

Association of TNF- α with severe respiratory syncytial virus infection and bronchial asthma

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Tumor necrosis factor (TNF- α) is a proinflammatory cytokine that is important in the innate host defence and thus in the defence of infectious agents. However, in excess it provokes the development of chronic inflammatory diseases. The aim of this study was to test association of TNF with severe RSV bronchiolitis as example of an infectious disease and asthma as representative for a chronic inflammatory condition. The following study populations were genotyped for 4 polymorphisms within TNF- β (rs909253) and TNF- α (rs1799964, rs1799724, rs1800629): 322 asthmatic children, 151 children with severe respiratory syncytial virus (RSV) bronchiolitis and 270 controls. Furthermore, serum TNF- α levels were measured by a FlowCytomix Assay. Asthma showed association with two TNF- α polymorphisms as well as with TNF haplotypes ($p = 0.0050$). In contrast, RSV bronchiolitis was associated with TNF haplotypes ($p < 0.00001$) but not with any single polymorphism. In addition, TNF- α serum levels correlated with rs1799724 ($p = 0.034$). A genetically mediated up-regulation of TNF- α expression might provoke a pronounced inflammation of the airways and thus a more severe course of RSV infection as well as the onset of asthma. It remains to be elucidated whether severe RSV bronchiolitis starts TNF- α upregulation and is one first step in the direction to asthma later in life, or whether both diseases are independent from each other and supported by TNF- α upregulation.

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Bronchiolitis caused by respiratory syncytial virus (RSV) and bronchial asthma are among the most common pediatric lung diseases. Whereas severe RSV bronchiolitis is most pronounced during early infancy – at the age of two, nearly all children had been infected with RSV at least once (1) – the onset of allergic asthma is typically later during childhood. Both diseases are characterized by bronchial hyperreactivity, reversible obstruction of the airways owing to a distinct inflammation of the bronchi. The observed analogy in clinical presentation and underlying pathophysiology led early to the speculation about a common origin of both diseases (2). Furthermore, it has been shown repetitively that severe RSV infection precedes

asthma in many children (3–5). Taken together this might implicate a common genetic background of both diseases as discussed previously. Another possible mechanism might be that the growing lung is severely damaged by an early RSV infection and thus more susceptible to asthma later in life.

Tumor necrosis factors (TNF- α and TNF- β) play key roles during the inflammatory response of either disease. Raised TNF- α levels have been detected in sputum, bronchoalveolar lavage and biopsy samples of asthmatic patients (6) as well as during RSV bronchiolitis (7–9). Inhalation of TNF- α provokes bronchial hyperreactivity in healthy volunteers (6). Indeed, a recent study in a mouse model suggests that TNF- α in concert

with interleukin (IL)-13 play a key role in mediating chronic inflammation of the bronchi after RSV infection, finally resulting in the development of asthma (10). Regarding these important functions in the pathophysiology of RSV-induced bronchiolitis and asthma TNF- α has been proposed as an ideal drug target for the therapy of both diseases (10, 11).

TNF- α and TNF- β have already been extensively investigated in the context of the genetics of asthma. Many studies have demonstrated that TNF polymorphisms, particularly TNF- α promoter polymorphisms are in association with asthma and related phenotypes like raised immunoglobulin E (IgE) levels, bronchial hyper-reactivity and wheezing (12–15). In fact TNF is among the nine ‘top asthma genes’ most often shown in association with asthma in ethnically diverse populations (16). In contrast, only one study investigated the impact of one TNF- α promoter polymorphism on the development of RSV-induced bronchiolitis, but did not find an association (17).

Because of the pivotal function of TNF in both diseases and the possible role of raised TNF- α expression during RSV infection as precursor of asthma we were interested whether one or both diseases were in association with TNF- α and/or TNF- β in the German population. We chose to study the TNF- β polymorphism 1+90C/T (rs909253) and three TNF- α polymorphisms – 1211C/T (rs 1799964), –1037C/T (rs 1799724) and –488A/G (rs 1800629). In addition, we measured serum TNF- α concentrations to investigate whether a correlation exists between genotypes and TNF- α levels.

