Identification and characterization of rare toll-like receptor 3 variants in patients with autoimmune Addison's disease

Sigrid Aslaksen a,b,*, Anette B. Wolff a,b, Magnus D. Vigeland c,d, Lars Breivik a,b,e, Ying Sheng d, Bergithe E. Oftedal a,b, Haydee Artaza a,b, Beate Skinningsrud d, Dag E. Undlien c,d, Kaja K. Selmer f,g, Eystein S. Husebye a,b,e, Eirik Bratland a,b

a Department of Clinical Science, University of Bergen, Norway
b KG Jebsen Center for Autoimmune Diseases, University of Bergen, Norway
c Institute of Clinical Medicine, University of Oslo, Norway
d Department of Medical Genetics, Oslo University Hospital, Norway
e Department of Medicine, Haukeland University Hospital, Norway
f Department of Research and Development, Division of Neuroscience, Oslo University Hospital and the University of Oslo, Norway
g National Centre for Epilepsy, Oslo University Hospital, Norway

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ABSTRACT

Autoimmune Addison's disease (AAD) is a classic organ-specific autoimmune disease characterized by an immune-mediated attack on the adrenal cortex. As most autoimmune diseases, AAD is believed to be caused by a combination of genetic and environmental factors, and probably interactions between the two. Persistent viral infections have been suggested to play a triggering role, by invoking inflammation and autoimmune destruction. The inability of clearing infections can be due to aberrations in innate immunity, including mutations in genes involved in the recognition of conserved microbial patterns. In a whole exome sequencing study of anonymized AAD patients, we discovered several rare variants predicted to be damaging in the gene encoding Toll-like receptor 3 (TLR3). TLR3 recognizes double stranded RNAs, and is therefore a major factor in antiviral defense. We here report the occurrence and functional characterization of five rare missense variants in TLR3 of patients with AAD. Most of these variants occurred together with a common TLR3 variant that has been associated with a wide range of immunopathologies. The biological implications of these variants on TLR3 function were evaluated in a cell-based assay, revealing a partial loss-of-function effect of three of the rare variants. In addition, rare mutations in other members of the TLR3-interferon (IFN) signaling pathway were detected in the AAD patients. Together, these findings indicate a potential role for TLR3 and downstream signaling proteins in the pathogenesis in a subset of AAD patients.

1. Introduction

Autoimmune Addison's disease (AAD), is a chronic endocrine disorder characterized by an immunological attack on the hormone-producing cells in the adrenal cortex [1]. The self-antigen 21-hydroxylase (21-OH) seems to be the dominant target of adrenal autoantibodies and autoreactive T cells [1–3]. The underlying causes of AAD are complex, involving both genetic and environmental factors. So far, the best characterized genetic factors are related to antigen presentation and T cell regulation, like certain HLA combinations and common variants in the genes CTLA4 and PTPN22 [4–6]. Most of these genetic risk factors are shared with other organ-specific autoimmune diseases, including type 1 diabetes (T1D), celiac disease and thyroditis, which often co-occur with AAD in patients with autoimmune polyendocrine syndrome type 2 (APS-2). On very rare occasions AAD can also be monogenic, as part of autoimmune polyendocrine syndrome type 1 (APS-1), and caused by mutations in the AIRE gene encoding the autoimmune regulator protein [7]. However, apart from HLA genes and AIRE, the genetic factors discovered so far are only moderately increasing the susceptibility to develop AAD. In particular, the clustering of AAD in some families suggest the existence of highly penetrant AAD susceptibility alleles [8,9], but the specific alleles remain elusive. The involvement of rare variants in the pathogenesis of AAD is in line with AAD being a rare disorder,
and may provide an explanation to the missing heritability problem of AAD. A possible strategy would therefore be to search for rare genetic variants (allele frequency <1% in the general population) affecting the phenotype [10].

Previous studies have demonstrated that errors in innate immunity are associated with autoimmune [11–14]. Recent findings by others and us have suggested that alterations in the innate immune response might be present in AAD as well [15–17]. Persistent viral infections have further been proposed to act as triggers for immunopathological tissue destruction, resulting in autoimmune disease [11,13]. To detect and eliminate infectious agents, the innate immune system uses several recognition systems, including pattern-recognition receptors (PRRs) [18,19]. Some of the best characterized PRRs are the toll-like receptors (TLRs), which are not only expressed in innate immune cells, but also in the parenchyma of different organs such as the endocrine glands [19–21]. In particular, amongst the ten human TLR paralogs, single nucleotide polymorphisms (SNPs) and rare variants in the TLR3 gene have been associated with several immune mediated diseases and different outcomes of infections [11,22–27]. TLR3 is localized intracellularly, within endosomes, and recognizes double-stranded RNA (dsRNA), which is the viral replication intermediate in most viruses and the actual genome in others [28,29]. Binding of dsRNA by TLR3’s ectodomain initiates a signaling cascade resulting in activation of interferon (IFN) regulatory factor (IRF)-3/IRF-7 and nuclear factor kappa B (NF-kB) that initiate a signaling cascade resulting in activation of interferon genes.

