NOVEL ALTERNATIVE TRANSCRIPTS OF TLR8 AND TLR9 REVEAL EVOLUTIONARY PRESSURE TO CONSERVE PROTEIN STRUCTURE

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Novel alternative transcripts of TLR8 and TLR9 reveal evolutionary pressure to conserve protein structure

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ABSTRACT

TLR8 and TLR9 are innate immune receptors belonging to the TLR family that are essential for viral recognition and early immune activation. Their dysfunction is linked to increased susceptibility to infections. TLR8 detects viral single- and double-stranded RNA, while TLR9 recognizes viral DNA molecules with CpG motifs. Six TLR8 alternative transcripts (V1, V2, V5, V6, V7, and V8) and nine TLR9 (V1, A, B, C, D, E, V8, V9, and V10) have been previously described in humans. In the present study, we have performed a comprehensive analysis of TLR8 and TLR9 transcripts in a healthy population and two new TLR8 transcripts (V3 and V4) and four new TLR9 transcripts (V2, V5, V6, and V7) were found. The main mechanisms for the generation of different mRNA variants were the insertion of non-coding regions and the loss of whole or partial exons. These changes result in the loss or insertion of new amino acids but only modify the initial leucine-rich repeat (LRR) region and preserve the rest of the receptor's complete structure. From the results obtained, we can deduce that there seems to be a strong evolutionary drive to maintain TLR8 and TLR9 functionality, unlike other innate immune response receptors.

Keywords: TLR8, TLR9; alternative RNA splicing; isoforms; evolution.

1-Introduction

Host pattern recognition receptors (PRRs) produce the innate immune response due to the recognition of pathogen-associated molecular patterns (PAMPs). This recognition generates an inflammatory response due to the production of type I (IFNα, IFNβ, IFNω, IFNε, or IFNκ) and type III interferons (IFNγ), inflammatory cytokines (IL-1, IL-6, TNFα, and IL-12) and chemokines (CCL3, CXCL8) [1, 2].

Within the different groups of PRRs, the Toll-like receptor (TLR) molecule has three-part in the protein consisting of an N-terminal domain with several tandem LRR (leucine-rich repeat) motifs that mediate ligand (MAMP, DAMP, etc.) recognition, a transmembrane (TM) domain, and a TIR domain that binds to adaptors to mediate downstream signaling [3]..

Ten functional TLRs have been described in humans and four of them (TLR3, TLR7, TLR8, and TLR9) are located intracellularly. These receptors recognize long double-stranded RNA (dsRNA) for TLR3; single-stranded RNA (ssRNA) and short double-stranded RNA for TLR7 and TLR8; and DNA molecules with CpG motifs for TLR9 [4, 5].

Given its involvement in immune responses and neurobiological processes, TLR8 is considered a potential target for the development of immunomodulatory therapies [6-8]. Two splice variants, termed TLR8v1 and TLR8v2, with alternative translation start sites have been described. Their proteins differ by 19 amino acids at the N-terminus. A third variant, termed the G or A/G allele of TLR8 (rs3764880), affects the coding region of TLR8v2, resulting in a truncated form. The A allele was associated with increased susceptibility to pulmonary tuberculosis (TB), whereas its G allele was associated with protection against active TB [9].

On the other hand, TLR9 dysregulation in humans is linked to autoimmunity, metabolic inflammation, and neurodegeneration [10]. Five human TLR9 isoforms (TLR9-A to TLR9-E) have been described. TLR9-A provides the canonical protein sequence, TLR9-C and TLR9-D, which include parts of intron 1 and the 5' untranslated region, with the corresponding addition of 23 or 24 amino acids, and two other truncated variants, TLR9-B and TLR9-E, which lack 57 and 15 amino acids, respectively (Figure 1 and Supplementary Figure 1). TLR9-A transcription has been reported to be three times higher in inflamed synovial membrane than in subcutaneous rheumatoid nodules. In contrast, TLR9-C is transcribed four times more in nodules than in synovial membrane [11].

2-Materials and Methods

In this study, TLR8 and TLR9 mRNA transcripts were studied in a total of 15 healthy, anonymous, unrelated blood donors obtained from the Transfusion Center of the Community of Madrid (Madrid, Spain). Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen Iberia, Madrid, Spain) and reverse transcribed to cDNA using the NZY first-strand cDNA synthesis RT kit (Nzytech, Portugal). cDNA PCR was performed using the NZYTaq II 2x colorless master mix (Nzytech, Portugal) and the corresponding primers (Table 1). PCR conditions were: 5 min at 95°C (initial denaturation), 35 cycles of denaturation (94°C, 45 s), annealing (60°C, 45 s), elongation (72°C, 3 min), and final elongation (72°C, 30 min).The PCR products obtained were direct sequenced in both directions on an ABI PRISM 3730 genetic analyzer (Applied Biosystems, Thermofiser, MA, USA) using BigDye terminator cycle sequencing kit (Applied Biosystems). The Blast© program (National Center for Biotechnology Information, Bethesda, MD, USA) and the MEGA 11 software [12] were used to analyze the sequence of the transcripts obtained.

