CONNECTING CLINICAL AND MOLECULAR DISEASE FEATURES IN COMMON VARIABLE IMMUNODEFICIENCY DISORDER

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Connecting Clinical and Molecular Disease Features in Common Variable Deficiency Disorder

ISBN: 978-94-028-0609-0

Deze publicatie is mogelijk gemaakt door | *This publication is made possible by*

Grafisch ontwerp | *Graphic design*
Juliet Campfens

Illustraties | *Illustrations*
Liron Zada

Drukwerk | *Print*
Ipskamp Printing

Uitgever | *Publisher*
Universiteit Utrecht

Connecting clinical and molecular disease features in Common Variable Immunodeficiency Disorder

Klinische en moleculaire kenmerken van Common Variable Immunodeficiency Disorder met elkaar verbonden

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 20 april 2017 des middags te 4.15 uur

door

Willemijn Jihane Mariëna Janssen
geboren op 21 mei 1983 te Nijmegen

Promotor:	Prof.dr. E.A.M. Sanders
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This research was supported by a non-restricted education grant from Baxalta B.V.

In liefdevolle herinnering aan mijn vader

CONTENT

CHAPTER 1

General Introduction

INTRODUCTION

This thesis focuses on the mechanisms of disease in primary immunodeficiency (PID) patients and in specific on common variable deficiency (CVID) patients. The defective immune system of these patients can be seen as experiments of nature and contribute to the understanding of the immune system in general.

Common Variable Immunodeficiency (CVID) disorder is the most prevalent symptomatic primary immunodeficiency in children and adults. Typically, a patient presents with recurrent pulmonary and/or gastrointestinal infections (with or without infection related complications), due to diminished immunoglobulin levels and insufficient specific antibody responses to encapsulated bacteria. Some patients present with autoimmune manifestations related to immune dysregulation[1-32,4,5]. In order to reach the diagnosis of CVID patients must meet certain diagnostic criteria as formulated by the American and European Society for Immunodeficiencies (ESID): A patient should have a marked decrease of IgG (at least 2 SD below the mean for age) and a marked decrease in at least one of the isotypes IgM or IgA, and all of the following criteria: 1) Onset of immunodeficiency at > 2 years of age 2) Absent isohemagglutinins and/or poor response to vaccines and 3) Defined causes of hypogammaglobulinemia have been excluded[6]. The current revised diagnostic ESID criteria for CVID are more detailed and furthermore include at least one of the following clinical symptoms of recurrent infections and/or autoimmunity and/or granulomatous disease or lymphoproliferation. Also, a patient should have poor vaccination responses or low switched memory B-cells. The onset of immunodeficiency is redefined to >4 years and no evidence of profound T-cell deficiency should be present[7].

Part I of this thesis: B- and T-cells in CVID patients

As included in the revised criteria, CVID patients may or may not have B-cell abnormalities. Several studies have addressed the B-cell subset phenotypes of CVID patients, overall the majority of patients have normal total B-cell numbers, however may display disturbed differentiation, with high naïve B-cell numbers, increased transitional B-cell percentages and low class switched memory B-cell percentages and peripheral plasmablasts[2,8,9]. Approximately half of the CVID patients are reported to have reduced class switched memory B-cell numbers (<=2%)[2]. Another feature of the disturbed B-cell compartment in CVID is the expansion of CD21low B-cells, associated with B-cell autoreactivity, splenomegaly and granulomatous disease[10,11]. Distinct B-cell differentiation populations in CVID patients were furthermore described by Driessen et al, where 5 B-cell differentiation patterns were identified based on somatic hypermutations and B-cell subset staining[12]. Next to B-cell abnormalities also T-cell abnormalities occur at a regular basis in CVID patients, in particular of the CD4 T-cell compartment. Patients display reduced T-cell proliferation, low CD3 T-cell counts

and a relative CD8 T-cell 'expansion' due to strongly diminished CD4 T-cell counts. Furthermore, CD4 naïve T-cells are then also diminished, with a dysfunctional CD4 T-cell compartment as a result[8,13–16].