In the present work we have identified several rare TLR3 variants in AAD patients, and characterized these with a novel NF-kB-based reporter assay. Some of these variants have not previously been functionally characterized. In addition, peripheral blood mononuclear cells (PBMCs) from patients with TLR3 variants were stimulated with dsRNA to examine any impairment of IFN production.

2. Materials and methods

2.1. Patients and controls

The initial patient cohort consisted of 142 AAD patients that were subjected to whole-exome sequencing (WES). All patients were diagnosed with AAD based on clinical criteria as recommended by the Endocrine Society [32], and were recruited from the National Registry of Organ-specific Autoimmune Diseases (ROAS) at Haukeland University Hospital, Norway. The control group consisted of an in house database containing 308 exomes sequenced in research projects with no link to AAD or related phenotypes. We also compared our results with those obtained from each patient and family member included in the study.

2.2. Ethics

The study was approved by the Regional Ethics Committee, project numbers 2013/1504, 2015/2053 and 2018/1417, and informed consent was obtained from each patient and family member included in the study.

2.3. Blood samples and DNA extraction

EDTA blood samples from patients and controls were used for purification of DNA and for the isolation of PBMCs. Genomic DNA was extracted from whole blood in house, using a QIAamp DNA Blood Mini Kit (Qiagen), or performed by the Nord Trondelag Health study (HUNT) biobank (Levanger, Norway). Blood samples were kept at −80 °C until DNA extraction using the MasterPure™ DNA purification kit version II B1 (Epicentre). Concentration of extracted DNA were normalized to 50 ng/µl. PBMCs were isolated from whole blood using Ficoll density gradient centrifugation, and were cryopreserved at −150 °C.

2.4. Whole-exome sequencing

WES was done on an Illumina HiSeq (Illumina), after exome capture with the Roche Nimblegen SeqCap EZ Exome Library V2 kit (Roche Diagnostics Corporation). The sequences were aligned to the reference human genome (hg19) by using bwa mem (v0.7.12, [34]). The alignments were then refined by the Genome Analysis Toolkit (GATK, Version 2.8, [35,36]) and PCR duplicates were marked by Picard (v1.124, http://broadinstitute.github.io/picard). The variants were called by UnifiedGenotyper by GATK (Version 2.8, [35,36]) and annotated by Annovar (v2017Jul16, [37]). Filtering of the exome variants was done in Filtus [38]. To identify rare variants, we applied filters removing variants with allele frequencies greater than 1% in any of the databases gnomAD, 1000 Genomes Project, or our in house database.

2.5. Validation of WES results and follow-up of patients

Confirmation of the detected TLR3 variants were obtained by Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Family members of selected patients were sequenced using the same sequencing kit. PCR was performed using AmpliTaq Gold™ DNA Polymerase with Gold Buffer and MgCl2 (Applied Biosystems). For one of the patients, no DNA from family members was available. To determine the haplotype of this patient, exon 4 of TLR3 was amplified from genomic DNA and cloned into pCRTM2.1-TOPO vector, using TOPO TA cloning kit (Invitrogen) following the manufacturer’s instructions. One Shot TOP10 chemically competent E. coli cells (Invitrogen) were transformed with cloned vector according to the manufacturer’s recommendations, and plated onto ampicillin containing plates and grown overnight at 37 °C. Plasmid DNA from single colonies were then amplified, purified using ExoProStar 1-Step enzymatic clean up system (GE Healthcare Life Sciences), and sequenced as described above. All primer sequences used for PCR and Sanger sequencing were designed using Primer3web version 4.0.0 and are available upon request.

2.6. In silico predictions

To estimate the degree of evolutionary conservation of the identified TLR3 variants, 18 protein sequence homologs to the human TLR3 protein were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) method [39] and ran by Jalview multiple sequence analysis workbench version 2.10.4b1. The specific areas containing the variants of interest were aligned separately to avoid using gaps in the alignment.

Furthermore, the variants of TLR3 and the TLR3/IFN signaling pathway detected in patients were classified by the in silico variant pathogenicity predictors SIFT [40], PolyPhen2 [41], MutationTaster2 [42], PROVEAN [43] and CADD [44]. In particular, CADD score > 20 was used as a threshold for classifying variants as potentially damaging. The structural implications of the F531I mutant were also analyzed using The PyMOL Molecular Graphics System v. 1.5 [45]. The x-ray crystallography structures of the ectodomains of human and mouse TLR3 [46,47] were used as templates.

