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TLR-4 and *CD*14 polymorphisms in respiratory syncytial virus associated disease

Beena Puthothu[a], Johannes Forster[a,b], Andrea Heinzmann[a,*] and Marcus Krueger[a]
[a]*University Children's Hospital, University of Freiburg, Mathildenstrasse 1, D-79106 Freiburg, Germany*
[b]*St. Josef's Hospital, Sautier Str. 1, D-79104 Freiburg, Germany*

Abstract. Respiratory syncytial virus (RSV) is the most common viral respiratory pathogen during infancy world wide. It induces innate and adaptive immune response in host cells. The toll like receptor 4 (TLR4)/CD14 complex is particularly important for the initiation of an innate immune response to RSV. Thus we were interested whether an association exists between severe RSV associated diseases and polymorphisms within TLR4 and CD14.
We genotyped the CD14 promotor polymorphism -C159T and the two common TLR4 amino acid variants (D259G, and T359I) in 131 infants with severe RSV associated diseases and 270 controls. Statistical analyses of single polymorphisms made use of the Armitage's trend test, haplotypes were calculated by FAMHAP, FASTEHPLUS and Arlequin.
All polymorphisms were in Hardy Weinberg Equilibrium. We found marginal association between amino acid exchange D259G in TLR4 with RSV infection ($p = 0.0545$). Furthermore, haplotypes analysis of the two TLR4 polymorphisms by three independent programs revealed association of haplotypes with severe RSV infection ($p \leqslant 0.0010$). In contrast, the promotor polymorphism within CD14 was not associated with susceptibility to RSV disease. We conclude from our study, that TLR4 polymorphisms, and particularly the haplotypes, may influence the genetic predisposition to severe RSV infection.

Keywords: Association, CD14, genetics, RSV, TLR4

1. Introduction

The innate immune response, or first-line host defense, mainly serves to prevent the infectious challenge during the period required for the development of adaptive immunity. Toll-like receptors (TLRs) play an pivotal role in the innate immune recognition of microbes, mycobacteria and fungi. They are also important for the interplay between the innate and adaptive immune response [1,2].

The TLRs, an evolutionary conserved transmembrane receptor group, belong to the interleukin-(IL) 1 superfamily that recognizes pathogen associated molecular patterns [3]. The extracellular domain of the TLRs bares leucine rich repeats, its intracellular domain contains Toll/IL-1R motifs, which are conserved in all TLRs. Ten different TLR-members have been identified in the human genome so far.

The conserved nature of the TLRs and their role in innate immunity lead to the hypothesis that other infectious pathogens, such as viruses, may also activate the innate immune response via the toll signaling pathway. In addition, it is currently well accepted that the innate immune response is an important component for the immunity to respiratory syncytial virus (RSV) [4].

RSV, the most common viral respiratory pathogen world wide, is involved in respiratory tract diseases in both infants and children [5]. RSV is a member of the family Paramyxoviridae, existing as an enveloped virus containing a negative sense single stranded RNA genome (ss-RNA virus). The two major surface glycoproteins, which are primarily responsible for the formation of neutralizing antibodies are the fusion protein (F) and the attachment protein (G). The G protein leads to an adaptive immune response, which is

*Corresponding author: Dr. Andrea Heinzmann, University Children's Hospital, University of Freiburg, Mathildenstr. 1, D-79106 Freiburg, Germany. Tel.: +49 761 2706371; Fax: +49 761 2706372; E-mail: andrea.heinzmann@uniklinik-freiburg.de.

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dominated by T-Helper cell (TH)-2 and is associated with disease progression. Murine and human *in vitro* experiments demonstrated that the F protein induces pro-inflammatory cytokines and that this response is dependent on expression of TLR4 and CD14 [6,7].

CD14 is expressed as a glycosylphosphatidylinositol-linked membrane protein on macrophages and monocytes and is essential for most responses to lipopolysaccharide (LPS) [8,9]. Thus it is assumed that CD14 forms a receptor complex with TLR4.

Several studies, including our former studies, showed association of genes involved in the TH2 immune response with severity of RSV bronchiolitis [10]. Other studies demonstrated that polymorphisms in partner genes of the innate immune response, such as surfactant protein A or/and D, are in association with severe RSV bronchiolitis [11–13].

Due to the importance of the TLR4/CD14 complex for the initiation of an innate immune response to RSV, we were interested in the relationship between severity of RSV disease and polymorphisms within *TLR4* and *CD14*. We have chosen for our study two common amino acid exchanges in *TLR4*, D259G (also refered to as Asp299Gly in the literature) and T359I (also refered to as Thr399Ile). The different numbering is due to the signal peptide. In *CD14* we studied the promoter polymorphism C-159T. These polymorphisms represent the best investigated polymorphisms within both genes and have repetitively been described in association with different diseases.

