

MBL2 variants in relation to common childhood infections and atopy-related phenotypes in a large German birth cohort

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Mannose-binding lectin (MBL) is considered an important component of innate immunity. Four functional *MBL2* alterations in codons 52, 54, 57 and in the promoter at position c.1-290 are correlated with significantly lowered MBL serum levels. These variants have been associated with susceptibility to a variety of infectious agents as well as with various immunologic disorders including asthma. To reassess these observations, we analysed the four above mentioned *MBL2* variants in 749 children, who were recruited by the German Multicenter Allergy Study and were prospectively evaluated for common respiratory childhood infections and atopy-related phenotypes from birth up to the age of 11 yr. We performed genotyping by melting curve analysis using fluorescence resonance energy transfer probes and the LightCycler. In contrast to previous studies, we found an association of *MBL2* variants neither with the frequency of common respiratory childhood infections at any age nor with asthma or other atopy-related phenotypes. Our data suggest that MBL deficiency does not represent a pre-disposing factor for respiratory infections or atopic disorders in infants and children.

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Mannose-binding lectin (MBL), also designated as mannan-binding protein, elicits complement activation by binding to mannose- and *N*-acetylglucosamine sugar groups on various microorganisms. Activation of complement through MBL, an acute-phase reactant secreted by the liver, has been established as the third pathway of complement activation, which is referred to as 'lectin pathway' (1). Complement binding to the surface of bacteria promotes phagocytosis by granulocytes and macrophages. The process is independent of acquired immunity and believed to be crucial for host defence against gram-positive bacteria (2). Whenever acquired immunity is not fully established as in infancy or

immunosuppression, MBL mediated complement activation is thought to be of importance (3).

The gene encoding MBL is located on chromosome 10 and is designated as *MBL2*. Four frequent functional *MBL2* variants have been identified (4): three are located in exon 1 at codons 52 (c.154C > T; p.R52C), 54 (c.161g > A; p.G54D) and 57 (c.170g > A; p.G57E) and each of them impairs the formation of the triple helix in the collagenous tail leading to functional deficiency by disrupted polymerization and enhanced enzymatic degradation (5). The wild type allele is designated A and the deficiency alleles together are designated O (specifically

54-B, 57-C, 52-D). All exon 1 alleles reduce the MBL serum level in heterozygotes 5- to 10-fold, whereas in homozygotes or compound heterozygotes MBL is commonly undetectable (6). The c.1-290C>G transversion in the *MBL2* promoter region, designated X/Y, may also influence MBL serum levels (7). An advantage of *MBL* genotyping for disease association studies instead of measuring MBL serum levels is that the genotype is not influenced by disease processes, surgery or infections (8).

The frequencies of the different *MBL2* alleles vary significantly between ethnic groups with the highest occurrence in African populations and the lowest in Greenland Eskimos (7, 9). In Europeans, 33% of subjects are heterozygous and 5% homozygous or compound heterozygous for one of the functional *MBL2* variants and therefore presumably MBL-deficient (10). *MBL2* genotypes have been associated with a multitude of immunologic and infectious disorders [for review see (5, 8)], however, MBL deficiency has presumably most pronounced effects in patients with impaired immunity e.g., immunodeficiency, cystic fibrosis during chemotherapy or after transplantation. Neth et al. genotyped 100 children receiving chemotherapy and observed a significant effect of *MBL2* genotypes on the duration of febrile neutropenic episodes (11). Also, *MBL2* genotypes have been correlated with the severity of pulmonary disease in patients with cystic fibrosis (3). Garred et al. investigated 228 patients with various non-HIV-related immunodeficiencies and found an increased frequency of homozygous *MBL2* deficiency alleles (12). Moreover, an increased risk for common respiratory childhood infections in non-immune comprised individuals with two *MBL2* deficiency alleles has been reported (9, 13–16).