3-Results and Discussion

Six TLR8 alternative transcripts had been previously described in humans: TLR8-V1: NM016610; -V2: NM138636; -V5: AY358296; -V6: AF246971; -V7: AF245703; -V8: AB445666 and nine TLR9, TLR9-V1: NM017442; -A: AF259262 (renamed as V3); -B: AF259263 (renamed as V4); -C: AF246972 (partial RNA); -D: AF246973 (partial RNA); -E: AF246974 (partial RNA); -V8: AB045180; -V9: AB445673; -V10: AF245704. Here we described two new TLR8 transcripts: -V3: OQ842539; -V4: OQ982474; and four new TLR9 transcripts: -V2: PQ540979, -V5: PV693343 (complete sequence of C); -V6: PV693344 (complete sequence of D); and -V7: PV693345 (complete sequence of E) (Figure 1 and Supplementary Figure 1). In order to give a homogeneus name, TLR9 isoforms A and B were renamed as V3 and V4, respectively; and the partial sequences C, D, and E were sequenced completely and named V5, V6, and V7, respectively. The alternative transcripts obtained in the present work have been detected in the 15 individuals analyzed; it would be interesting to test the presence of the different transcripts in a higher number of individuals.

The TLR8-V1 transcript contains all exons and generates the complete protein (isoform IS1). TLR8-V2 is similar to V1 but lacks the first exon, resulting in a new isoform (IS2) with the loss of 18 amino acids. The TLR8-V3 transcript is similar to V2 with a nucleotide change A → G (rs3764880) at the third position before the end of exon 1 resulting in a new isoform (IS3) in which 21 amino acids are lost. TLR8-V4 is identical to v1, but with a partial insertion of 156 nucleotides from intron 1, a change in the last nucleotide of intron 1 (G → C) (respect to genomic sequence NG012882) and a 2-nucleotide change in exon 1 (AG → GA) generating the IS1 isoform. TLR8-V5 is identical to V2, but lacks the first 31 nucleotides of exon 1, which modifies the reading frame and generates the IS2 isoform. TLR8-V6 is identical to V1 but presents the A →

G change (rs3764880) and the loss of the first 19 nucleotides of exon 1, without a change in the reading frame, generating the IS1 isoform. TLR8-V7 is the same as V2 but with the loss of 39 nucleotides from exon 1, generating the IS2 isoform. Finally, TLR8-V8 is the same as V2 but with the loss of 87 nucleotides from exon 1, giving rise to the IS2 isoform (Figure 1 and Supplementary Figure 1).

TLR9-V1 transcript generates the full-length protein (IS1 isoform). TLR9-V2 is like V1 but with a loss of 45 nucleotides at the beginning of exon 2, resulting in a shift in the reading frame, losing 18 amino acids (isoform IS2). TLR9-V3 (previously named isoform A), has a partial insertion of 518 nucleotides from the 5' untranslated region but gives rise to the IS1 isoform. TLR9-V4 (previously named isoform B), compared to V1, loses all of exon 1 and 123 nucleotides from the beginning of exon 2. This results in a different isoform (IS3) with 57 fewer amino acids. TLR9-V5 (previously named isoform C), compared to V1, lacks the entire exon 1 and 72 nucleotides from the end of intron 1 are inserted, creating a different isoform (IS4) with 23 more amino acids than IS1. TLR9-V6 (previously named isoform D), is similar to V1, but it loses all of exon 1 and has two nucleotide sequences of 23 and 123 nucleotides inserted from the 5' untranslated region. This insertion creates a new isoform (IS5) with 24 more amino acids than IS1. TLR9-V7 (previously named isoform E), similar to V1 but with the insertion of 113 nucleotides from the 5´untranslated region, and the loss of 10 nucleotides from the beginning of exon 1, and 45 nucleotides from the begining of exon 2, losing 15 amino acids and generating a new one isoform (IS6). TLR9-V8 and –V9 are the same as V1 but with the loss of 10 and 116 nucleotides, respectively, from the beginning of exon 1, which does not modify the complete protein (isoform IS1). TLR9-V10 is the same as V1, but with an insertion of 28 nucleotides from the end of the 5' untranslated region, that generates the IS1 isoform.