2. Material and methods

2.1. Subjects

2.1.1. Population of children with severe RSV infection

The sample population was recruited at the Centre for Pediatrics and Adolescent Medicine of the Freiburg University Hospital; and in the Community Children's hospital, St Josef Hospital, both hospital are located in the city of Freiburg, Germany. Infants and children less than two years of age were eligible for the study if they had been hospitalised and given a final diagnosis of severe RSV infection between September 1998 and March 2005. RSV infection was confirmed if antigen test and/or RSV-specific PCR were positive [14]. The diagnosis of severe RSV infection was given if children had symptoms of small airway disease, such as wheezing and tachypnoe, and needed either supplementary oxygen and/or gavage feeding and/or intravenous fluid due to weakness or tachypnoe. Children with congenital heart defects, known immune deficiency or chromosomal aberrations were excluded. DNA samples were obtained either from peripheral leucocytes after venipuncture or from buccal smears done with steril cotton sticks. In total 131 children were included in the study.

2.2. Control population

Two hundred and seventy randomly chosen individuals served as controls (aged 19 to 40 years). They originate from the same area in the South-Western part of Germany. No medical history was taken and no medical testing was performed on controls.

2.3. Genotyping

DNA was extracted from peripheral blood leucocytes or buccal smears following standard protocols and column purified (DNA midikit, Qiagen, Hilden, Germany). Genotyping was performed by restriction fragment length polymorphism (RFLP).

In both genes we selected those common polymorphisms, which have been described repetitively in association with different diseases.

CD14-C159T was typed as described previously [15].

TLR4 D259G was typed using the primer pair 5'-GTT TAA ATG TAA TGA AAA CTT GT- 3'and 5'-CAA ACA ATT AAA TAA GTC AAT AAG A- 3'. After PCR (annealing temp: 50°C for 1min and 40cycles) the product was digested with 1 unit of Dpn II (New England Biolabs, Frankfurt, Germany) at 37° over night and the fragments resolved on a 4% NuSieve gel.

TLR4 T359I was typed using the primer pair 5' -CTG TAA ATT TGG ACA GTT TC-3' and 5' -AGA TCT AAA TAC TTT AGCCG- 3'. After PCR (annealing temp: 50°C for 1 min and 35 cycles) the product was digested with 2 units of Msp I (New England Biolabs, Frankfurt, Germany) at 37°C over night and the fragments resolved on a 4% NuSieve gel.

2.4. Sequencing

For each polymorphism three controls (homozygous wildtype, heterozygous and homozygous mutation) were sequenced by the dideoxy chain termination method [16] using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Darmstadt, Germany). All the following studies included these reference individuals.

Table 1
Results of the genotyping

Polymorphism	Position	RSV		Controls	
		Frequency	Genotypes	Frequency	Genotypes
rs4986790	*TLR4:* D259G	0.981	126; 5; 0	0.952	245; 22; 2
rs4986791	*TLR4:* T359I	0.958	120; 11; 0	0.950	244; 23; 2
rs2569190	CD14: −C159T	0.555	41; 59; 27	0.540	79; 124; 58

Listed are the positions of the polymorphisms within the genes, the frequency of the wildtype allele and the genotype distribution (in the following order: homozygous wildtype, heterozygous and homozygous mutation) in both populations.

2.5. Statistical analysis

An association analysis, based on the case-control design, was performed for each single nucleotide polymorphism (SNP) using Armitage's trend test. This test takes into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni [17] as implemented in the program Finetti (Thomas F. Wienker, unpublished data; http://ihg.gsf.de/cgi-bin/hw/hwa1.pl and http://ihg.gsf.de/linkage/download/finetti.zip). The p-value for Hardy Weinberg equilibrium (HWE) was calculated for each polymorphism in both populations using also the program Finetti. In addition to analyses based on single polymorphisms, we performed haplotype frequency estimations by using the programs FASTEHPLUS [18], FAMHAP [19] and Arlequin [20]. The extent of linkage disequilibrium between the polymorphisms has been calculated using the Arlequin program.

