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Recurrent respiratory tract infections (RRTI) in the elderly: A late onset mild immunodeficiency?

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ABSTRACT

Elderly with late-onset recurrent respiratory tract infections (RRTI) often have specific anti-polysaccharide antibody deficiency (SPAD). We hypothesized that late-onset RRTI is caused by mild immunodeficiencies, such as SPAD, that remain hidden through adult life. We analyzed seventeen elderly RRTI patients and matched controls. We determined lymphocyte subsets, expression of BAFF receptors, serum immunoglobulins, complement pathways, Pneumovax-23 vaccination response and genetic variations in *BAFFR* and *MBL2*. Twelve patients (71%) and ten controls (59%) had SPAD. IgA was lower in patients than in controls, but other parameters did not differ. However, a high percentage of both patients (53%) and controls (65%) were MBL deficient, much more than in the general population. Often, *MBL2* secretor genotypes did not match functional deficiency, suggesting that functional MBL deficiency can be an acquired condition. In conclusion, we found SPAD and MBL deficiency in many elderly, and conjecture that at least the latter arises with age.

1. Introduction

Primary immunodeficiencies (PIDs) such as severe combined immunodeficiency (SCID) or chronic granulomatous disease (CGD) are generally associated with clinical manifest infections in the first stages of life and diagnosed by pediatric specialists. Infants and children with failure to thrive, a positive family history or an early need for intravenous antibiotics should be screened for PID [1].

In the elderly, 'wear and tear' of the human body results in reduced organ function, overstretches the system [2], and places greater demand on normally redundant elements of host defense to infection. This may reveal mild defects in the immune system such as skewed lyonization in female carriers of X-linked CGD [3]. We hypothesized that some mild PIDs, for example defective antibody production in response to pneumococcal polysaccharide antigens (PnPs) – also referred to as specific anti-polysaccharide antibody deficiency (SPAD) [4] —, may not emerge until older age with recurrent respiratory tract infections (RRTI). In our hypothesis, SPAD may be caused by a late onset diminished expression on B cells of B-cell activating factor (BAFF) receptors, namely the BAFF receptor (BAFF-R), the transmembrane activator and cyclophilin ligand interactor (TACI) and the B-cell maturation antigen (BCMA) (reviewed in [5]), and thus lead to RRTI in these elderly persons. One of the two ligands binding to these three receptors is BAFF, a cytokine that is induced by T-cell independent antigens such as PnPs [6]. The interaction of BAFF with its receptors, is essential for B cell maturation and subsequent antibody production. Therefore, it is possible that BAFF-R deficiency causes defective antibody production in SPAD. Indeed, children below the age of two are constitutively deficient in their responses to PnPs [7], which is likely due to low expression of these BAFF receptors [8].

Defects in BAFF-R play a role in known, serious antibody deficiencies with low levels of IgG, notably in common variable immunodeficiency

Abbreviations: BAFF, B-cell activating factor; BCMA, B-cell maturation antigen; CGD, chronic granulomatous disease; CVID, common variable immunodeficiency; MBL, mannose binding lectin; PID, primary immunodeficiency; PnPs, pneumococcal polysaccharide antigens; PPV-23, Pneumovax-23; RRTI, recurrent respiratory tract infections; SCID, severe combined immunodeficiency; SNP, single nucleotide polymorphism; SPAD, specific anti-polysaccharide antibody deficiency; TACI, transmembrane activator and cyclophilin ligand interactor.

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(CVID). CVID is a PID characterized by recurrent bacterial infections of the respiratory tract, low IgG (<5 g/L) and low IgA and/or IgM, and is generally diagnosed before the age of fifty [9]. First, some patients with CVID have reduced BAFF-R and increased TACI expression in association with high BAFF serum levels [10]. Second, a single nucleotide polymorphism in the BAFF-R contributes to CVID [11]. Third, BAFF-R deficiency led to adult-onset antibody deficiency with very low IgG in two siblings from a consanguineous marriage [12].

For our observational study we selected older adult RRTI patients who did not fulfill CVID criteria and either did not have RRTI earlier in life or experienced respiratory infections that were previously not viewed as important enough for analysis. We compared both their innate and adaptive immunity to age- and gender-matched controls without RRTI.

2. Materials and methods

2.1. Inclusion of patients and controls

Elderly patients (age > 45 years) with late-onset RRTI were selected from the LUMC outpatient clinic for infectious diseases from January 2010 to January 2014. The criteria for inclusion were: two or more bacterial or viral sino- and/or pulmonary infections in the previous year, and no RRTI diagnosed in early adulthood, adolescence or childhood. Exclusion criteria were a known primary immunodeficiency, including CVID (defined by a.o. IgG <5 g/L [9]), or secondary immunodeficiency such as malignancy or treatment with immunosuppressive agents. Three of the patients had cancer 14–20 years before the onset of RRTI, current comorbidities (such as hypertension and mild asthma in a few patients) were deemed not to affect the immune system. The patients did not have allergic diseases, and were all but two non-smokers (of three the smoker status was unknown). As part of the diagnostic work-up, patients were vaccinated with the 23-valent polysaccharide vaccine Pneumovax-23 (PPV-23) against *Streptococcus pneumoniae* and followed-up for the humoral response to PnPs vaccine components one month later.

