ORIGINAL ARTICLE

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

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ABSTRACT

BACKGROUND

Age-related macular degeneration is the most common cause of irreversible visual impairment in the developed world. Advanced age-related macular degeneration consists of geographic atrophy and choroidal neovascularization. The specific genetic variants that predispose patients to geographic atrophy are largely unknown.

METHODS

We tested for an association between the functional toll-like receptor 3 gene (*TLR3*) variant rs3775291 (involving the substitution of phenylalanine for leucine at amino acid 412) and age-related macular degeneration in Americans of European descent. We also tested for the effect of TLR3 Leu and Phe variants on the viability of human retinal pigment epithelial cells in vitro and on apoptosis of retinal pigment epithelial cells from wild-type mice and *Tlr3*-knockout ($Tlr3^{-/-}$) mice.

RESULTS

The Phe variant (encoded by the T allele at rs3775291) was associated with protection against geographic atrophy ($P=0.005$). This association was replicated in two independent case–control series of geographic atrophy ($P=5.43\times10^{-4}$ and $P=0.002$). No association was found between *TLR3* variants and choroidal neovascularization. A prototypic TLR3 ligand induced apoptosis in a greater fraction of human retinal pigment epithelial cells with the Leu–Leu genotype than those with the Leu–Phe genotype and in a greater fraction of wild-type mice than $Tlr3^{-/-}$ mice.

CONCLUSIONS

The TLR3 412Phe variant confers protection against geographic atrophy, probably by suppressing the death of retinal pigment epithelial cells. Since double-stranded RNA (dsRNA) can activate TLR3-mediated apoptosis, our results suggest a role of viral dsRNA in the development of geographic atrophy and point to the potential toxic effects of short-interfering-RNA therapies in the eye.

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Age-related macular degeneration 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, or deposits in the macula (central retina). When the drusen are confluent and "soft" in appearance, the affected person is considered to have early-to-intermediate age-related macular degeneration, even though vision is usually unaffected. The greater the number and size of the drusen, the greater the risk of progression to either form of advanced age-related macular degeneration: extensive atrophy of the retinal pigment epithelium and overlying photoreceptors (geographic atrophy, also called advanced "dry" age-related macular degeneration), or choroidal neovascularization (also called "wet" age-related macular degeneration).

Geographic atrophy is characterized by confluent areas of cell death in photoreceptors and retinal pigment epithelium, is bilateral in more than half of patients, and is responsible for 10% of cases of legal blindness from age-related macular degeneration.[1-3] Approximately 900,000 persons in the United States are affected.[4] Despite the prevalence of this disease, its cause remains largely unknown, and there is no approved treatment.

Loci at the genes encoding complement factor H (*CFH*), *LOC387715*–HtrA serine peptidase 1 (*HTRA1*), and complement components 2 and 3 (*C2* and *C3*, respectively) are associated with all phenotypic variants of age-related macular degeneration, including early age-related macular degeneration, geographic atrophy, and choroidal neovascularization.[5-18] However, the genetic basis and molecular mechanisms of geographic atrophy are not known.

There is an emerging consensus that perturbed inflammatory cascades cause susceptibility to age-related macular degeneration.[19,20] Because of the speculation that microbial and viral entities may provoke the pathologic inflammation that drives age-related macular degeneration, and given the previously reported potential association of variants in the toll-like receptor 4 gene (*TLR4*, a bacterial endotoxin receptor)[21] with age-related macular degeneration,[22] we tested for associations between polymorphisms in the toll-like receptor 3 gene (*TLR3*) — which encodes a viral sensor that supports innate immunity and host defense[23] — and the manifestations of age-related macular degeneration: soft, confluent drusen or choroidal neovascularization and geographic atrophy. We then tested for a functional effect of an implicated TLR3 variant in human retinal pigment epithelial cells and in the retinal pigment epithelium of wild-type mice and *Tlr3*-knockout ($Tlr3^{-/-}$) mice.

Methods

Patients

The study was approved by the institutional review boards of the University of Utah, Johns Hopkins University, and Oregon Health & Science University; the institutional review board of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital; and the Age-Related Eye Disease Study (AREDS) Access Committee. All subjects provided written informed consent before participating. For details about recruitment of patients and information on the case–control series, see the Materials and Methods section in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

Genotyping

We genotyped single-nucleotide polymorphisms (SNPs) in *TLR3* and *TLR4* by using the SNaPshot Multiplex System and a 3100XL genetic analyzer (ABI), according to the manufacturer's instructions. The sequences of primers used for each SNP are provided in Table S2 in the Supplementary Appendix; a list of amplification conditions is available on request. Haploview software was also used to test for allelic associations.