FAMHAP and FASTEHPLUS calculate the p-value for the difference of the overall-haplotype distribution between two populations. They implement the Expectation-Maximization (EM) algorithm to estimate the maximum-likelihood haplotype frequencies, and perform a likelihood-ratio test to find out whether they differ between cases and controls. In addition, FAMHAP performs simulations to obtain a p-value which does not rely on asymptotic theory. In order to determine the correct p-value, FAMHAP permutes the disease status of the individuals for each simulated replicate, such that the numbers of cases and controls are the same as in the original sample. In our study we used 100.000 replications. In each replicate the haplotype frequencies are re-estimated by FAMHAP. The p-value equals the fraction of replicates which yield a test statistic greater than or equal to the one obtained for the original sample. Thus this method should result in more accurate p-values than those obtained with asymptotic theory.

2.6. Approval

The study was approved by the Ethical Committee of the University of Freiburg. A statement of informed consent was signed by all participants, or in the case of children, signed by their parents.

3. Results

3.1. Genotyping

Two coding polymorphisms within *TLR4* and one promotor polymorphism within *CD14* were genotyped on 131 children with severe RSV infection and 270 controls. The allelic frequencies of the polymorphisms and the genotype distributions in both populations are shown in Table 1. The amino acid exchanges in TLR4 (D259G and T359I) were very rare in both populations which is in accordance to the allelic frequencies given in the literature.

3.2. Association studies

As listed in Table 2 all polymorphisms were in Hardy Weinberg equilibrium in both populations. Thus genotyping errors or population stratification seem to be unlikely. The p-values for association, obtained by the Armitage's trend test, are also listed in Table 2. Weak association ($p = 0.054$) was observed between severe RSV infection and the amino acid exchange D259G in *TLR4*. The other polymorphisms, i.e. T359I in *TLR4* and – C159T in *CD14* showed no association with the disease.

3.3. Linkage disequilibrium and haplotype analyses

The polymorphisms within *TLR4* were in tight linkage disequilibrium as calculated by Arlequine (data not shown). Table 3 shows the haplotype distribution in both populations. Out of the three existing haplotypes, one was markedly over represented in children with

Table 2
Results of the association analysis

Polymorphism	HWE RSV	HWE controls	p-value for association
rs4986790	1.000	0.116	0.0545
rs4986791	1.000	0.133	0.6193
rs2569190	0.503	0.481	0.7020

The results of the association study is given for each polymorphism under investigation. Listed are the p-values for Hardy Weinberg equilibrium as given by Finetti as well as the p-values for association with RSV infection as calculated by Armitage's trend test.

Table 3
Haplotype distribution in both populations

Haplotype	RSV	Controls
1 1	0.9646	0.9496
1 2	0.0236	0.0018
2 2	0.0118	0.0485

This table shows the frequencies of the *TLR4* haplotypes in both populations. Listed are haplotypes with a frequency of at least 0.01 in either population. The polymorphisms are listed in the following order: rs4986790 and rs4986791. 1 refers to the wildtype allele, 2 refers to the mutation.

severe RSV infection in comparison to controls (frequency of 0.0236 in the RSV population compared to 0.0018 in the control population). The haplotype bearing the wildtype allele for both polymorphisms showed a similar frequency in the RSV and control population.

The haplotype distribution differed significantly between both populations as calculated by three independent programmes: Haplotypes were associated with severe RSV infection by $p = 0.0010$ (FAMHAP), $p = 0.0009$ (FASTEHPLUS) and $p = 0.0010$ (Arlequin).

4. Discussion

The clinical manifestation of RSV infections in infants ranges from no symptoms to severe lower respiratory tract infection, with or without wheezing. It is currently well accepted that individual differences in the immune response explains some of the variation in the severity of RSV bronchiolitis. Thus we focused our study on variants in genes involved in innate immune signaling.

So far only few studies on RSV infection have investigated the heterogeneity of genes involved in immune response in general and in the innate immune response in particular. Löfgren and colleagues found that polymorphisms in surfactant protein A are associated with severe RSV infection [13]. Another study demonstrated a correlation of severe RSV infection with surfactant protein D, which also participates in the innate immune response. In our study, we concentrated on gene variants, which are part of the innate immune response, by investigating two amino acid variants in *TLR4*, and a promoter polymorphism in *CD14*. The importance of TLR4 in the response to LPS was firstly described by Poltorak et al., who observed that LPS-hyporesponsive mice have a point mutation in *TLR4* [21]. In addition Arbour et al. demonstrated the presence of *TLR4* mutations in humans [22].