We recruited age- and gender-matched controls without RRTI from visitors of the LUMC travel clinic and hospital staff. Exclusion criteria were a history of RRTI, previous vaccination with PPV-23 or conjugated Prevenar and/or a known primary or secondary immunodeficiency. Controls completed a questionnaire, were immunized with PPV-23 and blood was sampled before, and one month after vaccination. The controls did not have allergic diseases, they were not asked whether they were smokers or not. All participants provided informed consent and the study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol P15-058).

2.2. DNA isolation and genotyping

DNA was isolated from blood using standard methods [13]. The single nucleotide polymorphism (SNP) rs77874543 in *TNFRSF13C* (encoding BAFF-R) was genotyped as described by Pieper et al. [11]. *MBL2* secretor genotypes were determined by PCR (primers and conditions available on request) and sequencing.

2.3. Phenotyping of lymphocytes

We determined B cells, NK cells and CD4 + and CD8 + T-cell subsets, differentiation stages within the B-cell population and the T-cell subsets, and receptor expression of the BAFF receptors BAFF-R, BCMA and TACI on B-cell differentiation stages by flow cytometry. Peripheral blood mononuclear cells (PBMC) were obtained from patients and healthy donors by Ficoll density gradient separation and stored in liquid nitrogen until analysis. Thawed PBMC were stained with fluorochrome-labeled antibodies against the indicated cell surface antigen (see supplemental Table 1). To facilitate lymphocyte gating PBMC samples were stained for CD45, CD14, CD33, CD235a (glycophorin A). Fc-receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to manufacturer's instructions to prevent aspecific binding of antibodies. We added DAPI (4′,6-diamidino-2-phenylindole) to discriminate between live and dead cells. Samples were measured on a BD Biosciences LSR II flow cytometer (San Jose, CA) with DIVA software, and data analyzed by Kaluza software (Beckman-Coulter, Brea, CA).

Phenotypical definitions of T-cell differentiation stages were: naïve T-cells: $CD45RA^{+}CCR7^{+}$; central memory T-cells: $CD45RA^{-}CCR7^{+}$; antigen experienced $CD4^{+}$ T-cells $CD45RA^{+/-}CCR7^{-}$: early $CD28^{+}CD27^{+}$; intermediate $CD28^{+}CD27^{-}$; late $CD28^{-}CD27^{-}$; antigen experienced $CD8^{+}$ T-cells $CD45RA^{+/-}CCR7^{-}$: early $CD28^{+}CD27^{+}$; intermediate $CD28^{-}CD27^{+}$; late $CD28^{-}CD27^{-}$. Phenotypical definitions of B-cell differentiation stages were: immature B-cells: $CD24^{++}CD38^{++}IgM^{+}IgD^{-}$; transitional B-cells: $CD24^{++}CD38^{++}IgM^{+}IgD^{+}$; naïve B-cells: $IgM^{dull}IgD^{++}CD27^{-}$; natural effector B-cells: $IgM^{++}IgD^{dull}CD27^{+}$; double negative memory B-cells: $IgM^{+/-}IgD^{-}CD27^{-}$ and within this subset IgA^{+} and IgG^{+} cells; IgM committed memory B cells: $IgM^{+}IgD^{-}CD27^{+}$; switched memory B-cells: $IgM^{-}IgD^{-}CD27^{+}$ and within this subset IgA switched (IgA^{+}) and IgG switched (IgG^{+}) cells. Within B-cell differentiation stages expression of three BAFF receptors was measured: BAFF-R (CD268), TACI (CD267) and BCMA (CD269).

2.4. Laboratory parameters

Levels of serum immunoglobulins (IgG, IgG subclasses, IgA and IgM); activity of the classical pathway (CP), of the alternative pathway (AP) and of Mannose binding lectin (MBL); concentrations of complement components (C1q, C4 and C3); IgG antibody titers to PnPs (11 antigens measured before 2014: type 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23; 9 antigens measured from 2014 onwards: type 6B, 8, 9V, 14, 15B, 19F, 20, 23, 33F) were measured by standard assays [14–16].

A normal response to PPV-23 is defined as responses to >70% (*e.g.* 9 out of 11) of the vaccine components with an antibody titer ≥1 μg/mL after vaccination. Presence of antibodies against VZV, EBV, Rubella, and CMV were determined in various blood samples according to standard diagnostic procedures. MBL protein was measured in serum samples with the MBL DuoSet ELISA (DY2307, R&D Systems, Minneapolis, MN).

2.5. Statistical analyses

Data were analyzed by SPSS version 20 (IBM SPSS Inc., Chicago, IL). The non-parametric Mann-Whitney *U* test was used for comparison of continuous data. For categorical data, the Pearson χ^2 test was used except where indicated otherwise. Differences with a p-value < 0.05 were considered statistically significant.

3. Results

3.1. Response to polysaccharide antigens is deficient in many elderly individuals

Before vaccination 14 out of 15 patients (2 not determined) and all 17 controls lacked adequate responses to PnPs. Twelve out of 17 elderly patients with late-onset RRTI and 10 out of 17 healthy age- and gender-matched controls had inadequate IgG antibody production to PnPs after vaccination with PPV-23 (Table 1). Every individual was seropositive for some of the antigens, the responses are on a continuum rather than an 'all or nothing' effect and were not correlated with age or presentation (only upper, only lower, or combined upper and lower RRTI). To evaluate whether the individuals with an inadequate response to PnPs were overall deficient in antibody production we measured the production of antibodies against protein antigens from common viruses (VZV, EBV,

Table 1
Characteristics and vaccination responses of RRTI patients and controls.