In Vitro Viability Assay for Human Retinal Pigment Epithelial Cells

Primary human retinal pigment epithelial cells were isolated from eyes obtained from Advanced Bioscience Resources and were passed through 70-μm and 40-μm nylon mesh filters (Falcon Plastics). After centrifugation at 1500 rpm for 5 minutes, 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 (VWR International) with fetal-calf serum (25% for primary culture and 10% for subsequent cultures) (Omega Scientific), 100 U of penicillin per milliliter, 100 μg of streptomycin per milliliter, and 2 mM L-glutamine (Omega Scientific) at 37°C under 95% air and 5% carbon dioxide. At confluence, cells were detached with the use

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of 0.05% trypsin and 0.02% EDTA (VWR International), collected by centrifugation, and suspended. The purity of the retinal pigment epithelial-cell culture exceeded 95%, as confirmed by immunohistologic positivity for cytokeratin and the absence of CD11b-positive macrophages and of von Willebrand factor–positive endothelial cells.

Retinal pigment epithelial cells with a homozygous genotype (412Leu–Leu) or a heterozygous genotype (412Leu–Phe) of passage 3 or 4 were synchronized for cell cycle by cultivation in high-glucose Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal-calf serum (GIBCO) to achieve confluence and then by overnight serum starvation. They were cultured on 96-well plates at a density of 10,000 cells per well (60 to 70% confluence), followed by stimulation for 24 hours with interferon-α/β (1000 U per milliliter, Pestka Biomedical Laboratories Interferon Source). Cultures were then treated with polyinosine–polycytidylic acid (InvivoGen) or polydeoxyinosine–polydeoxycytidylic acid (Sigma-Aldrich). At 48 hours, cell viability was measured with the use of a bromodeoxyuridine enzyme-linked immunosorbent assay (Chemicon) according to the manufacturer's instructions. Optical densities of the 96-well plates were analyzed on a plate reader (SpectraMax, Molecular Devices) at 450 nm with the use of Softmax Pro software, version 4.3. Cell numbers were compared by means of the Mann–Whitney U test (SPSS software, version 15.0 for Windows). For descriptions of additional in vivo and in vitro manipulations of these cells, see the Materials and Methods section in the Supplementary Appendix.

FUNDUS PHOTOGRAPHY

We performed dilated-fundus examination in wild-type mice and *Tlr3*$^{-/-}$ mice (using 1% tropicamide, Alcon) at baseline and at 2 weeks after intravitreous injection of polyinosine–polycytidylic acid (2 μg). Retinal photographs were obtained with the use of a camera (TRC-50 IX, Topcon) with a digital imaging system (Sony) and were reviewed by two physicians who were unaware of the phenotype of the mice.

MORPHOLOGIC CHARACTERISTICS OF THE RETINA

Eyes were enucleated from wild-type mice and *Tlr3*$^{-/-}$ mice. The retinas were either snap-frozen in Tissue-Tek O.C.T. (Qiagen) and stained with hematoxylin and eosin (Richard Allen Scientific), for basic histologic examination with an inverted light microscope (Nikon), or fixed in 3.5% glutaraldehyde and 4% paraformaldehyde for 2 hours, followed by preparation of ultrathin sections stained with uranyl acetate and lead citrate, for studies with a transmission electron microscope (Biotwin 12, Phillips).