In this study, we found weak association of the amino acid exchange D259G in *TLR4* with severe RSV infection. The other two investigated polymorphisms showed no association. However, haplotype analyses of the two polymorphisms within *TLR4* suggest an influence of these gene variants on the genetic predisposition to RSV disease in our population. The p-values gained by three different haplotype analysing programs ($p \leqslant 0.001$) lead to the hypothesis that *TLR4* haplotypes might be more important for severe RSV disease than the single polymorphisms.

On the other hand, this result does not necessarily mean that haplotypes of these two polymorphisms confer the demonstrated association between severe RSV infection and *TLR4*. Several other explanations may exist: it might be possible that the polymorphisms are not directly linked with the immune response to RSV. Instead they might induce another independent pathway, which is important for the immune response in general. Besides, it may be possible that these common *TLR4* polymorphisms are in linkage disequilibrium with other polymorphisms or genetic abnormalities in near proximity. However, none of the genes near the *TLR4* region on chromosome 9 have been identified to be relevant to RSV bronchiolitis.

The amino acid variants within *TLR4* are less common in children with severe RSV infection than in controls. Thus it seems that these variants protect against RSV, whereas the wildtype allele enhances the risk to severe infections. There are some studies reporting parallels to this hypothesis with respect to other diseases. For example one study demonstrated that *TLR4* polymorphisms are associated with resistance to Legionnaire's disease – another respiratory tract infection −, whereas individuals with the wildtype alleles have more often infections [23].

There are only two studies that investigated the effect of *TLR4* and *CD14* polymorphisms on severe RSV

infection. In contrast to the results of our study Inoue et al. did not find association of *TLR4* and *CD14* with severe RSV in a small population of Japanese children (poster 1537, World allergy congress in Munich, 2005). Tal et al. described association of the same polymorphisms within *TLR4* with RSV, whereas the CD14 polymorphism showed no association [24]. Interestingly, Tal et al. found the opposite effect of the polymorphisms, i.e. the wildtype allele being more common in controls. There are two main differences between the study population of Tal et al. and our own investigation which might explain the contrary results: Their population was of Jewish origin and significance was only seen with infants less than 6 months, whereas we used a Caucasian population in an age of less than two years.

When the *CD14* promotor polymorphism C-159T was described, it was also found that that children who were homozygous for the wildtype allele had a higher level of soluble CD14 than the children who were homozygous for the mutant allele [25]. Besides, CD14 was already found in association with positive skin prick tests and total IgE levels in different ethnic groups [26,27]. Considering this and the fact that the TLR4/CD14 complex plays an important role in innate immunity, leads to the hypothesis that this polymorphism could be associated with RSV severity. However, we found no association of severe RSV infection with -C159T. In fact, the polymorphism was nearly equally distributed in both populations.

In conclusion, our results indicate a possible association between common *TLR4* amino acid variants and severe RSV disease, and more strongly suggest that certain haplotypes composed of both *TLR4* polymorphisms may contribute to determine an individual's genetic predisposition to develop severe disease after RSV infection.

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References

[1] M.G. Netea, J.W. Van der Meer and B.J. Kullberg, Toll-like receptors as an escape mechanism from the host defense, *Trends Microbiol* **12** (2004), 484–488.

[2] S.A. Vaidya and G. Cheng, Toll-like receptors and innate antiviral responses, *Curr Opin Immunol* **15** (2003), 402–407.

[3] K. Takeda and S. Akira, Toll receptors and pathogen resistance, *Cell Microbiol* **5** (2003), 143–153.

[4] R.A. Tripp, L. Jones and L.J. Anderson, Respiratory syncytial virus G and/or SH glycoproteins modify CC and CXC chemokine mRNA expression in the BALB/c mouse, *J Virol* **74** (2000), 6227–6229.

[5] T. Darville and T. Yamauchi, Respiratory syncytial virus, *Pediatr Rev* **19** (1998), 55–61.

[6] E.A. Kurt-Jones, L. Popova, L. Kwinn, L.M. Haynes, L.P. Jones, R.A. Tripp, E.E. Walsh, M.W. Freeman, D.T. Golenbock, L.J. Anderson and R.W. Finberg, Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus, *Nat Immunol* **1** (2000), 398–401.

[7] A. Gagro, M. Tominac, V. Krsulovic-Hresic, A. Bace, M. Matic, V. Drazenovic, G. Mlinaric-Galinovic, E. Kosor, K. Gotovac, I. Bolanca, S. Batinica and S. Rabatic, Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis, *Clin Exp Immunol* **135** (2004), 267–272.

[8] E. Lien and R.R. Ingalls, Toll-like receptors, *Crit Care Med* **30** (2002), 1–11.