	RRTI Patients $n = 17$	Controls $n = 17$	p-Value
Gender, male (%)	6 (35%)	6 (35%)	
Age, mean (median; range)	63.0 (61; 46–90)	62.6 (61; 48–78)	
Respiratory tract infections[a]		None	
Chronic rhinosinusitis	14		
(Recurrent) pneumonia	8		
(Recurrent) bronchitis	5		
Chronic otitis media with effusion	4		
Response to pneumococcal antigens			
Normal[b]	5	7	
Low or absent	12	10	
Positive responses, mean % ± sem	55 ± 6%	72 ± 4%	0.034
Response to protein antigens[c]			
Positive[d]	17 out of 17	16 out of 16[e]	

[a] Late onset recurrent respiratory tract infections, some patients have more than one problem.
[b] Positive responses (IgG antibody titer ≥1 μg/mL) to >70% of the vaccine antigens after Pneumovax-23 vaccination.
[c] Antibodies analyzed against: VZV, EBV, Rubella, CMV.
[d] One or more positive responses.
[e] Not determined in one control.

Rubella, CMV). All patients and controls tested had specific antibodies to one or more protein antigens (Table 1).

3.2. IgG and IgM are comparable in patients and controls, IgA is lower in patients

Inability to produce certain immunoglobulin isotypes can result in an increased susceptibility to infections. Levels of serum IgG, IgM and IgG subclasses did not differ between patients and controls (Fig. 1A, C–G), while IgA was significantly lower ($p = 0.008$) in RRTI patients than in controls (Fig. 1B and Table 2). IgA was undetectable in two patients and above the normal range (0.7–4.0 g/L) in only one patient. All controls had IgA levels within the normal range or above (the latter in two individuals) (Fig. 1B). There was no correlation between age and IgA concentration.

3.3. Haematological data are comparable between patients and controls

Cell numbers in blood from RRTI patients and controls were overall comparable, except for thrombocytes that were marginally higher in RRTI patients (Table 2). Counts of lymphocyte populations, *i.e.*, T cells ($CD3^+$), NK cells ($CD3^-$, $CD7^+$, $CD16^{+/-}$, $CD56^+$) and B cells ($CD19^+CD20^+$), were comparable between patients and controls (Table 2).

3.4. B cells, T cell subsets and differentiation stages are comparable between patients and controls

B cells, T-cell subsets and B- and T-cell differentiation stages were analyzed by flow cytometry. Cell numbers at differentiation stages within the $CD4^+$ and $CD8^+$ T-cell subsets, *i.e.*, naïve, central memory, effector memory and end-stage effector cells were similar in patients and controls (data not shown). Likewise, numbers of all B-cell differentiation stages, *i.e.*, transitional, naïve, natural effector, IgM committed memory; switched memory (SMB) IgA^+, switched memory (SMB) IgG^+, double-negative (DN) IgA^+ memory, double-negative (DN) IgG^+ memory (Fig. 2A–H), immature and switched memory (data not shown) were similar in both groups. The two lowest measurements in the switched memory IgA^+ B cells in Fig. 2E and G correspond, as is to be expected, with the two patients with undetectable IgA in their serum.

3.5. Expression intensity of BAFF binding receptors is similar between patients and controls

The BAFF receptors TACI, BCMA and BAFF-R are known to play an important role in B-cell differentiation. To determine whether altered expression may account for RRTI, or at least for the reduced response to PnPs in many of our RRTI patients, we measured expression of these receptors on various B-cell differentiation stages by flow cytometry. BCMA was not at all detectable on any B cell from the transitional to the switched memory stage (data not shown). While TACI expression was low but detectable on all B cell differentiation stages analyzed, *i.e.*, transitional, naïve, natural effector, double-negative, IgM committed and switched B cells – as exemplified for naive B cells and switched memory B cells (Fig. 3A–B)— expression was not different between patients and controls. BAFF-R was also expressed on all B-cell differentiation stages with no differences between patients and controls (Fig. 3C–D and data not shown). While TACI expression was highest on switched memory B cells and lowest on naïve B cells, this was the other way around for BAFF-R expression (Fig. 3A-D).

A variant in *TNFRSF13C*, rs77874543, that results in an amino acid change (P21R) in BAFF-R, is known to affect BAFF binding and, thereby, BAFF-induced NF-κB activation that is needed for B-cell activation [11]. We genotyped this variation to determine whether it is more prevalent in RRTI patients than in controls. Two individuals in each group were heterozygous for this variation (Table 3). BAFF-R expression for these individuals was always below the median in the various B-cell differentiation stages (Fig. 3C-D and data not shown).

3.6. MBL deficiency in many elderly controls

Various components of the complement pathways were analyzed to determine whether a deficiency in any of these could contribute to RRTI observed in the patients. Concentrations of C1q, C4 and C3 were not significantly different between the two groups (Table 2). Percentages of complement activation through the classical and alternative pathways were also similar between the groups, with only one patient having alternative pathway activation below the normal range (Table 2).

