellular transmission of viral intermediates or transcripts that activate TLR3 mediate GA pathogenesis in some patients. Alternatively, RNA from adjacent damaged/dying cells could trigger TLR3 activation.<sup>32</sup> Given our findings, it would be important to search for the existence and nature of dsRNAs (viral or otherwise) in GA-affected eyes. Retina and RPE cell death and apoptosis in response to poly (I:C) were reduced but not abolished in *Tlr3*<sup>-/-</sup> mice (Figure 2d,e,f), suggesting that other dsRNA sensors<sup>33-35</sup> might have been activated.

dsRNAs as short as 21 base pairs (bps) have also been shown to bind TLR3 *in vitro*.<sup>33-35</sup> Indeed, we have shown recently that 21-bp short interfering RNAs (siRNAs), including those currently in advanced stages of FDA clinical trials for CNV in AMD, bind and activate TLR3 *in vivo*.<sup>36</sup> Our findings suggest that TLR3 activation induces cell death in human and mouse RPE cells and raise the possibility that siRNA therapeutics might increase the risk of patients to GA depending on *TLR3* genotype. We speculate that chemical modifications to siRNA that abolish TLR3 activation could enhance their therapeutic specificity. The presence of a protective allele in *TLR3* against GA supports a rationale for targeting TLR3 as a preventive or therapeutic strategy for GA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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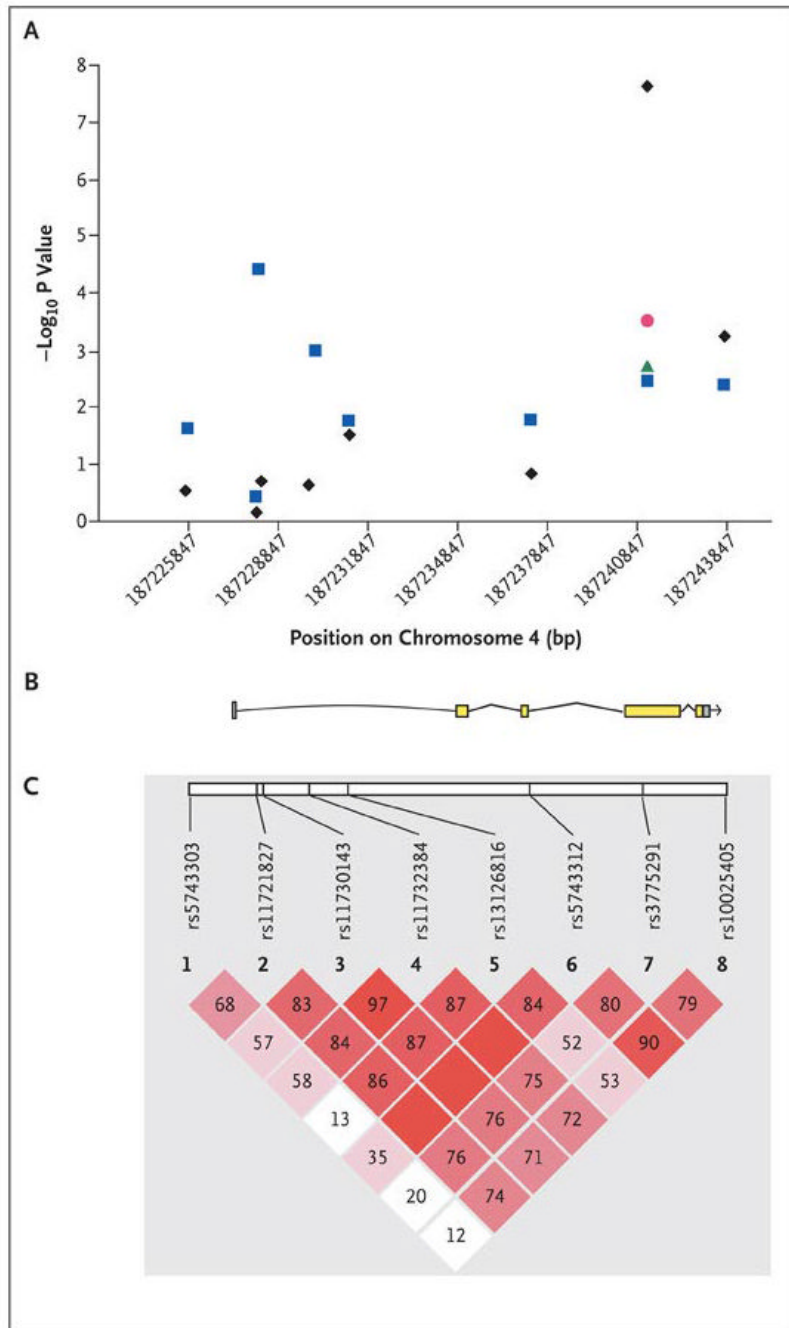
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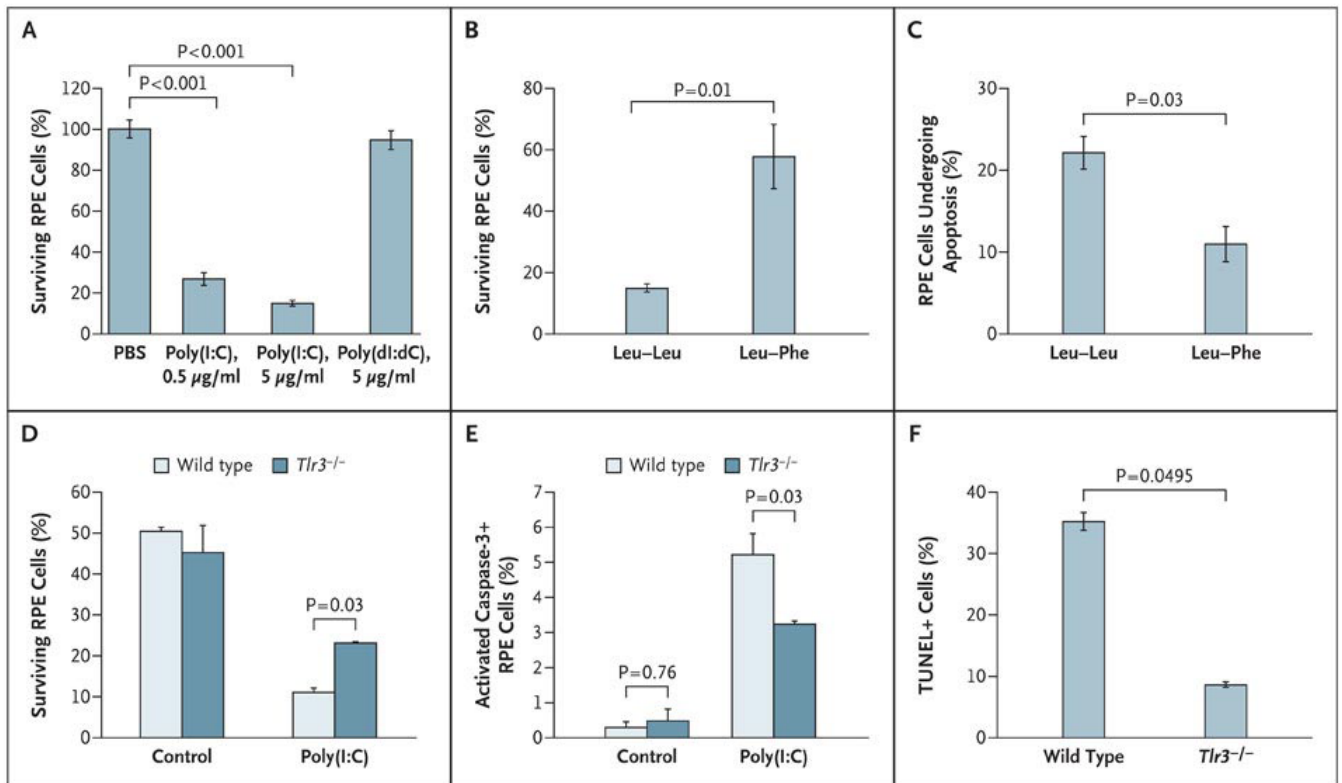
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**Figure 1.** Negative log p-values from *TLR3* rs3775291 for association with GA. A. Negative log p-values (Y-axis) from association analyses for eight SNPs in the *TLR3* region on 4q35 (also see table S3). Blue squares represent  $-\log p$  values of all SNPs around rs3775291 of the Utah case-control series. The red circle represents rs3775291 for the first replication case-control series, while the green triangle represents rs3775291 for the AREDS replication case-control series. The black diamonds represent association for case and control in the three case-control series combined. B. Genomic structure and locations of genes between 187222847 base pairs and 187243847 base pairs (NCBI build 36). C. Pairwise D'Haploview plots for SNPs around *TLR3* using combined GA case and normal control data.