The dysfunctional B- and T-cells in common variable immunodeficiency contribute to autoimmune manifestations, although the precise mechanism are still not known for the majority of patients. Suggested mechanisms are proposed through altered B- and T-cell receptor signaling, increased survival of autoreactive B-cells through eg increased BAFF expression and TACI mutations and altered inflammatory cytokine production[10,16,17].

Another approach to decipher the molecular mechanisms of CVID is through RNA- and next gen sequencing methods[18–24]. Since the last decade these techniques have become readily available for screening of patients, and thus rapidly increasing new gene mutations related to PID's are found. To date, 269 PID related genes are identified and the rate is ongoing. For CVID patients however, monogenetic causes of disease are still scarcely identified.

Thus the first part of this thesis focuses on possible new gene mutations and molecular mechanisms contributing to the B- and T-cell defects in CVID patients. To this end, we screened 30 primary immunodeficiency patients with a next gen sequencing technique for 170 known PID genes and 300 candidate genes and performed functional experiments to prove disease association for potentially pathogenic new gene mutations.

Part II of this thesis: Diagnostic and clinical aspects of CVID patients.

The diagnostic criteria of CVID include the evaluation of T-cell dependent (outer membrane proteins eg HiB, DKTP) and independent (eg polysaccharides) vaccination responses[6,25–27]. Especially the pneumococcal polysaccharide (PnPS) T-cell independent responses are valuable to assess since they offer an opportunity to evaluate the isolated B-cell functioning, although the exact nature of these marginal zone B-cells is still under debate[28–31]. The measurement of anti-PnPS IgG responses upon vaccination can be performed through a combined measurement of all PnPS serotypes or through evaluation of individual PnPS serotype responses. Both methods are in use and discussion is ongoing as to which method is superior for the correct evaluation of anti-PnPS responses[32–35,27,36]. In general, anti-PnPS IgG responses are considered insufficient when <50% or <75% of responses are above 1ug/ml (depending on age)[37–39]. The insufficient specific antibody responses against encapsulated bacteria (and low immunoglobulin levels), make the CVID patient group prone for recurrent respiratory tract infections and subsequent pulmonary complications such as bronchiectasis[40]. Pulmonary complications occur in approximately 30% of CVID patients and may result in chronic lung failure, therewith significantly contributing to the increased mortality rate of CVID patients. Besides acute clinical infections and development of malignancies,

pulmonary comorbidity can be divided into two categories, structural airway disease due to recurrent pulmonary infections and interstitial lung disease (ILD), associated with autoimmune phenomena[40].

Another organ system often affected in CVID is the gastrointestinal tract, the prevalence of gastrointestinal manifestations is reported between 20-60%[41,42], Roughly four different forms of gastrointestinal disease can be distinguished: infectious-, autoimmune-, malignant and inflammatory disease. Although CVID gastrointestinal disease mimics well-established diseases such as inflammatory bowel disease and celiac disease, gastrointestinal disease in CVID however is characterized by an absence of plasma cells and follicular lymphoid hyperplasia and the pathogenesis is not well known.

Finally, treatment of CVID patients consists of Ig replacement therapy, with the purpose to prevent recurrent infections and subsequent complications. However the optimal IgG trough levels for protection of recurrent infections and reduction of disease related (infectious) complications are still not proven[43–47]. Other treatment options to prevent recurrent pulmonary bacterial infections include antibiotic prophylaxis, while for treatment of autoimmune manifestations immunosuppressive therapy (e.g. systemic corticosteroids) may be considered.

In summary, CVID patients may suffer from a variety of clinical symptoms and different organ systems may be affected. Furthermore the measurement of pneumococcal polysaccharide vaccination responses as part of the diagnostic criteria is not fully agreed on. Therefore the second part of this thesis looks further into the local pulmonary and gastrointestinal homeostasis of CVID patients and evaluates the use of pneumococcal polysaccharide vaccination responses as a diagnostic criterium for CVID.