2.7. Cell culture

HEK-Dual Null (NF/L8) cells (InvivoGen) were grown in DMEM (1X) + GlutaMax (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma Aldrich) and 100 µg/ml Normocin (InvivoGen) at 37 °C with 5% CO2 in a humidified incubator. These cells are stably transfected with herpes simplex virus type-1 (HSV-1) encephalitis (HSE) [31]. Both dominant and recessive mutations have been described, but incomplete clinical penetrance has been observed for most genetic etiologies of HSE.

Mutations in TLR3 or associated key-members of the TLR3-IFN pathway, may cause sporadic cases of herpes simplex virus type-1 (HSV-1) encephalitis (HSE) [31]. Both dominant and recessive mutations have been described, but incomplete clinical penetrance has been observed for most genetic etiologies of HSE.
with two different reporter genes (Secreted embryonic alkaline phosphatase (SEAP) and the *Lucia* luciferase) inducible by NF-κB and IL-8, respectively, and are triply knocked out for TLR3, TLR5 and the TNF receptor genes.

### 2.8. Plasmids and site-directed mutagenesis

The following SNPs in Human TLR3 cDNA in pCMV6-Entry/TLR3 (Origene) were produced using the QuickChange site-directed mutagenesis kit (Strategene): T59N, G221R F351I, L412F, L742F and R867Q. The mutagenic primers were designed with PrimerX and are available upon request. All SNPs were confirmed by Sanger sequencing.

### 2.9. Reporter gene assay

HEK-Dual Null (NF/L8) cells were harvested and plated in 6-well plates (6 × 10^5 cells/well) for 24 h. The cells were then transiently transfected with a mixture of the Lipofectamine 2000 reagent (Thermo Fisher Scientific) and plasmids pCMV6-Entry/TLR3 (Origene) encoding the full-length wild type (WT) gene, and/or TLR3 mutants. The cells were then incubated for 48 h, before being harvested and plated in a Micro-Amp Optical 96-well Reaction Plate. 100 μg/ml of polyinosinic-polycytidylic acid (poly(I:C)) (Invivogen) were then added to each well. The cells were allowed to incubate for 22–24 h to reach 70–80% confluency. 20 μl of cell supernatant of each well were then mixed with 180 μl of SEAP-inducer compound (Invivogen) according to the manufacturer’s protocol. The SEAP activity was assessed by reading the optical density (OD) at 620–655 nm after 2 h. The units of SEAP activity were normalized to untreated controls within the same transfection, and the activity of the mutant TLR3 proteins was measured as a percentage of activity relative to the WT protein.

### 2.10. Western blots

HEK-Dual Null (NF/L8) cells were transiently transfected with WT or mutant TLR3 as described above. The cells were then harvested and lysed using complete Lysis-M buffer 0 (Sigma Aldrich), the lysates were centrifuged, and the supernatants obtained. The protein concentration of the supernatants was estimated using a DC protein assay kit (Bio-Rad) –655 nm after 2 h. The units of SEAP activity were normalized to untreated controls within the same transfection, and the activity of the mutant TLR3 proteins was measured as a percentage of activity relative to the WT protein.

### 2.11. PBMC stimulations

Cryopreserved PBMCs from patients, family members and controls, were thawed and plated onto 24-well plates (2 × 10^6 cells/well) and stimulated with either poly(I:C) (100 μg/ml) or polyadenylic-polyuridylic acid (poly(A-U)) (10, 50 and 100 μg/ml) for 24 h. The production of cytokines in the supernatants was determined by ELISA kits according to the manufacturer’s instructions. These included IL28A/IFN-λ2, IFN-γ (both from BioLegend) and IFN-β (R&D systems).

### 2.12. Statistical analysis

In order to estimate whether rare and possibly damaging variants in TLR3 were enriched among AAD patients, we compared the proportion of individuals carrying variants with CADD scores above 20 in 20 of our AAD cohort with the appropriate ethnic subpopulations of the Genome Aggregation Database (gnomAD), using a two-sided Fisher’s exact test. Only exomes from controls were used for the analysis. All data from experiments using HEK-Dual Null (NF/L8) cells are expressed as means of at least three independent experiments. All collected data were then tested by an ordinary one-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparisons test. To test for IL28A/IFN-λ2 differences between controls and patients, multiple t-tests were used followed by the Holm-Sidak method to correct for multiple comparisons. For all tests, P < 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism v.8.0.1 software.