Materials and methods

Subjects

Population of infants with severe RSV infection. The population was recruited at the Centre for Pediatrics and Adolescent Medicine, Freiburg, Germany and at the Community Children’s Hospital of Freiburg, St. Josefs Hospital. Infants were eligible when hospitalized as a result of RSV infection between September 1998 and March 2005. All included children were less than 2 yr of age at the time of RSV infection. In addition, only Caucasian children were asked to participate. Infection with RSV was confirmed by antigen test and/or RSV-specific polymerase chain reaction (PCR; 18). According to the case definition children had symptoms of bronchiolitis, such as wheezing and tachypnea and needed either supplementary oxygen and/or gavage

feeding and/or intravenous fluids. Children with congenital heart defects, immune deficiency or chromosomal aberrations were excluded. DNA samples were collected either by taking blood or buccal smears with sterile cotton sticks. In total 151 children were included.

Asthmatic population. Three hundred and twenty-two children with asthma (aged 5–18 yr) were recruited from the southwestern part of Germany between July 2000 and January 2005. The patients were also characterized at the Centre for Pediatrics and Adolescent Medicine, Freiburg, Germany, using a standardized clinical protocol. An extended medical history was recorded including occurrence and duration of wheezing symptoms; previous and acute medications; severity of previous asthma attacks; previous allergic rhinitis or conjunctivitis; atopic dermatitis and any family history of allergic diseases.

The diagnosis was based on a clear-cut history of asthmatic symptoms, the use of anti-asthmatic medication and the presence of bronchial hyper-reactivity. Bronchial hyperreactivity was defined as a fall in forced respiratory volume in 1 s (FEV₁) by at least 15% in histamine testing or exercise provocation using standard protocols (19). The exact recruitment procedure has been described in detail previously (20). Again, only Caucasian individuals were included to ensure a common genetic background of the study populations.

Control population. Two hundred and seventy randomly chosen individuals were used as controls (aged 19–40 yr). They originate from the same area in the southwestern part of Germany as both the other populations. No medical history was taken and no medical testing was performed on controls. Thus, they represent a cross-section of the population.

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). The polymorphisms, rs1799964 and rs909253 were typed as described previously (13). The primer pairs 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and 5'-GAG CGT CTG CTG GCT GGG TG-3' for rs1800629 and the primer pairs 5'-GTA TGG GGA CCC CCC CGT AA-3' and 5'-GAC CCG

GAG ACT CAT AAT GC-3' for rs1799724 were used for PCR (the mutated nucleotide is under-scored). The expected product sizes for these polymorphisms were 345 bp and 284 bp, respectively. For genotyping of rs1800629 the PCR product was digested with 1U NcoI (NEB, Frankfurt, Germany) at 37°C over night and the fragments were resolved on a 4% agarose gel. The PCR product for rs1799964 was digested with 1U MaeIII (Roche, Grenzach, Germany) at 55°C for 2 h and the fragments were also resolved on a 4% agarose gel.

Sequencing

For each polymorphism three controls (homozygous wildtype, heterozygous and homozygous mutation) were sequenced by the dideoxy chain termination method (21) using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA). All the following studies included these reference individuals.

Statistical analysis

For each single nucleotide polymorphism (SNP) association analysis, based on the case-control design, was performed by using the Armitage's trend test. This test takes into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni (22), as implemented in the program Finetti (Thomas F. Wienker, unpublished data; <http://ihg.gsf.de/cgi-bin/hw/hwal.pl> and <http://ihg.gsf.de/linkage/download/finetti.zip>). The program Finetti was also used to calculate the Hardy-Weinberg equilibrium (HWE) for each polymorphism in all populations. A significant deviation from HWE could be a hint to population admixture (23). In addition, we performed haplotype frequency estimations with the programs FASTEH-PLUS (24) and FAMHAP (25). The extent of linkage disequilibrium between polymorphisms has been calculated using the Arlequin program.

Measurement of TNF- α levels in sera

TNF- α levels were determined by a commercially available FlowCytomix Assay (Bender Medsystems, Wien, Austria) according to the manufacturer's instructions. In total, sera of 58 asthmatic children were investigated. All children were in stable condition without current symptoms of asthma.

Statistical analysis was performed by using the SPSS 11.0 package (SPSS Inc., Chicago, IL,

USA). The Kruskal-Wallis test was used to determine association between levels of TNF- α and the polymorphisms rs1799964 and rs1799724.

Approval

The collection of blood and the experimental procedures were 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.