OUTLINE OF THIS THESIS

PART I
B- and T-cell disturbances in CVID patients

Chapter 2: describes the functional effects of a novel BLK mutation in B-cells of two CVID patients

Chapter 3: explores the effect of a novel STAT3 mutation on T- and B-cells of a primary immunodeficiency patient with autoimmunity and hypergammaglobulinemia

Chapter 4: focuses on the functional effects of two novel PSTPIP1 mutations in T-cells of two CVID patients

Chapter 5: reviews the literature on primary immunodeficiency related T-cell defects and the effects on f-actin remodeling

PART II
Diagnostic and clinical aspects of CVID disease

Chapter 6: compares different methods for measurement of pneumococcal polysaccharide serotype IgG responses

Chapter 7: describes the use of anti-PnPS IgM, IgA and IgG responses to discriminate transient from persistent antibody deficiency

Chapter 8: quantifies the presence of gastrointestinal viruses and secreted IgA levels in faeces of CVID patients and healthy controls

Chapter 9: focuses on the progression of pulmonary airway disease in CVID patients and correlation to Ig replacement therapy

Chapter 10: Summary and General discussion of the chapters

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CHAPTER 2

Dysfunctional BLK in Common Variable Immunodeficiency perturbs B-cell proliferation and ability to elicit antigen-specific CD4+ T-cell help

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Published in: Oncotarget 2015

ABSTRACT

Common Variable Immunodeficiency (CVID) is the most prevalent primary antibody deficiency, and characterized by defective generation of high-affinity antibodies. Patients have therefore increased risk to recurrent infections of the respiratory and intestinal tract. Development of high-affinity antigen-specific antibodies involves two key actions of B-cell receptors (BCR): transmembrane signaling through BCR-complexes to induce B-cell differentiation and proliferation, and BCR-mediated antigen internalization for class-II MHC-mediated presentation to acquire antigen-specific $CD4^+$ T-cell help.

We identified a variant (L3P) in the B-lymphoid tyrosine kinase (BLK) gene of 2 related CVID-patients, which was absent in healthy relatives. BLK belongs to the Src- kinases family and involved in BCR-signaling. Here, we sought to clarify BLK function in healthy human B-cells and its association to CVID.

BLK expression was comparable in patient and healthy B-cells. Functional analysis of L3P-BLK showed reduced BCR crosslinking-induced Syk phosphorylation and proliferation, in both primary B-cells and B-LCLs. B-cells expressing L3P-BLK showed accelerated destruction of BCR-internalized antigen and reduced ability to elicit CD40L-expression on antigen-specific $CD4^+$ T-cells.

In conclusion, we found a novel BLK gene variant in CVID-patients that causes suppressed B-cell proliferation and reduced ability of B-cells to elicit antigen-specific $CD4^+$ T-cell responses. Both these mechanisms may contribute to hypogammaglobulinemia in CVID-patients.