### 3. Results

#### 3.1. General findings of WES

Initially, we searched for variants in a candidate list of 194 genes (available upon request) related to 1) APS-1 disease manifestations apart from AAD (e.g. chronic mucocutaneous candidiasis), 2) AIRE, molecular partners of AIRE, or related genes, 3) other autoimmune diseases or syndromes consisting of autoimmune manifestations, and 4) steroids-genesis, vitamin D metabolism, or adrenal disorders. A total number of 135 variants with CADD scores > 20 in 76 different genes was detected in the patients. 94 patients carried at least one variant (ranging from 1 to 6), while 38 patients did not carry any rare variants. The results from the last 10 patients were excluded from further analysis as the sequencing data failed to pass quality control. No significant findings were noted with regard to total number of coding variants, or enrichment of rare variants in any of the list of 194 candidate genes. However, when searching for rare and potentially damaging variants, we identified several interesting hits in TLR3. A complete list of all detected rare variants with CADD

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Autoimmune disease</th>
<th>Age of onset</th>
<th>HLA status</th>
<th>Affected relatives</th>
<th>TLR3 variants (NM_003265.2)</th>
<th>Zygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>M</td>
<td>Isolated AAD</td>
<td>22</td>
<td>High risk</td>
<td>c.176C&gt;A (p.Thr59Ala)</td>
<td>Het</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.1051T&gt;A (p.Phe351Ile)</td>
<td>Hет</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>M</td>
<td>Isolated AAD</td>
<td>24</td>
<td>Neutral</td>
<td>c.1051T&gt;A (p.Phe351Ile)</td>
<td>Hет</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.1234C&gt;T (p.Leu412Phe)</td>
<td>Het</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>F</td>
<td>Isolated AAD</td>
<td>33</td>
<td>High risk</td>
<td>c.1234C&gt;T (p.Leu412Phe)</td>
<td>Het</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.2600G&gt;A (p.Arg867Gln)</td>
<td>Hom</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>M</td>
<td>Isolated AAD</td>
<td>47</td>
<td>Neutral</td>
<td>c.1234C&gt;T (p.Leu412Phe)</td>
<td>Het</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>c.2242C&gt;T (p.Leu742Phe)</td>
<td>Het</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>F</td>
<td>APS-2 + celiac disease</td>
<td>19</td>
<td>High risk</td>
<td>c.661G&gt;A (p.Gly221Arg)</td>
<td>Het</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1** Overview of patients with TLR3 variants. Abbreviations: AAD = autoimmune Addison’s disease; APS = autoimmune polyendocrine syndrome type 2; F = female; Het = heterozygous; Hom = homozygous; M = male; T1D = type 1 diabetes.
scores > 20.0 can be found in Supplementary Table 1.

3.2. Five TLR3 missense variants in five unrelated patients with AAD

WES revealed five rare TLR3 (transcript NM_003265.2) missense variants in five AAD patients: c.176C > A, c.661G > A, c.1051T > A (in two patients), c.2224C > T and c.2600G > A, leading to the predicted amino acid changes p.Thr59Asn, p.Gly221Arg, p.Phe351Ile, p.Leu742Phe and p.Arg867Gln, respectively. In the following these variants will be referred to as T59N, G221R, F351I, L742F and R867Q. None of these variants were detected among the 308 exomes in our in house database, but some were present at low frequencies in the NCGR exome database (summarized in Table 2). Using Sanger sequencing to validate the variants, we discovered that three patients carrying the F351I, L742F and R867Q single nucleotide variants (SNVs), respectively, also carried the more common TLR3 variant c.1234C > T (p.Leu412Phe), from here on referred to as L412F. One patient carried both the T59N and the F351I variant. Therefore, four of the five patients had two variants each. All variants, except G221R, were present at low frequencies in gnomAD, which includes data from ~140,000 unrelated individuals [48]. G221R was absent in gnomAD, but was detected in a single allele in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort as part of the UK10K consortium [49]. The F351I variant detected in two AAD patients, is extremely rare, with only one allele (detected in Finland) deposited in gnomAD. T59N and R867Q have previously been implicated in HSE. R867Q has been described in homozygous combination in a Finnish patient with HSE, and was shown to be a hypomorphic allele resulting in a partial form of autosomal recessive TLR3 deficiency [31], and T59N was detected in a HSE patient along with a rare variant in the TANK-binding kinase 1 (TBK1) gene, but did not confer any impairment on TLR3 function in vitro [50].

3.3. Clinical features of patients with rare TLR3 variants

The five patients carrying TLR3 variants, three males and two females, were all typical AAD patients (Fig. 1 and Table 1): All had autoantibodies against 21-OH and 3/5 had the HLA class II genotype (DR3-DQ2/DR4-DQ8) conferring the highest risk of developing AAD [5]. Patient 1, 2, 3 and 4 (P1–P4) had isolated AAD, while Patient 5 (P5) had APS-2 consisting of AAD, TID and hypothyroidism, as well as celiac disease. P1–P3 and P5 reported a family history of organ-specific autoimmunity, including AAD in a brother of P1. P1–P3 did not report any serious viral infections, but P1 and P2 had records of recurrent upper respiratory tract infections requiring antibiotics. P1 had also been suffering from chronic sinusitis that required surgery. P4 died in 2016 at the age of 87, probably due to cardiac arrest, and no follow-up study could be done for this patient. The patient had been admitted to hospital care with possible L2-L3 fracture and lower respiratory tract and urinary infections shortly prior to his death.