Based on the functional activity of MBL, two patients were partial and seven were completely MBL deficient (9/17 patients were

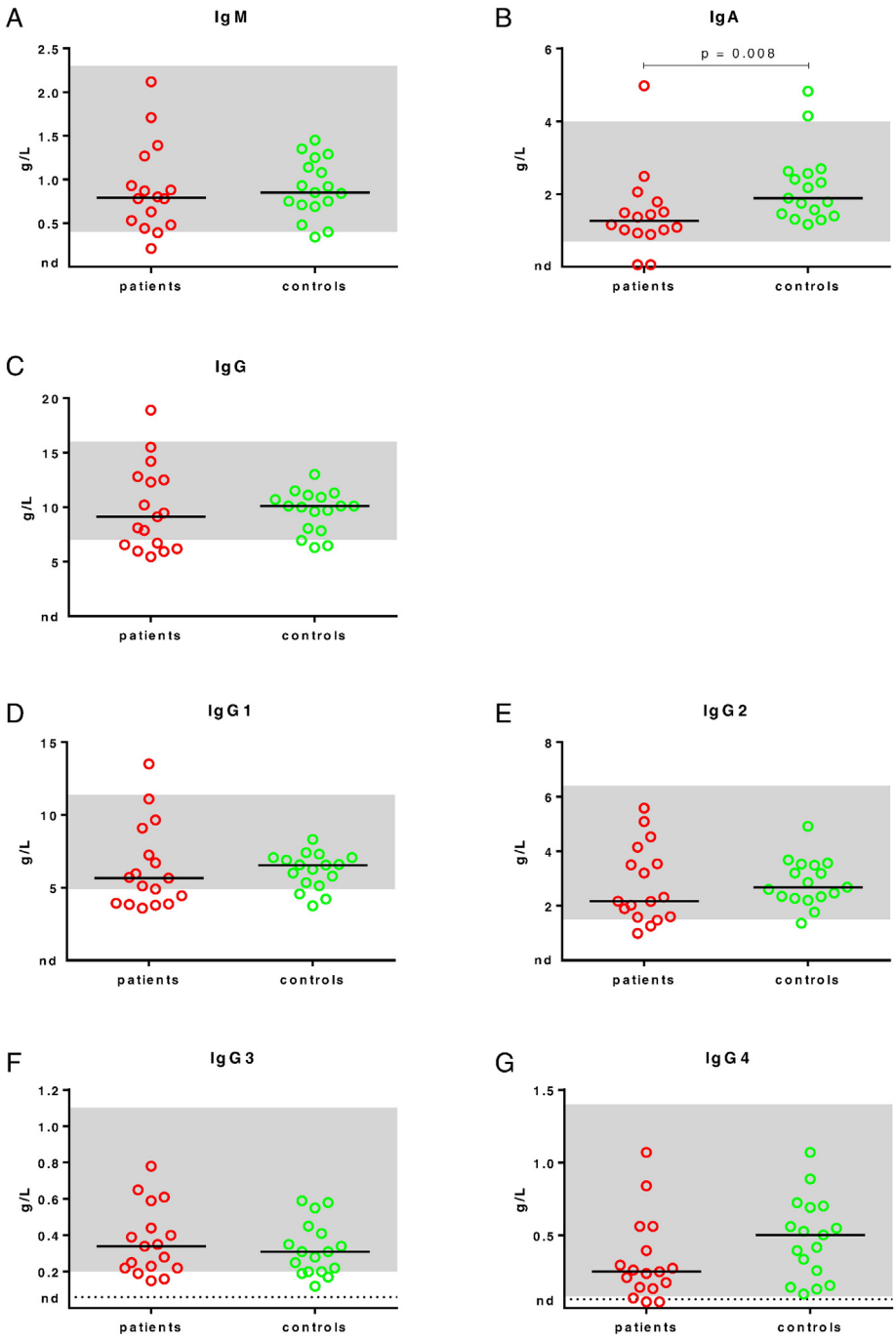

Fig. 1. Immunoglobulins are overall low in the elderly, IgA is lower in patients. Immunoglobulins (in g/L) were determined in serum from 17 RRTI patients and 17 controls (C–G) except for IgM and IgA (A–B) where only data for 16 patients are available. The black horizontal line indicates the median, grey areas indicate the reference values for adults.

deficient); of the seventeen controls one was partial and ten were completely MBL deficient (11/17 controls were deficient). These low MBL activation percentages were not significantly different between the two groups (Table 2). Since such a striking number of individuals in both groups had no MBL activity, we repeated MBL measurements in most samples, but this did not alter the outcomes. Subsequently, we determined *MBL2* secretor genotypes by sequencing the promoter region and exon 1 of the *MBL2* gene. These two parts of *MBL2* contain the polymorphisms and mutations that determine the *MBL2* secretor genotype. Interestingly, we found a significant difference in distribution of *MBL2* genotypes ($p = 0.003$). Low or deficient genotypes were more frequent in controls ($n = 10$) than in patients ($n = 2$) (Table 3). Even more surprising, in many of the individuals, *i.e.*, 9 RRTI patients and 6 controls, the secretor genotype (high, intermediate or low) did not match the observed partial or complete functional deficiency (Fig. 4, supplemental Table 2). Of note, *MBL2* secretor genotype was nonetheless associated with MBL activity ($p = 0.012$).

To rule out an acquired inhibiting factor in the serum that could cause the discrepancy between *MBL2* secretor genotypes and MBL activity, we measured MBL activity of a pool of individuals with high MBL activity combined with various dilutions of sera from four elderly individuals with high secretor genotype and low or absent MBL activity. Inhibition of MBL activity by any of the sera was not detected (data not shown). To determine whether MBL protein production is reduced in elderly individuals at advanced age, we measured the MBL protein in the sera of 22 of the individuals. Indeed, we found that MBL protein in the

Table 2
Haematological and immunological variables of RRTI patients and controls.