INTRODUCTION

Common variable immunodeficiency (CVID) is the most common primary immunodeficiency (PID), with an estimated prevalence of 1 in 25.000-50.000 adult Caucasians [1]. CVID patients are characterized by defective generation of high affinity antibodies by B-cells and therefore suffer from recurrent infections of the respiratory- and intestinal tract [1]–[3]. The vast majority of B-cells recirculate through the blood and the follicular regions of the lymphoid tissues. These quiescent B-cells require sustained expression of functional B-cell receptors (BCRs) to transduce antigen- independent tonic signals for their survival. B-cells may capture encountered antigen with their specific BCR that is composed of the Igα/Igβ dimer. Productive BCR- antigen engagement initiates a signaling cascade that starts with the activation of tyrosine kinases from the Src family. Src kinases phosphorylate the ITAM motif of Igα and Igβ. For example, the Src kinase family member B-lymphoid tyrosine kinase (BLK) phosphorylates the ITAM motif of Igα [4]. This leads to the recruitment and subsequent activation of Spleen tyrosine kinase (Syk) [5]. Ultimately this leads to functional B-cell activation and clonal expansion. For the development of high affinity antigen-specific antibodies, in addition BCR expression and signaling is necessary to internalize antigen into endosomes for processing by endosomal proteases. Antigen-derived peptides thereby generated are subsequently presented as antigen-specific peptide/class II MHC complexes to acquire antigen-specific $CD4^+$ T-cell help in form of cytokines and co-stimulatory molecules, such as IL-4 and CD40L respectively. Therefore it is not surprising that in patients showing characteristics of CVID, monogenetic defects are found in genes relevant to BCR signaling or the elicitation of $CD4^+$ T-cell help, including CD19 [6], CD20 [7], CD81 [8], or CD27 [9], CD40L [10] and ICOS-L [11]. Using our recently developed Primary Immunodeficiency (PID) targeted Next-Generation Sequencing-based approach [12], we screened patients with CVID of unknown origin for (putative) PID- associated gene variants. Here we report a Leucine to Proline replacement at position 3 in B-lymphoid tyrosine kinase (BLK) in 2 patients with CVID. BLK was initially thought to be expressed solely in B-lineage cells [13], but is now known to be also expressed in both human and mouse pancreatic β-cells [14], in murine plasmacytoid dendritic cells [15], and is required for the development of T-cells and IL-17-producing γδ T-cells in mice [16]. BLK belongs to the Src family of tyrosine kinases that phosphorylate Igα subunit of BCR signalling complex [4, 17]. We sought to clarify BLK function in human B-cells and its association to CVID. To this end, we researched the effect of L3P-BLK gene variant expression on BLK function and B-cell function in primary CVID-patient B-cells and immortalized B-LCLs. Unlike in mice, where to a certain extent functional redundancy exists between Src kinases [18–20], in human B-cells the L3P-BLK mutation had functional consequences to BCR signaling. We propose that BLK is pivotal to normal B-cell function as antigen presenting cells. Moreover, BLK disease variants may contribute to CVID disease pathology by perturbing B-cells to proliferate and adequately elicit $CD4^+$ T-cell help,

known to support B-cells class-switching of Ig heavy chain regions and eventually the sufficient production of high affinity IgG and IgA antibodies.

RESULTS

The index patient presented in our hospital at age 7, with a history of severe recurrent pulmonary infections from the age of 8 months onward, requiring frequent hospitalizations. Laboratory investigations showed hypogammaglobulinemia with persistent low levels of IgG (5g/L), low IgA levels (0.42g/L), and relatively normal IgM levels (0.39g/L) (Table 1). The patient was vaccinated according to the national vaccination program in the Netherlands, however showed insufficient vaccination responses for Haemophilus Influenza B (Hib), Meningococci type C, and pneumococcal polysaccharide antigens 4-6 weeks after re-vaccination. Based on these findings the patient was diagnosed with CVID [1] and treated with monthly infusion of intravenous immunoglobulin (Ig), and antibiotic prophylaxis.To study potential causes of the antibody deficiency, flow cytometric analysis was performed on peripheral blood of the patient. This showed normal numbers of monocytes ($0{,}4x10^9$/L), lymphocytes ($2{,}47x10^9$/L) and NK cells (11%), but slightly reduced B-cell numbers (Table 2). Within the B-cell compartment, IgG memory B-cells were in the low normal range (2.6% of B-cells), but IgM- (5.6%) and IgA-positive memory B cells (0.2%) were significantly reduced[21]. There were no signs for autoimmunity or lymphoproliferative disease. The patient's father had a disease history comprising of recurrent respiratory tract infections and episodes of bacteriaemia upon small skin lesions. Moreover, he had relative low IgM (0,28g/L) and IgG (6,65g/L) levels (shown in Table 1). In contrast to both the index patient and his father, the mother, sister and another brother showed no clinical symptoms related to antibody deficiency. The mother was excluded from functional experiments, because she was treated with immunosuppressive therapy for recently diagnosed ulcerative colitis.