For P1–P3, DNA was available from family members (Fig. 1B). The brother of P1 was diagnosed with AAD and carried T59N, whereas the brother diagnosed with TID harbored two WT alleles. The unaffected father carried F351I. Four additional healthy siblings were also sequenced; three were heterozygous for T59N and one of them carried F351I. The latter, a seemingly healthy brother of P1, tested negative for any serious viral infections, but P1 and P2 had records of recurrent upper respiratory tract infections requiring antibiotics. P1 had also been suffering from chronic sinusitis that required surgery. P4 died in 2016 at the age of 87, probably due to cardiac arrest, and no follow-up study could be done for this patient. The patient had been admitted to hospital care with possible L2-L3 fracture and lower respiratory tract and urinary infections shortly prior to his death.

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predicted to be damaging (CADD score difference was also significant). This difference was significant when comparing rare variants in AAD patients (6/264, 2.27%) and Norwegian exomes of the NCGC database and our in house database (19/2206, 0.86%): Odds ratio, 2.68 – 6.77, two-sided P value ¼ 0.043. The F351I, L742F and R867Q TLR3 proteins combined with L412F showed a significant reduction in receptor activity by 40–45% (P < 0.001 for all combinations).

3.4. In silico studies of the variants

Sequence alignment analysis of the six TLR3 variants indicated that F351I, L412F, L742F and R867 were highly conserved in the 18 species studied, whereas T59 and G221 were less conserved, especially among the fish species (Fig. 2). Thus, based on evolutionary conservation, F351I, L412F, L742F and R867Q would be expected to be more damaging than T59N and G221R. T59N, G221I, F351I, L742F and R867Q were all classified by the in silico pathogenicity prediction tools SIFT, PolyPhen2, MutationTaster, PROVEAN and CADD, through the respective web services (Table 2). A schematic diagram of the TLR3 protein is presented in Fig. 3. T59N, G221R, F351I and L412F are all located in different parts of the ecto-domain of TLR3, which is essential for the recognition and direct binding of dsRNA at acidic pH [51,52]. L742F is located in the linker region, which bridges the transmembrane domain and the TIR domain [31]. Furthermore, R867Q is located in the TIR domain essential for the interaction with TRIF and thus the intracellular signal transduction upon binding of dsRNA [51,52]. All these variants, especially F351I, L412F, L742F and R867Q could potentially have a negative impact on the function of the TLR3 protein.

When taking into account all the alleles containing TLR3 variants predicted to be damaging (CADD score > 20), we found 6/264 (2.27%) AAD patients and 213/26926 (0.79%) controls of Swedish and North Western European origin. This difference was significantly different (Odds ratio, 2.92 (95% CI 1.38–6.37), two-sided P value = 0.02). The difference was also significant when comparing rare variants in TLR3 in AAD patients (6/264, 2.27%) and Norwegian exomes of the NCGC database and our in house database (19/2206, 0.86%): Odds ratio, 2.68 (95% CI 1.12–6.77, two-sided P value = 0.043).

3.5. Effects of variants on TLR3 activity

To determine the functional impact of the six variants, we studied the response to poly(I:C) stimulation in cells transfected with constructs encoding the WT or variant TLR3 proteins. This was achieved using Hek Dual Null cells, stably transfected with a SEAP reporter gene induced by NF-kB. As the endogenous TLR3 gene is specifically knocked out, all expression and activity of the receptor is therefore dependent on transfected TLR3. Stimulation of TLR3, and subsequent activation of NF-kB, is achieved by treating the cells with a TLR3 ligand such as poly(I:C). The units of SEAP activity were normalized to untreated controls within the same transfection, and the activity of the mutant TLR3 proteins was measured as a percentage of activity relative to the WT protein. T59N, G221R and L412F demonstrated approximately the same level of activity as WT TLR3 (Fig. 4A). F351I had reduced activity by ~70% (P < 0.001), L742F had reduced activity by ~ 50% (P < 0.01), and R867Q had reduced reporter activity by ~ 55% (P < 0.01) which increased to ~ 60% together with L412F (P < 0.001) compared to the WT. Thus, consistent with the in silico studies, the F351I, L742F and R867Q TLR3 variants were dysfunctional.