	RRTI Patients $n = 17$	Controls $n = 17$	p-Value
Haematological data, mean			
Leukocytes, in 10^9/L	6.3 ± 0.6	5.7 ± 0.5	n.s.
Eosinophils, in 10^9/L	0.17 ± 0.02	0.19 ± 0.04	n.s.
Basophils, in 10^{12}/L	31 ± 5	32 ± 5	n.s.
Neutrophils, in 10^9/L	3.88 ± 0.46	3.37 ± 0.38	n.s.
Lymphocytes, in 10^9/L	1.75 ± 0.20	1.66 ± 0.15	n.s.
Monocytes, in 10^9/L	0.48 ± 0.05	0.46 ± 0.05	n.s.
Erythrocytes, in 10^{12}/L	4.5 ± 0.1	4.6 ± 0.1	n.s.
Thrombocytes, in 10^9/L	260 ± 12	232 ± 11	0.049
Haemoglobin, in mmol/L	8.3 ± 0.2	9.0 ± 0.2	n.s.
Cell subsets, mean per μL			
Lymphocytes	1753 ± 201	1661 ± 154	n.s.
T cells	1298 ± 168	1114 ± 108	n.s.
NK cells	213 ± 25	294 ± 37	n.s.
B cells	208 ± 55	223 ± 43	n.s.
Immunoglobulins, mean in g/L			
IgA[a]	1.5 ± 0.3	2.2 ± 0.2	0.008
IgG	9.8 ± 0.9	9.6 ± 0.5	n.s.
IgG1	6.4 ± 0.7	6.2 ± 0.3	n.s.
IgG2	2.8 ± 0.3	2.9 ± 0.2	n.s.
IgG3	0.4 ± 0.05	0.3 ± 0.04	n.s.
IgG4	0.3 ± 0.1	0.5 ± 0.1	n.s.
IgM[a]	0.9 ± 0.1	0.9 ± 0.1	n.s.
Complement, mean			
C3, in g/L[a]	1.1 ± 0.05	1.0 ± 0.04	n.s.
C4, in mg/L[a]	229 ± 14.3	217 ± 13	n.s.
C1q, in mg/L[a]	136 ± 10.5	147 ± 5	n.s.
CP activation, in %	97 ± 2.6	92 ± 2.4	n.s.
AP activation, in %	73 ± 5.1[b]	67 ± 3.5	n.s.
MBL activation, in %	30 ± 9.1	20 ± 8.7	n.s.
MBL low or absent[c]	9 out of 17	11 out of 17	n.s.

All means are indicated as mean ± the standard error of the mean. MBL = mannose binding lectin; CP = classical pathway; AP = alternative pathway; n.s. = not significant.
[a] Data from 1 patient are lacking.
[b] Only one patient was deficient.
[c] Normal is defined as >10% activation, low as 1–10% activation, absent as 0% activation.

serum was low in many individuals with low or absent MBL activity despite their high or intermediate *MBL2* secretor genotype (Supplemental Fig. 1). There was no correlation between age and MBL concentration or MBL activity in these elderly individuals.

4. Discussion

In this observational study we analyzed 17 elderly patients with late onset RRTI and 17 age- and gender-matched controls to identify a subtle immunodeficiency in the RRTI patients. Although the number of positive responses one month after PPV-23 vaccination was significantly lower in patients than in controls, we found many elderly with deficient responses in both groups. The presence of SPAD in such a high number of patients and controls (71% and 59% respectively) suggests that this deficiency does not offer an explanation for late onset RRTI in our patients. In search for further clarification, we assessed for, but did not find, differences between patients and controls, neither in various blood cells and lymphocyte subsets, nor in B- and T-cell differentiation stages and BAFF receptor expression. Surprisingly, we did find a higher frequency of deficient/low *MBL2* secretor genotypes in controls than in patients, suggesting a protective effect of low MBL activity against RRTI. Furthermore, functional MBL activity was much lower than we expected in our elderly population at large, in patients and controls, often paralleling low MBL protein serum concentrations but not *MBL2* secretor genotypes.

In some countries, but not in The Netherlands, it is common practice to immunize elderly (≥65 years old) with PPV-23 to protect them from infection with *S. pneumoniae*, a frequently found airway pathogen. Dutch immunologists and infectious disease specialists use PPV-23 to diagnose potential antibody deficiency syndromes in RRTI patients. We found that the response to the pneumococcal polysaccharide antigens in PPV-23 was deficient not only in patients but also in many controls. To determine whether these elderly people, similar to CVID patients, have a general inability to produce antibodies, we analyzed overall immunoglobulin production and antibody production in response to protein antigens such as CMV and EBV. Production of immunoglobulin (sub)classes was comparable between patients and controls except for IgA which was significantly lower in the patients. None of the individuals had CVID or a generalized hypogammaglobulinemia. In addition, antibodies against protein antigens were present in all individuals. Admittedly, it would be interesting to assess the response to a neoantigen (such as tick-borne encephalitis virus) as well, as the protein antibodies we assessed may be secreted by long-lived plasma cells; this will be subject of further study. In addition, it is interesting to determine whether the positive responses to pneumococcal antigens that are present 1 month after vaccination are still detectable at levels similar between the patients and controls 6 to 12 months after vaccination, as it is conceivable that in the patients the response is more transient. Regardless, our data indicate that the deficient antigen responses in the twelve patients and ten controls are specific for pneumococcal antigens (SPAD), and not representative of a general problem in antibody production.