Screening of Common Variable Immunodeficiency-associated candidate genes uncovers L3P point mutation in the BLK gene

Regular genetic analysis of the index patient revealed no mutations in known CVID-associated genes. Hence, we exploited our recently developed Primary Immunodeficiency (PID)-targeted Next-Generation Sequencing-strategy based on 170 PID-related (IUIS) and 350 candidate PID-genes. This strategy allows detection of point mutations with a sensitivity and specificity >99% in covered regions [12], and revealed that both CVID patients but not their healthy relatives had a heterozygous point mutation (NM_001715.2 c.8T>C) in the BLK gene. No mutations were found in the other known or candidate PID-genes present on the chip [12]. The point mutation in BLK was subsequently confirmed by Sanger sequencing, as shown in Figure 1A. The L3P-BLK gene variant is unique to these CVID patients, with healthy family members not carrying

Table 1

Serum antibody titers of L3P-BLK carrying individuals in comparison to age-matched controls.

Immunoglobulins		**patient (L3P-BLK)**	**reference values**	**father (L3P-BLK)**	**reference values**
IgM-total	g/L	0.39	0.28-1.9	0.28	0.40-2.3
IgA-total	g/L	0.42	0.54-2.5	1.2	0.7-4.0
IgG-total	g/L	5	5.20-14.3	6.65	7.00-16.0
IgG1	g/L	3.4	3.5-9.1	4.1	4.9-11.4
IgG2	g/L	0.62	0.85-3.30	1.91	1.50-6.40
IgG3	g/L	0.34	0.20-1.04	0.20	0.20-1.10
IgG4	g/L	0.02	0.03-1.58	0.05	0.08-1.40
Post-vaccination: serum antibody titers		**patient (L3P-BLK)**	**father (L3P-BLK)**	**reference values**	
Meningococci C	mg/ml	0.33	<0.24	>1mg/ml	
HiB-BL	µg/mL	0.32	0.50	>1µg/ml	
PnPS1-BL	µg/mL	0.16	0.38	>1µg/ml*	
PnPS3-BL	µg/mL	0.22	3.2	>1µg/ml*	
PnPS4-BL	µg/mL	0.10	0.049	>1µg/ml*	
PnPS5-BL	µg/mL	0.26	0.072	>1µg/ml*	
PnPS6B-BL	µg/mL	0.27	0.061	>1µg/ml*	
PnPS7F-BL	µg/mL	0.72	>40	>1µg/ml*	
PnPS9V-BL	µg/mL	0.43	0.18	>1µg/ml*	
PnPS14-BL	µg/mL	1.4	4.5	>1µg/ml*	
PnPS18C-BL	µg/mL	4.9	0.051	>1µg/ml*	
PnPS19F-BL	µg/mL	1.2	0.38	>1µg/ml*	
PnPS23F-BL	µg/mL	0.13	0.082	>1µg/ml*	

*: >6 years of age, a normal response is defined as IgG reponses of >1µg/ml in at least 8/11 pneumococcal polyssacharide serotypes measured.

the mutation (Figure 1E) and it is not present in the dbSNP, or Dutch population- specific GoNL databases. Moreover the residue changed by the mutation is a highly conserved Leucine at the third position to a Proline (Figure 1B and 1C), predicted to be potentially damaging by PolyPhen-2, SIFT and Mutation Taster (Figure 1D). We determined by quantitative analysis that BLK RNA expression is not significantly different in human peripheral blood $CD19^+$ B-cells of healthy individuals and our CVID patients carrying L3P-BLK gene variant (figure 2A), but is absent in monocyte-derived dendritic cells (DCs) and human $CD4^+$ T-cells in circulation (our data not shown). Additionally, we seem to observe similar levels of BLK protein in human peripheral blood $CD19^+$ B-cells of healthy individuals and our index CVID patients (supplemental Figure 1A). Thus, L3P mutation appears to affect BLK function. Together this data prompted us to perform functional studies.