In addition, a potential role of MBL in the development of atopy has been discussed. It was suggested that MBL deficiency causes an imbalance in the normal immune response leading to impaired early antigen recognition (15). In a mouse model, MBL deficiency contributed to the development and maintenance of airway hyper-responsiveness during chronic fungal asthma (17). In humans, *MBL2* genotypes have been associated with non-atopic asthma in males (18).

In the present study, we evaluated in a large, prospectively well-characterized German birth cohort the relevance of *MBL2* variants in common respiratory childhood infections as well as atopy-related traits such as asthma, atopic dermatitis (AD), allergic rhinitis and allergic sensitization.

Methods

Study population

The Multicenter Allergy Study (MAS) has been described previously in detail (18). Briefly, 1314 children born in 1990 had initially been enrolled. The children of this cohort were followed up at the ages of 1, 3, 6, 12, 18 and 24 months, and from there on, at yearly intervals up to the age of 10 yr. The follow-up visits included structured interviews focusing on symptoms of asthma and atopy. Also, all other illnesses were documented in a standardized fashion at each follow-up visit. At 8, 9 and 11 yr of age only questionnaire data were collected. DNA was collected from 888 children. Parents gave informed consent and the study was approved by the local ethics committee.

Assessment of atopic phenotypes and respiratory infections

Atopy-related phenotypes were defined at the age of 7 and 10 yr as follows: Current asthma: one or more wheezing episode(s) during the previous 12 months. Allergic rhinitis: 'a runny, itchy nose and sneezing attacks without having a cold' during the previous 12 months plus specific IgE ≥ 0.7 kU/l against birch and/or grass pollen. Atopic dermatitis (AD; lifetime prevalence): modified criteria of Hanifin and Rajka as described in Ref. (19). Allergic sensitization: specific IgE ≥ 0.7 kU/l against at least one of nine food and/or inhalant allergens. Control subjects for each trait were selected based on no symptoms of asthma ever, no symptoms of allergic rhinitis ever, no diagnosis of AD ever, no specific IgE against any tested allergen (< 0.35 kU/l) at the age of 7 and 10 yr respectively.

Respiratory infections

Whenever parents reported that their child had been ill since the last follow-up, symptoms and diagnoses were noted. The diagnoses were encoded according to the Weidtmann code, an extended German language version of the ICD-9 (international classification of disease, ninth version) for paediatric use (20). The diagnoses of pseudocroup, rhinitis, pharyngitis, tonsillitis, sinusitis, otitis media, bronchitis, pneumonia, influenza-like infection and influenza were listed and the frequency of their occurrence was calculated for the following periods: 0–1, 0–2, 0–5 and 0–11 yr.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard proto-

cols. Primers flanking the promoter region and exon 1 of *MBL2* and fluorescence resonance energy transfer (FRET) probes were synthesized based on the published nucleotide sequence (GenBank#AF080508). Oligonucleotides used for PCR were 5'-CTCTGCCAGGGCCAACGTAG-3' and 5'-CTCCTCATATCCCCAGG-CAG-3'. We performed PCR using 0.75 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 400 μ M dNTPs, 1.5 mM MgCl₂ and 0.1 μ M of each primer in a final volume of 25 μ l. The reaction mix was denatured at 95°C for 12 min followed by 48 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, elongation at 72°C for 90 s and a final extension step for 2 min at 72°C in a thermocycler.

The detection of the mutant alleles was carried out by melting curve analysis with FRET probes in the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The sensor probe for exon 1 was complementary to the mutated c.170g>A (p.G57E) sequence resulting in allele specific melting curves (p.G54D: 60°C, p.R52C: 63°C, wild type: 66.5°C and p.G57E 67.5°C). The sequence of the LC Red 640 labelled sensor probe was 5'-LC 640-TCTTCCTTGGTGCCAT-CACGCCCA-3'; and that of the fluoresceine labelled anchor probe was 5'-CAG-CCCAACACGTACCTGGTTCCCCCT-FL. For identification of c.1-290C>G (X/Y alleles) within the promoter region we used a sensor probe complementary to the mutant allele (c.1-290C; X allele) leading to melting curve temperatures of 54°C (c.1-290G) and 63°C (c.1-290C). The sequences were 5'-TCTCACTGCCACCG-AAAGCAT-FL for the sensor and 5'-LC 705-TTTATAGTCTTCCAGCAGCAACGCCA-3' for the anchor probe. All FRET probes were designed and synthesized by TIB MOLBIOL, Berlin, Germany. We numbered the mutations according to the recommendations of the Nomenclature Working Group for human gene mutations (21). All mutations are numbered at the cDNA level, indicated by a 'c.' before the number. Position +1 corresponds to the A of the ATG translation initiation codon located at nucleotide 66 in the NM_000242.1 *MBL2* mRNA reference sequence.