Different transcripts that give rise to different isoforms directly influence the function of the immune response for both TLR8 and TLR9 [9, 11]. Regarding TLR8, all described transcripts generate only 3 isoforms characterized by the loss of the first 18 and 21 amino acids for the IS2 and IS3 isoforms, respectively, compared to the IS1 isoform. Regarding TLR9, 10 transcripts were detected in the present study that generate 6 isoforms. These isoforms result from the loss of 18 amino acids (IS2), 57 amino acids (IS3), and 15 amino acids (IS6), as well as the insertion of 23 amino acids (IS4) or 24 amino acids (IS5), modifying the initial LRR regions.

4-Conclusions

Since there are more isoforms in TLR9 than in TLR8, it could be postulated that TLR9 has evolved differently from TLR8. However, several different transcripts (V3, V8, V9 and V10) produce the same IS1 isoform. Therefore, the evolutionary pressure for these immune innate receptors appears to be strong in maintaining the full-length isoform and preventing the generation of non-functional isoforms, as described for other innate immune response receptors such as LPG2 or IFI-16 [13, 14].

As a next step, it seems essential to quantify transcripts in cell subpopulations under normal conditions, as well as in the context of infectious diseases, autoimmunity, or chronic inflammation. Since their relevance has already been demonstrated in certain cases, this could represent a first step toward developing therapies that may activate or block different transcripts and/or isoforms to modulate the immune response. Functional experiments to the protein level (isoforms) were not posible due to the lack of specific monoclonal antibodies to the different proteins. However, mRNA quantification “in vivo”, of the different transcripts in infectious diseases are being studied yielding a differential contribution of the different mRNA transcripts to the infectious response (Martinez-Laso et al., unpublished).

5-References

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FIGURE LEGENDS.

Legend to Figure 1. Genetic structure of the different TLR8 AND TLR9 RNA transcripts. TLR8 transcripts described V1: NM016610; V2: NM138636; TLR8 V3: OQ842539; V4: OQ982474; V5:AY358296; V6: AF246971; V7: AF245703; V8: AB445666. TLR9 transcripts described V1: NM017442; TLR9 V2: PQ540979; V3: AF259262 (named A); V4: AF259263 (named B); V5: PV693343, AF246972 (partial V5, named C); V6: PV693344, AF246973 (partial V6, named D); V7: PV693345, AF246974 (partial V7 named E); V8: AB045180; V9: AB445673; V10: AF245704. V: mRNA transcript Variant; IS: isoform; E1: exon 1; E2: exon 2; E3: exon 3; 5UT: 5 untranslated region; I1: intron 1; I2: intron 2; nt: nucleotides. One letter amino acid code is used for protein sequence. Dots: the sequence continue. *indicates the A→G change at the first position (RNA 88) of the last triplet of exon 1: ATG gives rise to the Methionine (M) start codon, while GTG does not, and translation begins later (see Figure 1 and Supplementary Figure 1). ** indicates the start position in the transcript of the corresponding isoform relative to the V1 transcript sequence; ***ga nucleotides 31 and 32 of exon 1 in V4, unlike the other transcripts that have ag (see also Supplementary Figure 1). **** indicates the start position in the transcript of the corresponding isoform relative to the genomic sequence.

The name of transcripts included in grey boxes represents the new transcripts described in this paper. The grey obliquely hatched box indicates a deletion of the corresponding fragment, and the black vertically hatched box indicates an insertion of an intron sequence into the corresponding transcript. Annotation style follows the reference of Yu et al., 2025 (ref. 15).

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CONFLICTS OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Jorge Martinez-Laso: Conceptualization, Methodology, Formal analysis, Writing-Original Draft, Supervision, Project administration. Isabel Cervera: Validation, Formal analysis, Investigation. Maria José Muñoz-Gómez: Formal analysis, Investigation, Software. Clara Sánchez-Menéndez: Formal analysis, Investigation, Software. Ana Salamanca-Soto: Validation, Investigation, Software. Montserrat Torres: Formal analysis, Investigation, Writing-Review and Editing. Mayte Coiras: Conceptualization, Formal analysis, Resources, Writing-Review and Editing, Supervision, Funding acquisition, Project administration.

All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

DATA AVAILABILITY STATEMENT:

The data of the manuscript has been registered in the Genbank with open access.

ETHICAL APPROVAL

Unrelated blood donors obtained from Transfusion Center of the Comunidad de Madrid (Madrid, Spain) with the corresponding informed consent were used for this study.