Our results show that in the elderly, a low or absent response to PPV-23 vaccination does not predispose to RRTI, and might be more justly interpreted as a sign of an advanced age. This suggests either that lacking responses to PPV-23 vaccination are not the cause of RRTI, or that the current definition of an adequate response (>70% of the antigens induced an antibody titer of ≥1 μg/mL) should be re-investigated in the elderly: since both the RRTI group and the healthy controls have very similar low responses, these low responses may actually be adequate.

In addition, the IgG antibody titers routinely measured in response to PPV-23 vaccination may not be the only relevant antibody titers. In

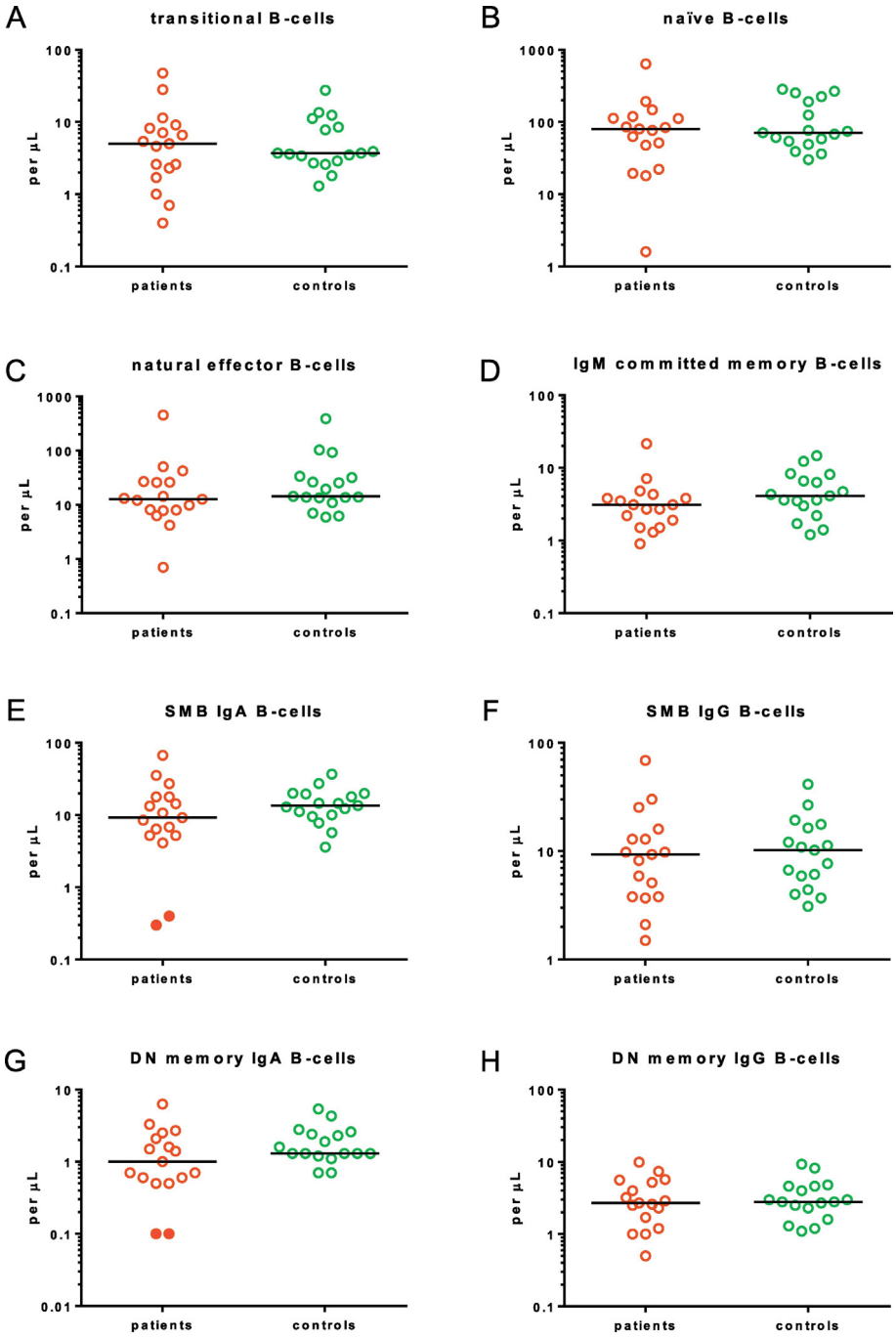

Fig. 2. B-cell differentiation stages are similar between patients and controls. B-cell differentiation stages in patients and controls (A–H). The two closed circles in E and G at the bottom of the graph correspond to the two patients who do not produce detectable IgA. The black horizontal line indicates the median. Lower detection level is 0.1 cells/µL for these graphs. SMB = switched memory B cells. DN = double-negative (IgD^-CD27^-).

both asplenic individuals and CVID patients, lack of natural effector B cells, also known as "innate" IgM memory B cells, and consequently lack of IgM antibodies, has been shown to be correlated with increased susceptibility to pneumococcal infections and lack of response to polysaccharide vaccines [17,18], suggesting IgM antibodies may be essential against pneumococcal infections. In our subjects, who are not asplenic and do not have CVID, we did not measure IgM antibodies against pneumococcal antigens. However, natural effector B cells (as well as germinal center-dependent IgM+ committed memory B cells) are present in normal amounts.