Statistical analysis

We evaluated all children participating in the longitudinal MAS study. This birth cohort study was primarily designed to examine the incidence and natural course of atopy-related phenotypes. The number of children included in the present analyses was based on study subjects with

available DNA as well as with data on the respiratory and atopic outcome parameters, for each time point during follow-up.

Structural and promoter variants were subdivided into three groups based on their effect on MBL levels according to Ref. (3): *MBL2*-1 = normal level, *MBL2*-2 = medium level, *MBL2*-3 = very low level (Table 1). For these three genotype subgroups we compared the mean number of all as well as of each of 10 specific infectious respiratory diseases, separately for four age groups (0–1 yr, 0–2 yr, 0–5 yr and 0–11 yr). The comparison of the atopy-related phenotypes (asthma, allergic rhinitis, AD) and sensitization to five inhalant allergens (mite, cat, dog, grass, birch) and four food allergens (cow's milk, hen's egg, wheat, soy) were assessed at age 7 and 10 yr. Furthermore, we analysed possible differences between sensitized and non-sensitized asthmatic children at these two age groups. For comparison of categorical data we used Pearson's Chi-squared test, for continuous non-parametric data analysis we used the Mann-Whitney *U*-test. Statistical significance was defined by a two-sided alpha-level of 0.05. All data were analysed with SPSS, version 13.0 (SPSS, Munich, Germany).

Results

All four variants were successfully genotyped in 749 children. The genotype distribution within the MAS cohort is shown in Table 2. The allele frequencies for the *MBL2* exon 1 alterations were 0.14 for allele B (p.G54D), 0.01 for allele C (p.G57E) and 0.07 for allele D (p.R52C). The allele frequency of the X promoter allele (c.1-290C) was 0.23. The distribution of all variants was in the Hardy-Weinberg equilibrium.

Common respiratory childhood infections

We detected no significant differences with regard to the overall frequency of infectious

Table 1. *MBL2* genotypes based on expected mannose-binding lectin (MBL) serum levels

Genotype subgroups	Serum level	Exon 1	Promoter
MBL-1	Normal	A/A	Y/Y
		A/A	Y/X
MBL-2	Medium-low	A/O (A/D, A/B, A/C)	Y/Y
		A/A	X/X
MBL-3	Very low/(undetectable)	O/O	Y/Y
		A/O	X/Y

A, exon 1 wild type allele; O, exon 1 deficiency allele (B, p.G54D [G>A]; C, p.G57E [G>C]; D, p.R52C [C>T]); Y, c.1-290G (wild type); X, c.1-290C.

Table 2. *MBL2* genotype distributions in the MAS cohort

Genotype		Genotype frequency
Exon 1	n = 816	
A/A	501	0.61
A/O	269	0.33
A/B (WT/G54D)	181	0.22
A/C (WT/G57E)	16	0.02
A/D (WT/R52C)	72	0.09
O/O	46	0.06
B/B (G54D/G54D)	21	0.03
C/C (G57E/G57E)	0	0.0
D/D (R52C/R52C)	6	0.01
B/C, B/D or C/D	19	0.02
Promoter	n = 805	
Y/Y (-290GG)	474	0.59
Y/X (-290GC)	286	0.35
X/X (-290CC)	45	0.06
Genotype subgroups	n = 749	
<i>MBL2</i> -1	423	0.57
<i>MBL2</i> -2	195	0.26
<i>MBL2</i> -3	131	0.17

episodes. Also, within each of the four age groups, the number of episodes of specific diseases (pseudo-croup, rhinitis, pharyngitis, tonsillitis, sinusitis, otitis media, bronchitis, pneumonia, influenza) did not differ significantly between genotype groups (Table 3; not all data shown).