STATISTICAL ANALYSIS

All results of SNP genotyping were screened for deviations from Hardy–Weinberg equilibrium; no SNPs showed significant deviation ($P \geq 0.01$). The chi-square test for allelic trend for an additive model or dominant-allele model across alleles was performed with the use of Programs for Epidemiologists software, version 4.0.[24] All SNP results from the same haplotype block were adjusted for multiple testing according to the false-discovery-rate (FDR) method: adjusted P value = no. of SNPs × [P value ÷ (rank × P value)]. Odds ratios and 95% confidence intervals were calculated by means of conditional logistic-regression analysis, performed with SPSS software, version 13.0. Linkage-disequilibrium structure was examined with the use of Haploview software, version 4.0.[25] Default settings were used, and 95% confidence intervals for the disequilibrium coefficient (D′) were calculated to identify pairwise SNPs in strong linkage disequilibrium.[26]

RESULTS

We tested for associations between various age-related macular degeneration phenotypes and two potentially functional variants in *TLR3* (the promoter SNP rs5743303 and the coding, nonsynonymous SNP rs3775291). Our first case–control series for age-related macular degeneration consisted of Americans of European descent from Utah: 441 patients with choroidal neovascularization, 232 with geographic atrophy, and 152 with soft, confluent drusen, as well as 359 unaffected controls (Table S1 in the Supplementary Appendix). We found no significant association between the SNP rs5743303 in *TLR3* and any age-related macular degeneration phenotype ($P>0.05$ for all comparisons, Table S3 in the Supplementary Appendix). We did find a significant association between the T allele of the nonsynonymous coding SNP rs3775291 and protection against geographic atrophy ($P=0.005$ with the additive allele-dosage model; odds ratio for geographic atrophy in heterozygotes, 0.712; 95% confidence interval [CI], 0.503 to 1.00; odds ratio in homozygotes, 0.437; 95% CI,

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0.227 to 0.839) (Table 1, and Table S3 in the Supplementary Appendix). This SNP was not significantly associated with choroidal neovascularization (P=0.06) or with soft confluent drusen (P=0.19) (Table 1, and Table S4 in the Supplementary Appendix).

To test for replication of the association, we genotyped participants in an independent case–control series of Americans of European descent, comprising 271 patients with geographic atrophy, 179 patients with choroidal neovascularization, and 421 unaffected controls. There was a significant association of rs3775291 with geographic atrophy ($P=5.43\times10^{-4}$) but not with choroidal neovascularization (P=0.18) (Table 1). We found no significant association between rs3775291 and choroidal neovascularization in a Han Chinese case–control series (P=0.51) (Table 1). A second test for replication yielded a significant association between rs3775291 and geographic atrophy in a case–control series consisting of AREDS participants of European descent, comprising 184 patients with geographic atrophy and 134 controls (subjects who were >60 years of age, had fewer than five small drusen, and had no retinal pigment epithelium abnormalities [AREDS category 1]) (P=0.002) (Fig. 1 and Table 1, and Table S4 in the Supplementary Appendix). Combined analysis of the three case–control series of European descent yielded a highly significant association between rs3775291 and geographic atrophy ($P=1.24\times10^{-7}$, adjusted according to the false-discovery-rate method). For all SNPs, the rate of genotyping success exceeded 98% and the accuracy exceeded 99%, as judged by the results of random resequencing of 20% of the samples in all case–control series.

We also tested for associations between the age-related macular degeneration phenotypes and two SNPs in *TLR4* (rs4986790 and rs4986791, which were previously reported to be associated with age-related macular degeneration).[22] We found no significant association of any phenotype with either SNP (P>0.05 for all comparisons) (Table S3 in the Supplementary Appendix).

To clarify the association between geographic atrophy and *TLR3*, we analyzed a linkage-disequilibrium block and haplotypes by genotyping six additional SNPs surrounding rs3775291 in the three case–control series of European descent. We found that rs10025405 was in strong linkage disequilibrium with rs3775291 (D′=0.79) and was also significantly associated with geographic

Table 1. Association between Phenotypes of Age-Related Macular Degeneration and the Toll-like Receptor 3 Gene (*TLR3*) Variant rs3775291.*

Value	Utah Case–Control Series				First Replication Case–Control Series			AREDS Case–Control Series†		Han Chinese Case–Control Series	
	Geographic Atrophy	Choroidal Neovascularization	Soft, Confluent Drusen	Controls	Geographic Atrophy	Choroidal Neovascularization	Controls	Geographic Atrophy	Controls	Choroidal Neovascularization	Controls
No. of participants	232	441	152	359	271	179	421	184	134	140	171
Frequency of protective T allele	0.26	0.29	0.30	0.34	0.25	0.29	0.33	0.21	0.31	0.26	0.23
P value											
Allele	0.005	0.06	0.19		5.43×10^{-4}	0.18		0.002		0.51	
Genotype	0.02	0.06	0.11		9.82×10^{-4}	0.35		0.004		0.37	
Trend	0.004	0.05	0.17		5.28×10^{-4}	0.17		0.001		0.52	
Dominant model	0.04	0.02	0.04		0.18	0.20		0.01		0.93	
Recessive model	0.01	0.22	0.59		2.03×10^{-4}	0.30		0.007		0.17	
Odds ratio for phenotype (95% CI)											
Heterozygote	0.712 (0.503–1.00)	0.912 (0.681–1.22)	1.01 (0.682–1.50)		0.565 (0.408–0.782)	0.879 (0.611–1.27)		0.597 (0.375–0.950)		1.093 (0.682–13753)	
Homozygote	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)	