[9] K. Ikejima, N. Enomoto, V. Seabra, A. Ikejima, D.A. Brenner and R.G. Thurman, Pronase destroys the lipopolysaccharide receptor CD14 on Kupffer cells, *Am J Physiol* **276** (1999), 591–598.

[10] B. Puthothu, M. Krueger, M. Bernhardt and A. Heinzmann, *ICAM1 amino acid variant K469E is associated with pediatric bronchial asthma and elevated sICAM levels*, Genes Immun, 2006, Epub ahead of print.

[11] B. Hoebee, E. Rietveld, L. Bont, M. Oosten, H.M. Hodemaekers, N.J. Nagelkerke, H.J. Neijens, J.L. Kimpen and T.G. Kimman, Association of severe respiratory syncytial virus bronchiolitis with interleukin-4 and interleukin-4 receptor alpha polymorphisms, *J Infect Dis* **187** (2003), 2–11.

[12] M. Lahti, J. Lofgren, R. Marttila, M. Renko, T. Klaavuniemi, R. Haataja, M. Ramet and M. Hallman, Surfactant protein D gene polymorphism associated with severe respiratory syncytial virus infection, *Pediatr Res* **51** (2002), 696–699.

[13] J. Lofgren, M. Ramet, M. Renko, R. Marttila and M. Hallman, Association between surfactant protein A gene locus and severe respiratory syncytial virus infection in infants, *J Infect Dis* **185** (2002), 283–289.

[14] J. Forster, G. Ihorst, C.H. Rieger, V. Stephan, H.D. Frank, H. Gurth, R. Berner, A. Rohwedder, H. Werchau, M. Schumacher, T. Tsai and G. Petersen, Prospective population-based study of viral lower respiratory tract infections in children under 3 years of age (the PRI.DE study), *Eur J Pediatr* **163** (2004), 709–716.

[15] A. Heinzmann, H. Dietrich, S.P. Jerkic, T. Kurz and K.A. Deichmann, Promoter polymorphisms of the CD14 gene are not associated with bronchial asthma in Caucasian children, *Eur J Immunogenet* **30** (2003), 345–348.

[16] F. Sanger, S. Nicklen and A.R. Coulson, DNA sequencing with chain-terminating inhibitors, *Biotechnology* **24** (1992), 104–108.

[17] P.D. Sasieni, From genotypes to genes: doubling the sample size, *Biometrics* **53** (1997), 1253–1261.

[18] J.H. Zhao and P.C. Sham, Faster haplotype frequency estimation using unrelated subjects, *Hum Hered* **53** (2002), 36–41.

[19] T. Becker and M. Knapp, Maximum-likelihood estimation of haplotype frequencies in nuclear families, *Genet Epidemiol* **27** (2004), 21–32.

[20] S. Schneider, D. Roessli and L. Excoffier, Arlequin Ver. 2000. A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
[21] A. Poltorak, X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler, Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, *Science* **282** (1998), 2085–2088.
[22] N.C. Arbour, E. Lorenz, B.C. Schutte, J. Zabner, J.N. Kline, M. Jones, K. Frees, J.L. Watt and D.A. Schwartz, TLR4 mutations are associated with endotoxin hyporesponsiveness in humans, *Nat Genet* **25** (2000), 187–191.
[23] T.R. Hawn, A. Verbon, M. Janer, L.P. Zhao, B. Beutler and A. Aderem, Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease, *Proc Natl Acad Sci USA* **102** (2005), 2487–2489.
[24] G. Tal, A. Mandelberg, I. Dalal, K. Cesar, E. Somekh, A. Tal, A. Oron, S. Itskovich, A. Ballin, S. Houri, A. Beigelman, O. Lider, G. Rechavi and N. Amariglio, Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease, *J Infect Dis* **189** (2004), 2057–2063.
[25] M. Baldini, I.C. Lohman, M. Halonen, R.P. Erickson, P.G. Holt and F.D.A. Martinez, Polymorphism in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E, *Am J Respir Cell Mol Biol* **20** (1999), 976–983.
[26] C. Ober, A. Tsalenko, R. Parry and N.J. Cox, A second-generation genomewide screen for asthma-susceptibility alleles in a founder population, *Am J Hum Genet* **67** (2000), 1154–1162.
[27] G.H. Koppelman, N.E. Reijmerink, O. Colin Stine, T.D. Howard, P.A. Whittaker, D.A. Meyers, D.S. Postma and E.R. Bleecker, Association of a promoter polymorphism of the CD14 gene and atopy, *Am J Respir Crit Care Med* **163** (2001), 965–969.