We did not find a difference between the controls and the RRTI patients. However, the RRTI group included patients with upper respiratory tract infections only ($n = 5$, recurrent otitis media and/or rhino sinusitis), patients with lower respiratory tract infections only ($n = 5$, bronchitis and/or recurrent pneumonia), as well as patients with combined lower and upper respiratory tract infections ($n = 7$). As the immune impairment in these RRTI subgroups may be different, we have analyzed the various B- and T-cell subsets and pneumovax responses in these subgroups. Pneumovax responses and T-cell subsets do not differ between the subgroups. We do observe a small but significant difference between controls and patients with combined upper and lower RRTI in total B cells ($p < 0.03$), natural effector B cells ($p < 0.03$), switched B cells ($p < 0.006$), SMB IgA B-cells ($p < 0.002$), and DN memory IgA B-cells ($p < 0.05$) but not between the controls and either of the lower or the upper RRTI subgroup (Supplemental Fig. 2).

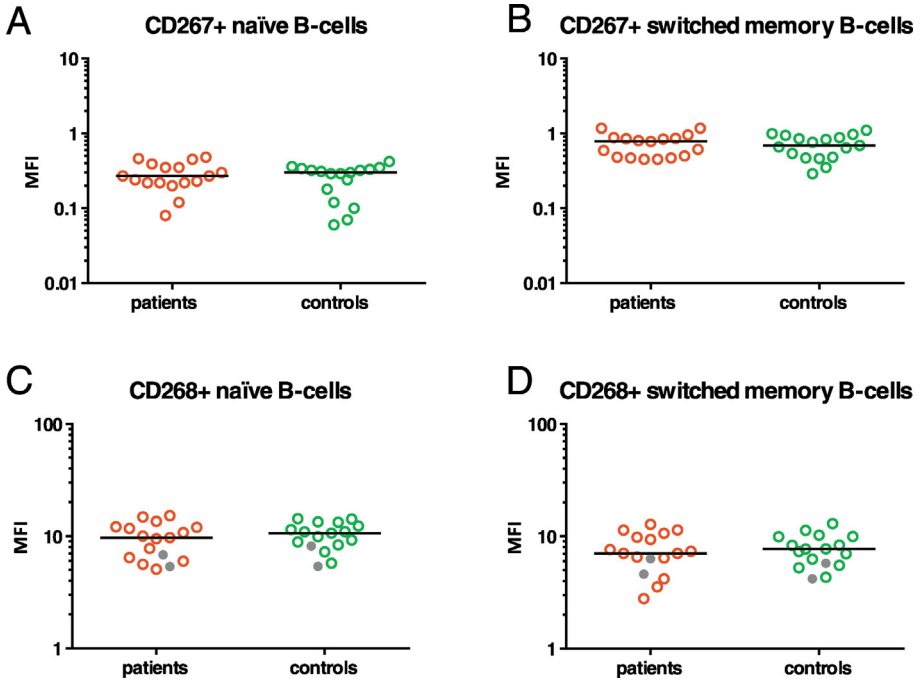

Fig. 3. TACI and BAFF-R are expressed on naive and switched memory B cells. Mean florescence intensity of naive B cells (A, C) and switched memory B cells (B, D) labeled with anti-CD267 (TACI) antibody (A, B) or anti-CD268 (BAFF-R) antibody (C, D). The black horizontal line indicates the median. MFI = mean fluorescence intensity. The filled grey circles depict individuals heterozygous for the SNP rs77874543 in *TNFRSF13C* (encoding BAFF-R).

B cells from CVID patients and young infants have different expressions of BAFF receptors, in particular BAFF-R and TACI, than controls [10,11]. Hence, we supposed that RRTI in our patients could be caused by lower expression of one of the BAFF receptors on their B cells. We were also interested in the cell types that express TACI and BCMA because, while BAFF receptors have different expression levels depending on the stage of B-cell differentiation and activation [19], it is controversial on exactly which cells TACI and BCMA are expressed (discussed in [19]). We were able to detect BAFF-R on various B-cell differentiation stages, with highest expression on naive B cells and lowest expression on memory B cells. Indeed, in normal ageing, memory B cells replace naive B cells within the B cell repertoire. However, we did not detect a difference in expression of BAFF-R between RRTI patients and controls. BCMA is reported to be expressed on CD138 + B cells isolated from tonsils and bone marrow [19], cells not analyzed in this study. Indeed, BCMA was not detectable on the various peripheral blood B-cell differentiation stages we analyzed. TACI is expressed predominantly by memory B cells [19], only on tonsillar B cells [20,21], or on mature naive and memory B cells [22]. In accordance, we detected a low amount of TACI expression on switched and natural effector B cells and expression at very low intensity on naive B cells from blood. Again, we did not detect a difference in TACI expression between RRTI patients and controls.

The BAFF receptor BAFF-R is essential for B-cell development and survival. Variations in the gene encoding BAFF-R, *TNFRSF13C*, may affect BAFF-R function and consequently the ability to generate an adequate antibody response. One BAFF-R variation, rs77874543 leading to the amino acid variation P21R and affecting BAFF binding, is quite common in the general population (7.5% in Europeans [23]) and slightly more frequent in individuals with CVID [11]. Once more, we did not find a difference between RRTI patients and controls in the presence of the rs77874543 variation.