* The data shown were calculated with the use of an additive model. AREDS denotes the Age-Related Eye Disease Study.
† Twenty-three of the case patients were not from the AREDS cohort but were referred by ophthalmologists.

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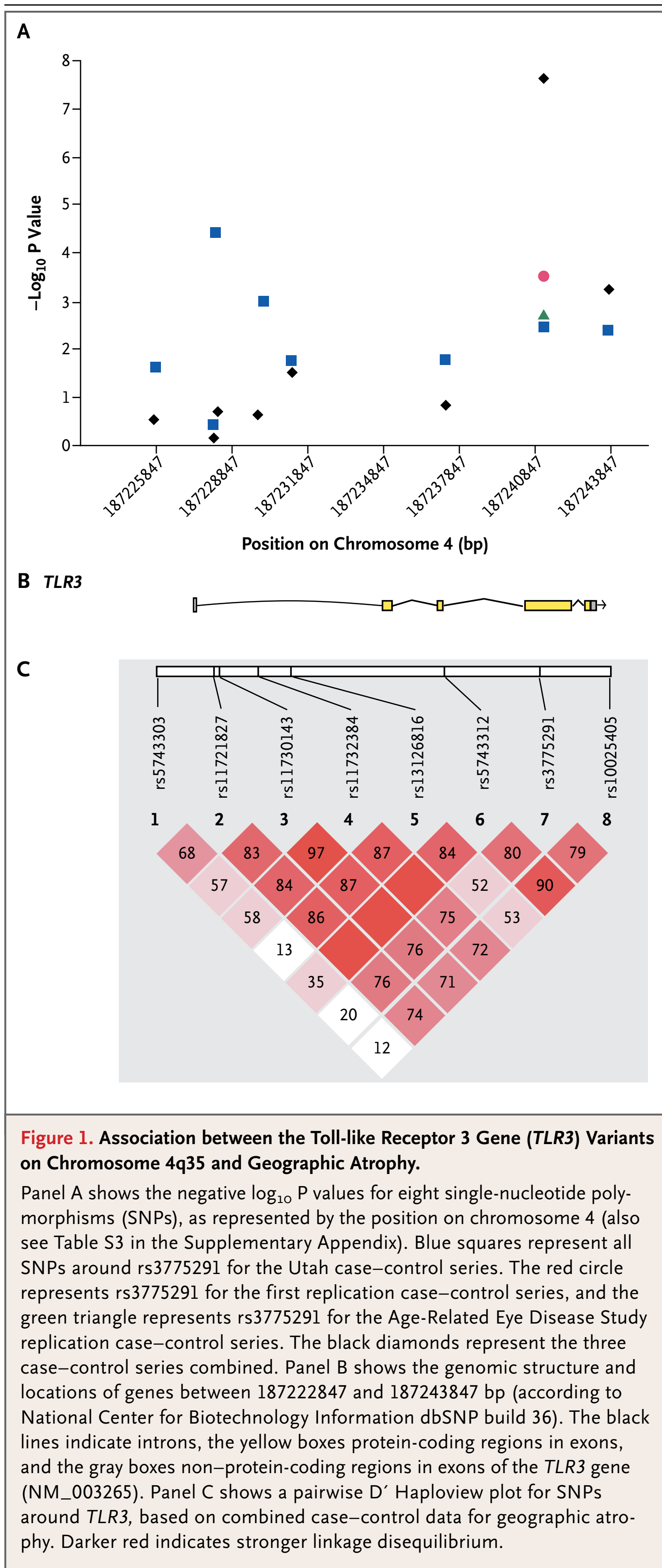

Figure 1. Association between the Toll-like Receptor 3 Gene (*TLR3*) Variants on Chromosome 4q35 and Geographic Atrophy.