Results

Genotyping of TNF- β and TNF- α

The TNF- β polymorphism rs909253 and the three TNF- α polymorphisms rs1799964, rs1799724 and rs1800629 were genotyped on 151 children with severe RSV infection, 270 controls and 322 asthmatic children. The rs number of the SNP and their designation used in previous studies are listed in Table 1. Also the allelic frequencies of the SNP and the p value of the HWE are given. As shown in Table 1 all SNPs were within the HWE ($p > 0.05$) in all three populations, except rs1799724 ($p < 0.01$) in the RSV population and rs1799964 in the asthmatic population ($p = 0.01$). Deviation of HWE could be because of genotyping errors. However, departure from HWE in this study is only seen in one population, respectively (once in the RSV and once in the asthmatic population for different SNPs), whereas the other populations fulfil HWE. Thus a systematic genotyping error seems unlikely. Furthermore, genotyping results were confirmed by sequencing. A second cause for deviation

Table 1. Results of genotyping of the TNF- β and TNF- α polymorphisms on the three populations. The rs number of the single nucleotide polymorphism (SNP) as well as the nomenclature used in previous studies (numbers in brackets) are given. In addition, the frequency for the wild-type allele and the Hardy-Weinberg equilibrium (HWE) as calculated by the Finetti program is listed

Gene	Polymorphism	Controls		RSV		Asthmatic	
		frequency	HWE	frequency	HWE	frequency	HWE
TNF- β	rs909253	0.677	0.18	0.709	0.47	0.661	0.06
TNF- α	1+90 C/T						
	rs1799964	0.827	0.20	0.792	0.75	0.782	0.01
	-1211C/T (-1031)						
	rs1799724	0.853	0.70	0.890	<0.01	0.896	0.08
	-1037 C/T (-857)						
	rs1800629	0.854	0.41	0.868	1.00	0.841	0.90
	-488A/G (-308)						

RSV, respiratory syncytial virus.

from HWE could be population admixture. As all three populations originate from the same geographic region in southwestern Germany this seems unlikely, too. In addition, all three populations have already been genotyped for several other SNPs without seeing departure from HWE. Thus, we assume that the deviation from HWE seen in this study just happened by chance.

Association studies

The p-values for association with severe RSV infection and bronchial asthma obtained by Armitage's trend test are listed in Table 2. Association was observed between bronchial asthma and the following polymorphisms: rs1799964 (also known as -1211C/T or -1031) showed an association of $p = 0.034$; polymorphism rs1799724 (also called -1037 C/T and -857 in previous studies) showed an association with $p = 0.028$. The other two evaluated polymorphisms showed no association with bronchial asthma.

None of the polymorphism was in association with severe RSV infection. Finally, there was no significant difference for any SNP when directly comparing the allelic frequencies of the polymorphisms between the asthmatic population and the RSV population.

Results of haplotype analyses

The haplotypes occurring in the populations are listed in Table 3. The following two haplotypes show the most distinct difference in their frequencies between the three populations. First, the haplotype bearing the wild-type allele for all polymorphisms (i.e. 1111) is over-represented in the RSV population (0.441), whereas is almost evenly distributed between asthmatics and controls (0.326 and 0.341). Second, the haplotype bearing the wild-type allele for all polymorphisms but rs1799724 (i.e. 1121) has a frequency of 0.150 in controls compared with 0.062 in the RSV group and 0.093 in the asthmatic popula-

Table 3. Haplotype distribution in all three investigated populations. Listed are all haplotypes with a frequency of at least 0.01 in either population (1 = wild-type allele and 2 = mutant allele). The polymorphisms are arranged according to Table 1

Haplotype	Controls	RSV population	Asthmatic population
1 1 1 1	0.341	0.441	0.326
1 1 1 2	0.021	0.000	0.030
1 1 2 1	0.143	0.062	0.093
1 2 1 1	0.171	0.157	0.189
1 2 2 1	0.000	0.039	0.088
2 1 1 1	0.200	0.167	0.190
2 1 1 2	0.120	0.125	0.132
2 2 1 1	0.000	0.000	0.024

tion. In addition to the association of single polymorphisms, bronchial asthma is also associated with haplotypes of TNF- α and TNF- β by $p = 0.0050$ (FAMHAP) and $p = 0.0302$ (FASTEHPUS).

Furthermore, haplotypes were even strongly associated with severe RSV infection ($p < 0.00001$ by FAMHAP and $p = 0.0009$ by FASTEHPUS). Finally, the distribution of haplotypes was significantly different between children with RSV infection and asthmatic children as calculated by both programs ($p = 0.0014$ and $p = 0.0004$, respectively).