Although TLR3 exists as a monomer in solution, dimerization is required in order for TLR3 to bind dsRNA [53]. To examine whether the WT allele could rescue the activity of dimers in presence of the mutants, we performed co-transfections of equal amounts of both variants and WT (Fig. 4B). The WT protein fully restored the function of TLR3 when expressed together with the variants, except from T59N and L742F, which still showed reduced activity, but without being statistically significant. To mimic the patients’ genotypes, in which all variants were located on opposite alleles, four different co-transfections were performed. These included (1) constructs encoding T59N together with constructs encoding F351I (P1), (2) constructs encoding F351I together with constructs encoding L412F (P2), (3) constructs encoding L412F together with constructs encoding both L412F and R867Q (P3), and (4) constructs encoding L412F together with constructs encoding L742F (P4) (Fig. 4C). The F351I, L742F and R867Q TLR3 proteins combined with L412F showed a significant reduction in receptor activity by 40–45% (P < 0.001 for all combinations).

3.6. Expression of the TLR3 variants

The reduction or complete loss of TLR3 activity caused by the mutations could be an effect of low protein expression/stability, or of an impairment of a specific function. To examine the protein expression of
Fig. 2. Sequence alignment of TLR3 from multiple species and conservation of the identified variants. Alignment of the TLR3 region in humans and the corresponding regions in the other 17 species studied, containing the six identified variants. The conserved amino acids where the variants are located are shaded in purple.
the TLR3 variants, we performed a Western blot using Anti-DDK (FLAG) as the primary antibody (Fig. 4D). The results from three independent experiments demonstrated that the expression levels of all variants (T59N, G221R, F351I, L412F, L742F and R867Q) were comparable with the WT. Thus, the decrease in activity was most likely not an effect of altered expression levels.

3.7. Impaired responses to dsRNA in PBMCs from patients with TLR3 variants

For P1–P3, we were able to collect live PBMCs for functional studies. For stimulation experiments with PBMCs, we chose to use the alternative TLR3 ligand poly(A:U) over poly(I:C). There are several additional receptors for dsRNA, in particular in PBMCs, and poly(A:U) has been shown to be more exclusive to TLR3 than poly(I:C) [54]. PBMCs from P1, P2 and P3, as well as three healthy blood donors with only WT TLR3 alleles, were stimulated with increasing amounts of poly(A:U). The secretions of different IFNs into the cell culture supernatant were then measured using ELISA. For IFNβ and IFNγ the production was either undetectable or no differences were observed between patients and controls (results not shown). The secreted levels of IL28A/IFNλ2 were also relatively low (1–10 pg/ml), but a dose dependent response was observed for the controls but not the patients (Fig. 5). For the highest dose of poly(A:U), 100 μg/ml, the difference between patients and controls was significant (P < 0.01 for fold change and P < 0.05 for absolute levels). Thus, the PBMCs from AAD patients with certain combinations of deleterious TLR3 variants are functionally impaired in response to dsRNA.

3.8. Structural analysis of the F351I TLR3 variant

Since the structure of the ligand-binding ectodomain of TLR3 has been solved by x-ray crystallography, we were able to analyze the structural implications of the rare and predicted deleterious variant,
The F351 residue is part of a phenylalanine spine that helps stabilize the interior of the convex face of the horseshoe-shaped tertiary structure of the TLR3 ectodomain (Fig. 6A, B). This particular structural feature are conserved in many of the TLR proteins, but also in other proteins containing leucine rich repeat (LRR) domains [55, 56]. Changing the phenylalanine at residue 351 to an isoleucine will not only disrupt the spine of consecutive phenylalanines, but perhaps also destabilize the convex shape of the TLR3 ectodomain by steric effects (Fig. 6C).

3.9. Additional rare variants in the TLR3-IFN signaling pathway in AAD patients

As mutations in a number of genes in the TLR3-IFN pathway have been described to result in impaired TLR3 signaling [57], we reexamined our WES data to search for rare variants in these genes in AAD patients. Specifically, we searched for variants in the genes IKBKE, IKBKG, IRF3, TBK1, TICAM1, TRAF3 and UNCO3B, as none of these were among our original candidate list of 194 genes. As with TLR3, we focused on variants with frequencies less than 1% in gnomAD, 1000GP and our in house exome database, that received a CADD score >20. Both novel and previously described variants were discovered in IRF3, IKBKE and TICAM1 (Table 3). Intriguingly, one AAD patient carried the previously described c.854G>A (p.Arg285Gln) mutation in IRF3, from here on referred to as R285Q. This autosomal dominant mutation, leading to haploinsufficiency, has previously been reported in an adolescent with HSE [58]. Furthermore, two AAD patients had a variant in TICAM1, whereby one of the variants (c.479C>T (p.Ser160Phe)) also has been described in an adult patient with HSE [50, 59].

4. Discussion

In the present study, we have identified and characterized several rare variants in TLR3 and associated members of the TLR3-IFN signaling pathway in patients with AAD. The discovery of mutations in these genes indicates that inborn errors of immunity could influence the genetic susceptibility to develop AAD.