Panel A shows the negative $\log_{10}$ P values for eight single-nucleotide polymorphisms (SNPs), as represented by the position on chromosome 4 (also see Table S3 in the Supplementary Appendix). Blue squares represent all SNPs around rs3775291 for the Utah case–control series. The red circle represents rs3775291 for the first replication case–control series, and the green triangle represents rs3775291 for the Age-Related Eye Disease Study replication case–control series. The black diamonds represent the three case–control series combined. Panel B shows the genomic structure and locations of genes between 187222847 and 187243847 bp (according to National Center for Biotechnology Information dbSNP build 36). The black lines indicate introns, the yellow boxes protein-coding regions in exons, and the gray boxes non–protein-coding regions in exons of the *TLR3* gene (NM_003265). Panel C shows a pairwise D′ Haploview plot for SNPs around *TLR3,* based on combined case–control data for geographic atrophy. Darker red indicates stronger linkage disequilibrium.

atrophy (P=0.003) (Table S3 in the Supplementary Appendix). A disease-associated haplotype made up of a cytosine residue at SNP rs3775291 and a guanine residue at SNP rs10025405 (CG) was present in 44.8% of case patients and 37.9% of controls (P=0.001). These SNPs were in strong linkage disequilibrium with each other in both the case and control groups (Fig. 1C). The protective haplotype consisting of a T allele and an A allele was present in 21.9% of case patients and 30.4% of controls ($P=5.92\times10^{-6}$).

Hidden subdivision (stratification) can generate false positive associations in case–control series.[27] We tried to keep this to a minimum by choosing case–control series of Americans of European descent who were from limited, distinct or localized geographic areas in the United States (for the Utah case–control series, Salt Lake City, and for the first replication case–control series, Baltimore, Salt Lake City, and Eugene, OR). In particular, those in our first series were all Utah residents of European descent.[28,29] The slight degree of subdivision therein[30] is unlikely to have caused the strong association that we found. Furthermore, it is improbable that stratification would underlie the associations found in both tests of replication. Finally, the AREDS case–control series was investigated previously for substructure, and no evidence of significant stratification was found.[14,18]

We also tested for an allelic effect of the *TLR3* variant rs3775291. The prototypic TLR3 ligand polyinosine–polycytidylic acid, a synthetic long double-stranded RNA (dsRNA) molecule that activates TLR3,[31] induced cell death in primary human retinal pigment epithelial cells that were homozygous for the 412Leu variant (which is encoded by the C allele at rs3775291) in a dose-dependent fashion (Fig. 2A), a finding that is consistent with the known cytotoxic effect of TLR3 activation. In contrast, polydeoxyinosine–polydeoxycytidylic acid, which does not activate TLR3,[31] did not affect the viability of retinal pigment epithelial cells (Fig. 2A). Cell death induced by polyinosine–polycytidylic acid was reduced by a mean (±SE) of 50±12% (P=0.02) in 412Leu–Phe human retinal pigment epithelial cells as compared with 412Leu–Leu cells (Fig. 2B). In addition, we found that induction of apoptosis (as indicated by annexin V+/propidium iodide– expression) was reduced by 50±9% in 412Leu–Phe cells as compared with 412Leu–Leu cells (P=0.03) (Fig. 2C).