Reduced syk phosphorylation upon BCR crosslinking in L3P-BLK patient B-cells

In B-cells, BLK protein associates with Syk and Igα upon activation [22, 23]. The latter is phosphorylate by BLK upon antigen-induced BCR-crosslinking [4]. However, experiments executed in COS cells using an overexpressed mutated L3C-BLK earlier demonstrated that BLK-mediated Igα phosphorylation was blocked [17], suggesting that in analogy, L3P- BLK in human cells may have a functional defect at phosphorylation. We tested this possibility by measuring Syk phosphorylation, the immediate downstream consequence of Igα phosphorylation. We induced BCR-crosslinking by addition of anti-IgM and IgG-F(ab')2 fragments, fixed PBMCs at indicated time points, and measured levels of phosphorylated (p)Syk in $CD20^+$ B-cells by flow cytometry as previously performed by others [5]. Instead of anti-IgM/G antibodies, anti-IgM/ anti-IgG F(ab')2 fragments were used. This approach ensures specific BCR targeting and eliminated non- specific binding with Fc receptors. In healthy controls, BCR crosslinking induced increased amounts of pSyk within 2 minutes, which corroborates with previous data [5]. In contrast, in L3P-BLK carrying CVID patient B-cells pSyk levels were induced later and remained at 50% lower levels in comparison to primary B-cells of healthy individuals (Figure 2C). Surface expression of B-cell (co)receptor complex molecules CD19, CD20, CD21, and membrane-bound IgM, was similar on B-cells from individuals carrying L3P- and common BLK variant (Figure 2D). Thus, BCR-mediated signaling rather than B-cell (co)receptor complex components expression levels appear affected in L3P-BLK carrying B-cells of our CVID patients.

Reduced Syk phosphorylation upon BCR- crosslinking in L3P-BLK-overexpressing B-cell lines

In B-LCLs, that are immortalized by Epstein-Bar virus (EBV), EBV-derived Latent Membrane Protein 2a (LMP2A) drives activation of α- and β-chains of the BCR [24]. Similarly as the BCR, LMP2a associates with Src family of tyrosine kinases [25]. This capacity of B-LCLs allowed us to study the effect of L3P-BLK variant expression on

Table 2

Lymphocytes in peripheral blood of L3P-BLK carrying individual in comparison to age-matched controls.

Lymphocyte subset		patient (L3P-BLK)	reference values
$CD3^+$	(% within Ly)	76	57-76
$CD3^+CD4^+$ T-cells	(% within Ly)	46.1	29-46
$CD3^+CD8^+$ T-cells	(% within Ly)	24.5	19-34
$CD19^+$ B-cells	(% within Ly)	11	12-26
NK cells	(% within Ly)	11	6-21
$IgM^+IgD^+CD27^-$ naïve B-cells	(% within B-cells)	74	52-73
$CD27^-CD21^{int}CD38^{++}CD10^+IgM^{++}$ trans. B-cells	(% within B-cells)	9	2.9-23.8
IgD^-CD27^+IgM memory B-cells	(% within B-cells)	5.6	6.5-22.2
IgD^-CD27^+IgG memory B-cells	(% within B-cells)	2.6	1.5-8.8
IgD^-CD27^+IgA memory B-cells	(% within B-cells)	0.2	1.3-6.1