**Figure 2.**

The T allele of rs3775291 (L412F) confers protection from poly (I:C)-induced cytotoxicity. A. The TLR3 ligand poly (I:C) dose-dependently (0.5-5 µg/ml) reduced the survival of primary human retinal pigmented epithelial cells (hRPECs) expressing the homozygous major allele, leading to a protein with an L at amino acid 412 of TLR3 (denoted as LL to indicate the product from each allele) compared to vehicle (phosphate buffered saline) or to poly (dI:dC), which does not activate TLR3. \*  $P < 0.01$ ; n=4. B. poly (I:C) (5 µg/ml) reduced viability of hRPECs expressing LL at amino acid 412 of TLR3 to a significantly greater degree than of hRPECs expressing the FF variant (15±1% vs 58±10%; \*  $P = 0.01$ ; n=4). C. The fraction of poly (I:C) (5 µg/ml) stimulated hRPECs undergoing apoptosis (Annexin+ PI-) was significantly greater poly (I:C) (5 µg/ml) in 412LL cells than in 412 LF cells (22±2% vs 11±2%; \*  $P = 0.03$ ; n=4). D. Intravitreal administration of poly (I:C) (2 µg) induced significantly less death of RPE cells (viability defined as fraction of CD147+CD31-cells in the RPE/choroid layers) in *Tlr3*<sup>-/-</sup> mice than in wild-type mice (48.6±0.4% vs 78.1±2.0%; \*  $P = 0.03$ ; n=4). E. Intravitreal administration of poly (I:C) (2 µg) induced activated caspase-3 expression in a greater fraction of RPE cells in wild-type mice than in *Tlr3*<sup>-/-</sup> mice (5.2±0.6% vs 3.3±0.1%; \*  $P = 0.03$ ; n=4). There was no significant difference in the fraction of activated caspase-3 expressing RPE cells between mice of different genotypes under control (baseline) conditions (\* $P = 0.76$ ; n=4-6). F. Intravitreal administration of poly (I:C) (2 µg) induced TUNEL expression in a greater fraction of RPE and outer retinal cells in wild-type mice than in *Tlr3*<sup>-/-</sup> mice (35.2±1.4% vs 8.6±0.5%; \*  $P = 0.05$ ; n=6). Values are shown as mean ±S.E.M. All  $P$  values were calculated by Mann Whitney U test.

**Table 1**  
 Association between subphenotypes of AMD and *TLR3* variant (rs3775291). Shown are calculations for the T allele of the *TLR3* variant (rs3775291) and the corresponding number and allelic frequency of cases and controls, *p*-value, and odds ratio (OR) assuming an additive model.

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	Control	GA	CNV	Control	GA	Control	CNV	Control
Number	232	441	152	359	271	179	421	184	134	140	171
Protective allele "T" frequency	0.26	0.29	0.30	0.34	0.25	0.29	0.33	0.21	0.31	0.26	0.23
Allelic p-value	0.005	0.06	0.19		$5.43 \times 10^{-04}$	0.18		0.002		0.51	
Genotypic p-value	0.02	0.06	0.11		$9.82 \times 10^{-04}$	0.35		0.004		0.368	
Trend p-value	0.004	0.05	0.17		$5.28 \times 10^{-04}$	0.17		0.001		0.518	
Dominant p-value	0.04	0.02	0.04		0.18	0.20		0.01		0.932	
Recessive p-value	0.01	0.22	0.59		$2.03 \times 10^{-04}$	0.30		0.007		0.173	
Heterozygote OR [95% CI]	0.712 (0.503, 1.00)	0.912 (0.681, 1.220)	1.01 (0.682, 1.500)		0.565 (0.408, 0.782)	0.879 (0.611, 1.27)		0.597 (0.375, 0.950)		1.093 (0.682, 1.3753)	
Homozygote OR [95% CI]	0.437 (0.227, 0.839)	0.536 (0.318, 0.902)	0.446 (0.198, 1.00)		0.527 (0.295, 0.944)	0.608 (0.305, 1.21)		0.198 (0.060, 0.648)		0.570 (0.233, 1.392)	