Our exome sequencing approach revealed five rare missense variants in TLR3 in five different AAD patients. All variants were absent in healthy Norwegian controls, whereas their allele frequencies in a cohort of Norwegian cancer patients were ranging from 0 to 0.0019. Similar frequencies were found for the TLR3 variants in the gnomAD database, ranging from 0 to 0.0017. To study if the identified variants displayed an impaired response against viral dsRNA in general, we designed and established a cell-based reporter assay using poly(I:C) as the stimulating factor. Of the five rare missense variants discovered, three were found to have significantly impaired function; F351I, an extremely rare variant carried by two of our patients, L742F, which has, together with F351I,

![Figure 5. PBMC responses to stimulation with dsRNA. IL28/IFNλ levels (fold change) were determined by ELISA in cell culture supernatants of PBMCs obtained from P1–P3 (red) and three healthy controls (blue). The cells were either unstimulated (NS) or stimulated with 10, 50 or 100 μg/ml poly(A:U) for 24 h. Broad and narrow horizontal bars show the mean and standard deviation, respectively, for each group per condition. Multiple t-tests were used to test for statistical differences between patients and controls, followed by the Holm-Sidak method to correct for multiple comparisons (**P < 0.01).](image)

![Figure 6. Consequences of the F351I variant on TLR3 ectodomain protein structure. (A) Overview of the TLR3 ectodomain with the phenylalanine spine in purple (F351 in red). The importance of consecutive aromatic amino acids, in particular phenylalanine, in this regard is probably due to the favorable action of π-electrons on top and below the aromatic rings. This excess of electrons gives the face of the ring a small net negative charge, while the hydrogen atoms on the edge of the ring gets a correspondingly small positive charge. The aromatic rings of the consecutive phenylalanines therefore interact with each other with their rings in parallel but slightly offset so that the edge of each ring is interacting with the face of the next. (B) Close-up view of F351 (in red) and two of its neighbouring phenylalanines. (C) Modelling of the F351I variant (in yellow), indicating both a disruption of the phenylalanine spine, but probably also steric hindrance in the interior of the TLR3 ectodomain.](image)
Table 3
Overview of rare variants of the TLR3-IFN signaling cascade (other than TLR3) found in AAD patients.

<table>
<thead>
<tr>
<th>Variant annotation</th>
<th>Frequencies</th>
</tr>
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<tbody>
<tr>
<td>In silico prediction tools</td>
<td></td>
</tr>
<tr>
<td>SIFT PolyPhen2 Mutation</td>
<td></td>
</tr>
<tr>
<td>Chromosomal location</td>
<td></td>
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<tr>
<td>Exon</td>
<td></td>
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<tr>
<td>AAD frequency</td>
<td></td>
</tr>
<tr>
<td>in human database frequency</td>
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</tbody>
</table>

**IRF3**
- c.613G > p.Glu205Lys NC_000019.9
  - Chromosomal location: Exon 6
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000018
  - SIFT: Tolerated
  - PolyPhen2: Possibly Damaging
   - a GnomAD score above 20 indicates that a variant is amongst the top 1% of deleterious variants in the human genome.

**IKBKE**
- p.Arg285Gln NC_000019.9
  - Chromosomal location: Exon 6
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000063
  - SIFT: Deleterious
  - PolyPhen2: Probably Damaging

**TICAM1**
- p.Leu465Phe NC_000019.9
  - Chromosomal location: Exon 2
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000028
  - SIFT: Deleterious
  - PolyPhen2: Probably Pathogenic

**NM_001571.5**
- g.50165574C > T
  - Chromosomal location: Exon 8
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000028
  - SIFT: Neutral
  - PolyPhen2: Deleterious

**NM_014002.3**
- g.206648254G > A
  - Chromosomal location: Exon 1
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000028
  - SIFT: Neutral
  - PolyPhen2: Deleterious

**NM_182919.3**
- g.4817911G > T
  - Chromosomal location: Exon 2
  - AAD frequency: 0.0038 (1/264)
  - In human database frequency: 0.000028
  - SIFT: Neutral
  - PolyPhen2: Deleterious