Association between TNF- α polymorphisms and TNF- α sera level

Because of the association of rs1799964 and rs1799724 with bronchial asthma in our study, we were interested whether serum concentrations of TNF- α correlate with these polymorphisms. Therefore we measured TNF- α serum levels in 58 children. As both polymorphisms are quite rare, we were only able to measure serum levels in individuals being homozygous for the wild-type allele and heterozygous probands.

Regarding rs1799964, we tested 33 sera of children being homozygous for the wild-type allele and 22 sera of children being heterozygous. There was no significant difference in serum concentrations between both groups ($p = 0.813$ using Kruskal-Wallis test).

For rs1799724, we analysed 49 sera of children being homozygous for the wild-type allele and 9 sera of children being heterozygous. Serum samples of children being homozygous for rs1799724 showed a mean concentration of 526.32 pg/ml of TNF- α whereas 135.9 pg/ml was measured in the serum of heterozygous children. Overall, there was a significant difference in serum concentrations between both

Table 2. The p-values for association with severe respiratory syncytial virus (RSV) infection and asthma is listed as given by Armitage's trend test. In addition, the RSV population was directly compared with the asthmatic population (last column)

Polymorphisms	RSV controls (p)	Asthma controls (p)	RSV asthma (p)
rs909253	0.345	0.560	0.122
rs1799964	0.200	0.034	0.691
rs1799724	0.160	0.028	0.820
rs1800629	0.562	0.519	0.279

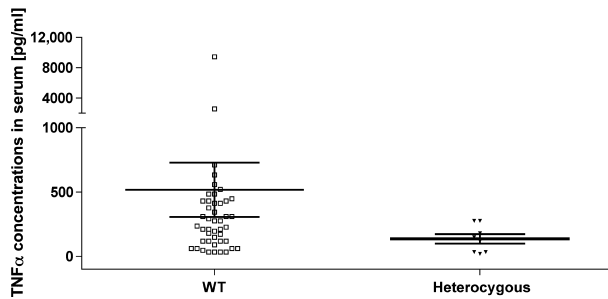


Fig. 1. Results of the measurement of TNF- α levels in sera of asthmatic patients with different genotypes (–1037CC homozygous for the wild-type allele and heterozygous, i.e. –1037CT). The difference between wild-type and heterozygous was significant by $p = 0.034$ (Kruskal–Wallis test).

groups by $p = 0.034$ using Kruskal–Wallis test (see also Fig. 1).

Discussion

TNF- α is a proinflammatory cytokine that provides a rapid form of host defence against various infectious agents. However, in excess TNF- α can provoke the development of chronic inflammatory disorders like inflammatory bowel diseases, arthritis and bronchial asthma. A tight regulation of TNF- α levels might therefore be essential to separate its beneficial from harmful effects. Previously it has been shown that TNF- α expression is influenced by genetic polymorphisms (15). Thus the aim of our study was to test for association of TNF with severe RSV bronchiolitis as example of an infectious disease and with asthma as representative for a chronic inflammatory condition. This might be of special interest as both diseases share many clinical and pathophysiological features and because TNF- α activity has already been proposed as an important link between both diseases (10).

In our study populations the distribution pattern of tested haplotypes of TNF- α and TNF- β is associated with severe RSV infection ($p < 0.00001$). In contrast, none of the four polymorphisms showed association with RSV bronchiolitis in single point analyses. Thus, it is not possible to decide which polymorphism has the key role in the development of the disease. Several reasons can account for the missing association of single polymorphisms despite the significant involvement of TNF haplotypes: the genetic basis of RSV bronchiolitis is highly complex and each SNP confers only a relative small risk factor (26). Most likely our RSV population is too small to detect such an effect. It may also be possible that the association seen

with TNF haplotypes is because of other genes in near proximity and not with TNF- α and TNF- β themselves. The genes are located on chromosome 6p21.3 within the human major histocompatibility cluster (MHC). In fact, two TNF- α polymorphisms used in our study [i.e. –1211T (=rs1799964) and –1037C (=rs1799724)] have been reported to be in linkage disequilibrium with some MHC alleles (27). An involvement of the MHC cluster in RSV bronchiolitis is highly likely as MHC molecules are important in antigen presentation and thus in the recognition of antigens and defence against viruses.