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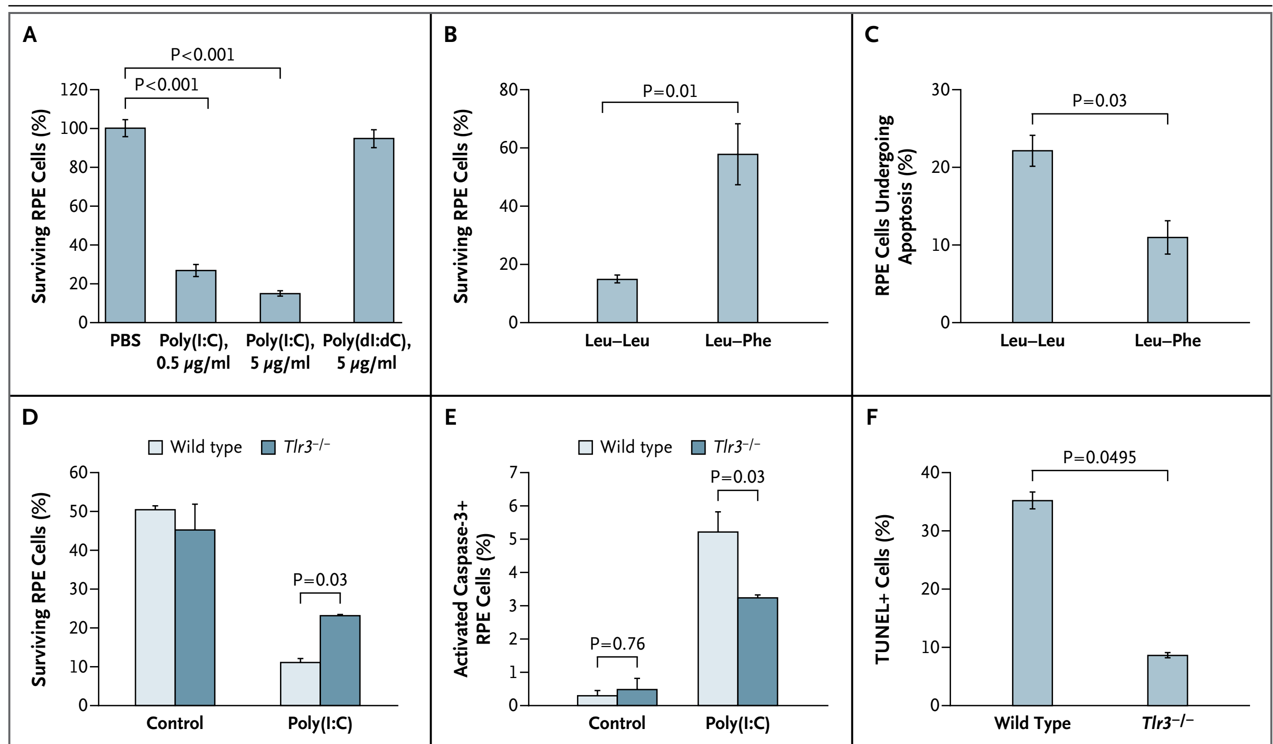

Figure 2. T Allele of rs3775291 (Leu412Phe) and Protection against Cytotoxicity Induced by Polyinosine–Polycytidylic Acid.

As compared with phosphate-buffered saline (PBS) or polydeoxyinosine–polydeoxycytidylic acid [poly(dI:dC)], which do not activate TLR3, the TLR3 ligand polyinosine–polycytidylic acid [poly(I:C)] significantly reduced the survival, in a dose-dependent manner, of primary human retinal pigmented epithelial (RPE) cells homozygous for the risk allele (encoding Leu at amino acid 412) of *TLR3* (Panel A). Poly(I:C) (5 μg/ml) reduced to a significantly greater degree the viability of human RPE cells homozygous for the risk allele than cells heterozygous at this amino acid position (412Leu–Phe cells) (percentage of surviving cells, 15±1% vs. 58±10%) (Panel B). As shown in Panel C, the fraction of human RPE cells stimulated by poly(I:C) (5 μg/ml) that underwent apoptosis (annexin V+/propidium iodide– cells) was significantly greater for 412Leu–Leu cells than for 412Leu–Phe cells (22±2% vs. 11±2%). As compared with control conditions, intravitreous administration of poly(I:C) (2 μg per microliter) induced significantly less apoptosis of RPE cells (with viability defined as the fraction of CD147+, CD31– cells in the RPE and choroid layers) in *Tlr3*$^{-/-}$ mice than in wild-type mice (percent reduction of surviving cells, 48.6±0.4% vs. 78.1±2.0%) (Panel D). Intravitreous administration of poly(I:C) (2 μg per microliter) induced activated caspase-3 expression in a greater fraction of RPE cells in wild-type mice than in *Tlr3*$^{-/-}$ mice (5.2±0.6% vs. 3.3±0.1%) (Panel E). The fraction of activated caspase-3–positive RPE cells did not differ significantly according to the genotype under control (baseline) conditions. As shown in Panel F, intravitreous administration of poly(I:C) (2 μg per microliter) induced terminal deoxynucleotidyl transferase–biotin–uridine triphosphate nicked-end (TUNEL) expression in a greater fraction of RPE and outer retinal cells in wild-type mice than in *Tlr3*$^{-/-}$ mice (35.2±1.4% vs. 8.6±0.5%). All values are means; I bars indicate standard errors. All P values were calculated with the use of the Mann–Whitney U test. The data shown are for analyses of four samples in all panels except Panel E (four to six samples) and Panel F (six samples).