Atopy-related phenotypes

Genotype distributions in individuals with asthma, allergic rhinitis, AD and allergic sensitization

vs. respective controls did not differ significantly, neither at the age of 7 yr nor at the age of 10 yr (Table 4; data for the 10 yr follow-up not shown). To evaluate if sensitized (to at least one inhalant allergen) asthmatic children differed from non-sensitized asthmatics, we performed subgroup analyses. There were only 22 non-sensitized asthmatic children at 7 yr; and only nine at 10 yr. No significant differences in genotype distributions were found between these two subgroups of asthmatic children at either 7 or 10 yr of age. Having one or two parent (s) with asthma, allergic rhinitis and/or atopic eczema, the strongest risk factor for allergies and asthma, was also not associated with the genotype distribution ($p = 0.225$).

Discussion

We analysed in a large German birth cohort the possible association of functional *MBL2* variants and the life-time incidence of common respiratory childhood infections or atopy-related phenotypes such as asthma, allergic rhinitis, AD and allergic sensitization in children. The *MBL2* allele frequencies in our cohort were similar to those reported in other Caucasian populations previously (3, 10, 16).

Infectious diseases

In our study, the prevalence of *MBL2* variants in children with common respiratory childhood

Table 3. Mean number $\pm$ s.d. of episodes for any and for selected infectious respiratory diseases in *MBL2* genotype groups during the first and the first 11 yr of life

Age in years	Number of children (n)		Episodes of any infectious respiratory illness; mean $\pm$ (s.d.)		Episodes of rhinitis; mean $\pm$ (s.d.)		Episodes of tonsillitis; mean $\pm$ (s.d.)		Episodes of otitis; mean $\pm$ (s.d.)	
	0–1	0–11	0–1	0–11	0–1	0–11	0–1	0–11	0–1	0–11
<i>MBL2</i> -1	423	242	3.1 (2.1)	19.5 (8.1)	2.4 (1.7)	14.1 (5.9)	0.04 (0.2)	0.9 (1.6)	0.3 (0.7)	2.0 (2.7)
<i>MBL2</i> -2	195	112	3.5 (2.3)	19.7 (8.1)	2.5 (1.7)	13.6 (5.5)	0.04 (0.2)	0.9 (1.6)	0.3 (0.9)	2.2 (2.8)
<i>MBL2</i> -3	131	75	3.2 (2.2)	19.9 (8.0)	2.5 (1.9)	13.2 (6.3)	0.04 (0.2)	1.1 (1.9)	0.3 (0.7)	2.2 (2.8)

Table 4. *MBL* genotype distribution in the MAS cohort in respect of allergic disease

MBL group	Asthma*		Allergic rhinitis*		Sensitization*		Atopic dermatitis*	
	No	Yes	No	Yes	No	Yes	No	Yes
Total	681	83	628	136	422	207	518	286
<i>MBL2</i> -1	383 (56.2)	46 (55.4)	352 (56.1)	77 (56.6)	236 (55.9)	114 (55.1)	294 (56.8)	158 (55.2)
<i>MBL2</i> -2	181 (26.6)	20 (24.1)	170 (27.1)	31 (22.8)	110 (26.1)	56 (27.1)	135 (26.1)	76 (26.6)
<i>MBL2</i> -3	117 (17.2)	17 (20.5)	106 (16.9)	28 (20.6)	76 (18.0)	37 (17.9)	89 (17.2)	52 (18.2)
	$p = 0.725$		$p = 0.436$		$p = 0.965$		$p = 0.906$	

*Phenotypes at the age of 7.

Values given in parentheses are percentages.

infections was not increased at any age. Thus, we found no evidence that *MBL2* deficiency alleles render non-immune comprised infants more susceptible to common infections.