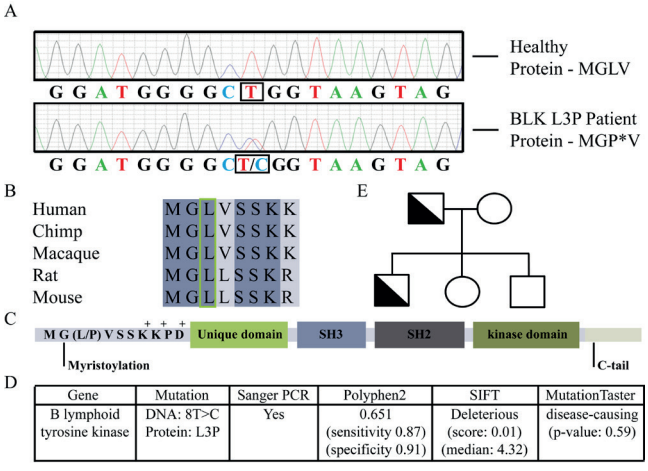


Figure 1

Classification of the novel L3P mutation in B lymphoid tyrosine kinase of Common Variable Immunodeficiency (CVID) patients

A. Confirmation of heterozygous mutation L3P in B lymphoid tyrosine kinase of CVID patient by Sanger PCR. B. The mutated Leucine indicated is an amino acid conserved amongst species. C. The mutation occurs at third position, between a myristoylated Glycine residue and a charged amino acid cluster. D. Three bioinformatics models predict a deleterious effect of the mutation on BLK function. E. Family tree showing CVID diagnosed L3P-BLK carrying patients (father and son) and non-carrying healthy family members.

B-cell signaling and function. To this end, we drove overexpression of either L3P- or common BLK variant in 3 separately derived B-lymphoblastoid cell lines (B-LCLs) by retroviral transduction. To enable comparison, we selected clones within each B-LCL cell line with similar overexpression levels of either L3P- or common BLK protein as determined by quantitative PCR (Figure 2B) and Western blot (Supplemental Figure 1B).

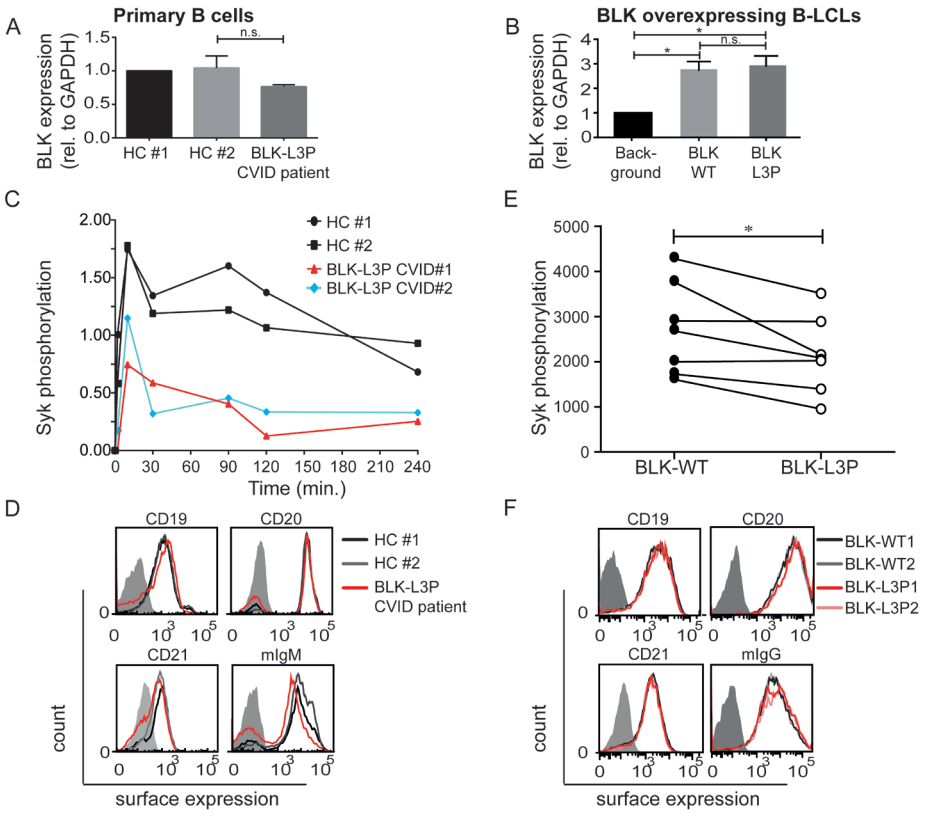


Figure 2

Reduced Spleen tyrosine kinase (Syk) phosphorylation upon B cell receptor crosslinking of CVID (L3P-BLK) patient cd20+ B cells.