We then tested the effect on TLR3 activation of injecting polyinosine–polycytidylic acid into the vitreous humor of wild-type or *Tlr3*$^{-/-}$ mice. The retinas of control (noninjected) wild-type and *Tlr3*$^{-/-}$ mice appeared normal on dilated-fundus examination (Fig. 1A and 1B in the Supplementary Appendix). Histologic evaluation revealed intact neural retinal layers, retinal pigment epithelium, and choroid (Fig. 1C and 1D in the Supplementary Appendix). Ultrastructural examination revealed orderly photoreceptor arrays and confluent retinal pigment epithelium in both mouse strains (Fig. 1E and 1F in the Supplementary Appendix).

Fundus examination 2 weeks after injection of polyinosine–polycytidylic acid revealed that wild-type mice had features that were consistent with geographic loss of photoreceptors and retinal pigment epithelial cells; such features were not evident in the *Tlr3*$^{-/-}$ mice (Fig. 2A through 2D in the Supplementary Appendix). In support of these observations, flow-cytometric analyses showed that 48 hours after injection of polyinosine–polycytidylic acid, there was a 61±4% greater

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loss of retinal pigment epithelial cells in wild-type mice than in $Tlr3^{-/-}$ mice (P=0.03) (Fig. 2D). Similarly, there was a 60±18% greater induction of apoptosis of retinal pigment epithelial cells (as indicated by caspase-3 activation[32-34]) in wild-type mice than in $Tlr3^{-/-}$ mice after injection of polyinosine–polycytidylic acid (P=0.03) (Fig. 2E). We also identified late apoptotic or necrotic cells through in situ terminal deoxynucleotidyl transferase–biotin–uridine triphosphate nicked-end labeling (TUNEL). Forty-eight hours after injection of polyinosine–polycytidylic acid, TUNEL-positive cells in the retina and retinal pigment epithelium were reduced by 75±1% in $Tlr3^{-/-}$ mice as compared with wild-type mice (P=0.05) (Fig. 2F, and Fig. 2E and 2F in the Supplementary Appendix), a finding that was consistent with the caspase-3 activation data.

DISCUSSION

Our data indicate that the T allele of SNP rs3775291 is associated with protection against geographic atrophy in patients with age-related macular degeneration and that this protective effect is probably mediated by a reduction of dsRNA-induced cell death in retinal pigment epithelial cells in vitro and in vivo. We did not find an association between SNPs in *TLR3* and choroidal neovascularization or early age-related macular degeneration. *TLR3* therefore seems to affect the geographic atrophy phenotype in particular. This association was evident only when controls were limited to persons with no drusen (as in the Utah case–control series) or fewer than five small drusen (as specified in AREDS category 1). Persons with five or more small drusen or changes in the retinal pigment epithelium (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 age-related macular degeneration. We speculate that although *HTRA1* and *CFH* predispose persons to early and late age-related macular degeneration, TLR3 activation (which is enhanced with the 412Leu variant) might promote progression to the geographic atrophy phenotype.

Once definite geographic atrophy appears, it generally progresses contiguously from preexisting areas of involvement. Such a consumptive course is consistent with previously healthy areas of retinal pigment epithelium being affected by adjacent diseased tissue. If activation of the viral dsRNA receptor TLR3 contributes to this progressive process, intercellular transmission of viral intermediates or transcripts that activate TLR3 could mediate the pathogenesis of geographic atrophy in some patients. Alternatively, RNA from adjacent damaged or dying cells could trigger TLR3 activation.[35,36] Given our findings, it is important to search for the existence and nature of dsRNA (viral or otherwise) in eyes affected with geographic atrophy. Cell death and apoptosis in the retina and retinal pigment epithelium in response to polyinosine–polycytidylic acid were reduced but not abolished in $Tlr3^{-/-}$ mice (Fig. 2D, 2E, and 2F), suggesting that other dsRNA receptors[37-39] might have been activated.

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