Although our study is the largest longitudinal prospective population-based study of *MBL2* variations in common respiratory childhood infections over a period from birth up to an age of 11 yr, we cannot exclude an effect of *MBL2* genotypes on severe bacterial infections due to a limited number of affected children.

In contrast to our findings, an association of *MBL2* genotypes and respiratory childhood infections was shown by Summerfield et al. in a retrospective, cross-sectional study of 617 children of diverse ethnicity attending a paediatric department in London (14). Children with infectious diseases were compared with children that attended the hospital for other reasons. As hospital attendance or admission for infectious disease was required, the children within this study were most likely more severely affected than children of our population based study (e.g. 11% of meningococcaemia within the study by Summerfield et al.). The incidence of severe respiratory illness in our study population is too small to assess the effect of *MBL2* genotypes on severe bacterial infections.

Koch et al. presented a prospective study on 252 children (including 204 Greenlandic Eskimos) up to the age of 2 yr. An increased risk for acute respiratory tract infections in carriers of *MBL2* deficiency alleles, most significant in the group aged 6–17 months, was described. All acute respiratory illnesses were subsumed, including predominantly viral (rhinitis) and bacterial (e.g., pneumonia) infections. However, the authors did not present data on life time incidence of infectious episodes for children within the *MBL2* genotype groups. Furthermore, the allele frequencies in the Greenlandic Eskimo children differed significantly from Caucasian children within the study population with a more frequent occurrence of MBL deficiency in Caucasians ($p = 0.03$). Thus, population admixture may, at least partly, explain the findings.

The following limitation of our study has to be considered: children of the MAS cohort were evaluated at regular follow-up visits (see above). Interviews and questionnaires included questions about all diseases that occurred since the previous visit. In addition, during the first year of life parents kept a diary and noted all infectious episodes. Therefore, infections were mostly defined and classified based on parents reports and not always confirmed by a physician.

Atopy-related traits

We did not observe an association of *MBL2* genotypes with atopy-related phenotypes such as asthma, allergic rhinitis, AD and allergic sensitization. Previously, Richardson et al. reported an association between defective yeast opsonization, which is thought to be a functional consequence of MBL deficiency (14) and atopy in a small study population (15). Aittoniemi et al. genotyped 243 asthmatic adults and 400 controls for the three exon 1 and the c.1-290 promoter alterations and did not find an association with asthma. Subgroup analyses showed an increased risk for asthma in non-atopic males with the c.1-290 variant, but not for three coding variants. However, the size of the subgroup was very small and data were not corrected for multiple testing (18).

Uguz et al. observed elevated MBL serum levels in asthmatics and a correlation with elevated eosinophils in a population of 70 asthmatics and 30 age-matched controls. They concluded that high MBL levels may contribute to airway inflammation (22). Thus providing evidence for the hypothesis that high MBL serum levels are associated with some biological disadvantages, which would explain the wide dissemination of MBL deficient individuals. The controversy about the role of different MBL levels emphasizes its questionable potential as a candidate gene in allergy.

There is no doubt that genetic inheritance has the largest influence on circulating MBL levels, but even in individuals with identical haplotypes a wide range of serum MBL is apparent. Thus, the serum concentration is also markedly influenced by non-genetic factors as shown by an up to threefold increase after infection or surgery (23). Also terms like MBL deficiency or insufficiency are not well defined and vary between <0.1 and 0.6 mg/l corresponding to an occurrence of 5% to about 40% in healthy adults. This may explain accidental findings of susceptibility to infections or to allergy with different *MBL2* genotypes. Thus, the genotype can be considered as a reliable predictor of MBL serum concentration in large populations only (8). Undoubtedly, the vast majority of individuals with low or very low MBL levels are healthy. When clinical symptoms occurred together with MBL deficiency, concomitant immunologic defects such as IgG subclass deficiency are often present (24). Taken together, our present study indicates that *MBL2* deficiency is not a risk factor for common respiratory infections or atopy in infancy and childhood in non-immune comprised individuals.

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