One previous study already investigated the TNF- α polymorphism rs1800629 (also called –488A/G or –308A/G in the literature) in the context of RSV bronchiolitis. An association with RSV bronchiolitis was not found (1). This is in accordance with the results of our study showing a p -value of 0.562 for association of this polymorphism with severe RSV infections.

Second, we found weak association of –1211C/T and –1037C/T with pediatric asthma ($p = 0.034$ and $p = 0.028$, respectively). The association was further supported by haplotype analyses, also demonstrating association with asthma ($p = 0.005$). Our results are in agreement with the majority of studies reporting association of TNF gene cluster with asthma in different populations. Randolph et al. (28) showed an association of TNF haplotype with asthma in family-based association tests. They examine four polymorphisms in total; two TNF- α and two TNF- β polymorphisms. Out of the four, three were the same that we used in our study (TNF- β -NcoI-G, TNF- α 308G and 1078G, respectively rs909253, rs1799724 and rs1800629 in our study).

Li et al. (29) found association of the polymorphism –308 G/A (corresponding to rs1800629) with childhood asthma and wheezing. In addition, they also showed that this SNP is protective against asthma and this protection mainly depends on outdoor ozone levels. This SNP did not show association in single polymorphism analysis in our asthma population, but our haplotype region comprised this SNP. Thus, it is very likely that this SNP is important in our population as well.

Sharma et al. (15) tested seven SNP, including the four SNP that we used in our study. The most informative SNP in this study was TNF- α -863C/A, which is only six nucleotides apart from –1037C/T (=857C/T), that showed the highest association in our study. Therefore, the association which we observed in our analysis of 1037C/T is also relevant to the SNP 863C/A.

One of the important factors for an ideal association study is the choice of suitable controls. Our control population comprises DNA from 270 randomly chosen adults, which have not been characterized for any allergic phenotype. Thus, it is high likely that our control population include some individuals who have asthma themselves. Thereby, the possibility of a type 2 error will be enhanced. On the other hand, there is a reduced risk of obtaining a false positive association in case-control studies by using these controls.

Thus we were interested in a possible correlation between serum levels of TNF- α and both polymorphisms. We measured TNF- α serum concentrations in groups of children being homozygous for the wild-type allele and compared them with children being heterozygous for the particular polymorphism. Unfortunately, as both SNP are quite rare we were not able to recruit enough children being homozygous for the mutant alleles for this investigation.

Statistical analyses showed that children bearing the -1037CT genotype had significantly higher TNF- α serum levels than children homozygous for -1037CC ($p = 0.034$ using Kruskal-Wallis test). One recent functional study investigated the expression of TNF- α in relation to TNF- α promotor polymorphisms by means of a luciferase assay. These authors showed that the transcriptional activity of the -1037T allele was significantly higher than the one of the wild-type allele (30). This is in accordance with our results showing lower TNF- α level in children homozygous for -1037CC in comparison with children heterozygous for -1037CT. To further support these results larger studies including children homozygous for -1037TT are needed.

Finally, we found a significant difference in haplotype distribution between the RSV population and asthmatic children ($p = 0.0004$). This might be confusing at the first sight as it may implicate that both diseases are inversely influenced by TNF haplotypes. However, looking at the allelic frequencies of the single polymorphisms it is evident that the two most important polymorphisms (i.e. -1211C/T and -1037C/T) show about the same difference when comparing both populations with controls (e.g. -1037C/T has a frequency of 0.853 in controls, but 0.890 in RSV population and 0.896 in asthmatics). Thus, both diseases might be indeed influenced by the same SNP but arranged in distinct haplotypes. Another explanation for the significant haplotype difference between both diseases could be the following. As already mentioned before, the

association of RSV with TNF haplotypes might be simulated by an association with the nearby MHC. In contrast asthma might be associated with TNF *per se*.

In conclusion, RSV bronchiolitis and bronchial asthma are both associated with TNF haplotypes. The results hint on a common genetic basis of both diseases concerning TNF. The expression of TNF- α is at least partially regulated by its promotor polymorphisms. The genetically mediated up-regulation of TNF- α expression might support a pronounced inflammation of the airways and thus provoke a more severe course of RSV infection as well as the onset of asthma. It remains to be elucidated whether severe RSV bronchiolitis starts TNF- α up-regulation and is thus one first step in the direction to the development of asthma later in life, or whether both diseases are independent from each other supported by TNF- α upregulation. Nevertheless, this study provides further evidence that TNF- α might represent a reasonable drug target for the therapy of either disease.

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