**Genetic variants with phenotype based on simple regression models are never been functionally characterized before, and R867Q, previously described to be causative (when homozygous) of HSE [31]. The reduced activity was probably not due to low levels of translated protein, as Western blot analyses revealed approximately the same levels of variant proteins as the WT. Instead, we believe that the variants displaying impaired function are disrupting key functional domains of the TLR3 protein. Using the available crystal structures of the TLR3 ectodomain, we were able to model the structural consequence of the F351I variant. It appears that F351 is a crucial part of the phenylalanine spine of the ectodomain, which serves to stabilize the interior of the convex side of the domain. Switching F351 into a non-aromatic amino residue, such as isoleucine, could therefore be detrimental to the stability of the ectodomain. Unfortunately, no structural analysis could be made of L742F and R867Q, as these residues are located in domains of TLR3 that have not yet been structurally solved. It is possible that the exchange of the leucine at position 742 with a phenylalanine may affect the protein’s stability, solubility, oligomeric state and proteolytic resistance [60]. Variants in this region have been reported as possibly pathogenic before, in particular the G743D variant in cis with the R8111 variant was shown to result in autosomal dominant TLR3 deficiency by haplosufficiency in a HSE patient [31]. The exchange of an arginine to an asparagine at position 867 makes the mutant protein partially dysfunctional [31]. The previous reporting of R867Q as a hypomorphic allele is consistent with our functional characterization of the variant.

Interestingly, four of the five patients carrying rare TLR3 variants were compound heterozygous for an additional TLR3 variant, except for the homozygous state of L412F in P3. For patients P2–P4, these genotypes resulted in an impaired signaling activity in our in vitro assay. For three of the patients the additional allele was the common L412F variant which has been associated with a wide range of aberrant immune responses, including an APS-1-like phenotype characterized by cutaneous candidiasis, increased susceptibility to cytomegalovirus infections and autoimmunity, including AAD and hypothyroidism [61]. However, the L412F variant was not found to be associated with AAD in a Polish cohort of 168 AAD patients [62]. In our functional characterization of the TLR3 variants, we could not detect any functional defect of L412F compared to the WT, which is in agreement with some previous reports [63], but in contrast to others [64,65]. On the other hand, L412F did not rescue the TLR3 activity when co-expressed with the hypomorphic alleles F351I, L742F and R867Q. Finally, we demonstrated that PBMCs from P1–P3 had impaired responses to dsRNA, as revealed by a significant loss in production of IL28A/IFNγ.
underpowered for rare variants [69]. Although numerous studies have demonstrated the ability of WES to identify low frequency variants associated with autoimmune disease, it is difficult to conclude any association when only a few individuals carry the variants. In addition, studying the genetic basis of complex diseases such as AAD is challenging because the genotype of a patient does not necessarily clearly correlate with the phenotype; phenocopies may occur, meaning that environmental factors could induce an AAD phenotype in a patient without known disease susceptible variants that matches the phenotype of a patient that does carry such variants. This may explain why none of our patients carrying TLR3 variants, exhibit a unique phenotype compared to the other 127 AAD patients. Hence, several different variants could induce the same phenotype, and multiple of these may be necessary to produce it [8]. On the other hand, even though the genotype does not correlate with the phenotype, we could speculate that it may predict the etiology of the disease. In that case, patients with damaging variants in innate immune genes may share the same triggers, such as viral infections, initiating or perpetuating the immunological attack of adrenal tissue [70].

Another caveat in our study is the presence of the TLR3 variants in unaffected carriers and family members, indicating incomplete penetrance, which is a common phenomenon for mutations associated with AAD [8]. The impact of rare TLR3 variants may therefore seem relative subtle when standing alone, but could confer an increased risk of developing AAD when present together with HLA risk alleles. In this regard, it should be noted that the patients (P1 and P3) sharing TLR3 genotypes with healthy siblings, both carried the HLA class II haplotypes giving the highest risk to develop AAD. However, incomplete penetrance seems to characterize the majority of mutations in the TLR3 pathway causing HSE, especially for the autosomal dominant mutations involving haploinsufficiency [57,58].

Taken together, our findings support that decreased or aberrant antiviral defense is associated with risk for AAD. This observation is consistent with previous research showing that patients with primary adrenal insufficiency have higher intake of antimicrobial agents, especially antifungal and antiviral drugs, and increased hospital admission as a result of infection compared to controls [17]. Moreover, the adrenal cortex has previously been shown to be permissive to a variety of viral, fungal and bacterial agents [71–75]. Decreased resistance or inappropriate responses to viruses can therefore lead to a persistent viral infection in the adrenal glands, resulting in destruction of adrenocortical cells, which may initiate an autoimmune process [1]. AAD may therefore only develop if there is an adrenal infection that effectively triggers the condition. The specific pathogens involved in this process, however, are yet to be discovered. Interestingly, experimental infections with HSV-1 in mice have shown that the adrenal cortex is among the first locations colonized by the virus [74]. Given the previous shown association between TLR3 mutations and HSE, we tested for an increased susceptibility to HSV-1 infections in AAD affected individuals. However, no significant difference in IgM and IgG antibodies against HSV-1 between patients and healthy controls was detected (data not shown).

In conclusion, this study provides the first identification of several rare and damaging variants in the TLR3-IFN pathway in patients with AAD and highlights the significance of studying the interplay between genes and viral infections in immune mediated diseases.

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Appendix A. Supplementary data

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References