Our finding that *MBL2* secretor genotypes were more often deficient/low in controls than in patients is counterintuitive. It suggests a protective effect of low MBL activity against RRTI, while airway infections are commonly linked to MBL deficiency [24,25]. In addition, numerous studies have shown that MBL deficiency is associated with increased severity and outcome of bacterial infection in critically ill individuals. For instance, in patients with systemic inflammatory response syndrome MBL insufficiency is associated with development of (severe) sepsis and septic shock [26]. In patients with community acquired pneumonia, MBL insufficiency is associated with sepsis severity and mortality [25]. MBL deficiency *alone* does, however, not affect the incidence of infectious diseases or mortality as concluded in a large cohort study of adults ($n = 9245$) in Denmark [27]. Importantly, in that study MBL deficiency was determined based on *MBL2* secretor

Table 3
Genetic variations in *MBL2* and *TNFRSF13C*.

	RRTI patients $N = 17$	Controls $n = 17$	p-Value
TNFRSF13C variation rs77874543			
GG	15	15	
GC	2	2	
MBL2 secretor genotypes[a]			0.003[b]
High YA/YA	9	7	
Intermediate YA/XA	6	0	
Low XA/XA or YA/O	2	5	
Deficient XA/O or O/O	0	5	

[a] *MBL2* secretor genotypes according to Bernig et al. [32].
[b] Fisher's exact test.

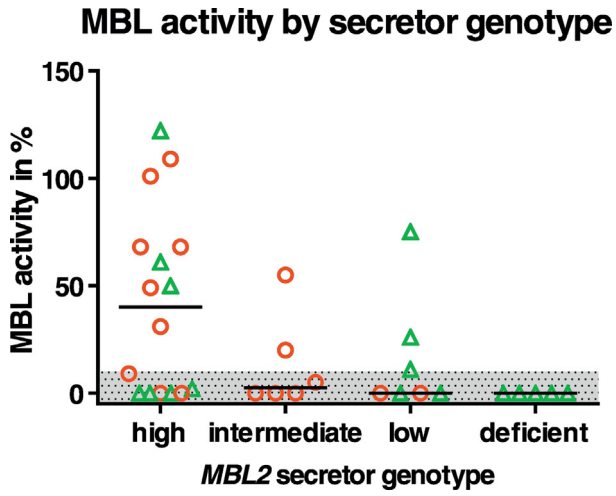

Fig. 4. In many elderly individuals MBL activity does not correspond with *MBL2* secretor genotype. MBL activity was measured in serum of all individuals and *MBL2* secretor genotypes (high, intermediate, low and deficient) were determined by sequencing the *MBL2* gene. Red circles indicate RRTI patients, green triangles indicate controls. The black horizontal line indicates the median. The grey pattern indicates the area where samples are completely (0% activity) or partially (activity between 0 and 10%) deficient.

genotypes. Our study shows that at least in elderly people (>45 years old) MBL production and activity is declined and may no longer correlate with *MBL2* secretor genotype.

This potential protective effect of low MBL activity against RRTI obviously needs verification in a study involving large numbers of patients and controls. However, it is illustrative that we not only found significantly more controls than RRTI patients with low or deficient *MBL2* secretor genotypes, but also a somewhat higher percentage of controls than patients with low or absent MBL functional activity. A relation between an intermediate *versus* high (or low) *MBL2* secretor genotype and lifespan in an elderly population, not selected for any disease phenotype, has been reported previously [28]. The authors suggest that having an intermediate haplotype is an evolutionary advantage. Indeed, the relatively high frequency of deficient alleles in the general population suggests that this must be the case [29]. Importantly, low MBL activity has been associated with less severe malaria [30] and less frequent and less severe leishmaniasis [31]. Beneficial effects of MBL deficiency may exist for other diseases that have not been investigated thus far, where higher MBL activity contributes to mortality, resulting in selection at an advanced age of individuals carrying null alleles.

Despite the above described difference in MBL functional activity between controls and patients, we found high percentages of functional MBL deficiency in our study population as a whole, both in patients (53%) and in controls (65%). These percentages were much higher than expected based on the prevalence of functional MBL deficiency in the general population (20–25% in all populations analyzed, [29]). *MBL2* secretor genotypes often did not match this MBL deficiency, neither in controls nor in patients, although we did find an overall correlation between *MBL2* secretor genotype and MBL activity. Altogether, our findings suggest that in elderly people the activity of the MBL protein becomes impaired. We excluded presence of an inhibitory serum factor as an underlying mechanism, and will focus in future studies on other options explaining this acquired MBL deficiency in the elderly population at large.

This pilot study explores an underlying cause of disease in elderly patients presenting with late-onset RRTI, which would disqualify this possible sign of dysfunctioning immunity as a typical old age ailment. Within the limits of the study we did not obtain clear evidence for an immune defect discriminative for late-onset RRTI. It is of course possible that many of these patients have unknown combinations of polymorphisms in other genes involved, each leading to RRTI in a different way.

In summary, we found that elderly people in general are often MBL deficient, a condition that is potentially acquired in older age, and that many have low vaccination responses to PPV-23 which do not necessarily culminate in clinically overt SPAD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2017.05.008.

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