Quantitative PCR demonstrates that heterozygous L3P-BLK CVID patient comparable levels of BLK mRNA (p=0.25) in A. CD19+ B cells relative to healthy controls or B. BLK overexpressing B-LCLs. Data of 3 independent experiments represented as mean +/- SEM. C. B cell receptors are crosslinked by incubation of PBMCs from CVID (L3P-BLK) patient or healthy controls with excess amounts of anti-IgM and IgG F(ab')2 fragments for the indicated duration (0, 2, 10, 30, 90, 120, and 240 minutes). At the end of incubation the cells are fixed and surface-stained with CD20 antibody. Subsequently, the cells are permeabilized with methanol and stained for intracellular

presence of phosphorylated Syk (pSyk), followed by flow cytometric analysis on live (FSC/SSC) CD20+ B cells. Representative of 3 independent experiments, presented as mean fluorescent intensity levels of phosphorylated Syk over time in CD20+ B cells of healthy controls (Black), CVID (L3P-BLK) patient (Red), and CVID patient #2 (Blue) also heterozygous for L3P-BLK mutation. D. Expression of B cell (co)receptor molecules CD19, CD20, CD21, and membrane-bound IgM as B cell receptor (mIgM) in CD20+ B cells of healthy controls (Black and Grey) and CVID (L3P-BLK) patient (Red). Representative of 3 independent experiments. Filled grey graphs are non-stained negative controls. E. LMP2a driven phosphorylation of Syk in B-LCLs overexpressing either L3P- or common BLK variant in simultaneously executed experiments. F. Expression of B cell (co) receptor molecules CD19, CD20, CD21, and membrane-bound IgM as B cell receptor (mIgM) in B-LCLs overexpressing L3P-BLK (Red) or common BLK variant (black/grey). Representative of 3 independent experiments. Grey filled graphs are non-stained B-LCLs. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

To address whether L3P mutation in BLK is solely responsible for the reduced levels of Syk phosphorylation observed in the primary $CD19^+$ B-cells of our CVID patients (Figure 2C), we analyzed B-LCLs overexpressing BLK and L3P-BLK for relative levels of pSyk similarly as described above. In analogy of the L3P-BLK primary B-cell data, also B-LCLs that overexpressed L3P-BLK exhibited reduced Syk phosphorylation without affecting expression levels of the B-cell (co)receptor molecules CD19, CD20, CD21, and membrane-bound IgG (Figure 2E and 2F). Thus, the L3P-BLK variant suppresses BCR crosslinking-induced Syk phosphorylation, when compared to the common BLK protein.

L3P-BLK variant negatively affects tonic signaling-dependent B-cell proliferation

B-cell proliferation requires tonic BCR signaling via Src family kinases to which BLK belongs[4]. We considered this finding in light that our L3P-BLK CVID index patient shows reduced numbers of circulating IgD^- $CD27^+$IgM and IgD^-CD27^+IgA memory B-cells (Table 2). Therefore, we investigated whether and to which extend L3P-BLK may affect B-cell proliferation. We stained B-LCLs overexpressing either the L3P-BLK or common BLK variant with Cell Tracer Violet at day 1, to assess the B-cell proliferation rate. During 4 days, cell tracer violet fluorescence was monitored using flow cytometry (Figure 3A). Already after the first day and continuing until the last day, B-LCLs overexpressing L3P-BLK had a small but significant delay in B-cell proliferation when compared to common BLK carrying B-LCLs (Figure 3B). All together, these data support that L3P-BLK has less proficiency than the common BLK variant to transduce tonic BCR signaling towards B-cell proliferation, which may contribute to the reduced B-cell number phenotype in the L3P-BLK CVID patient.

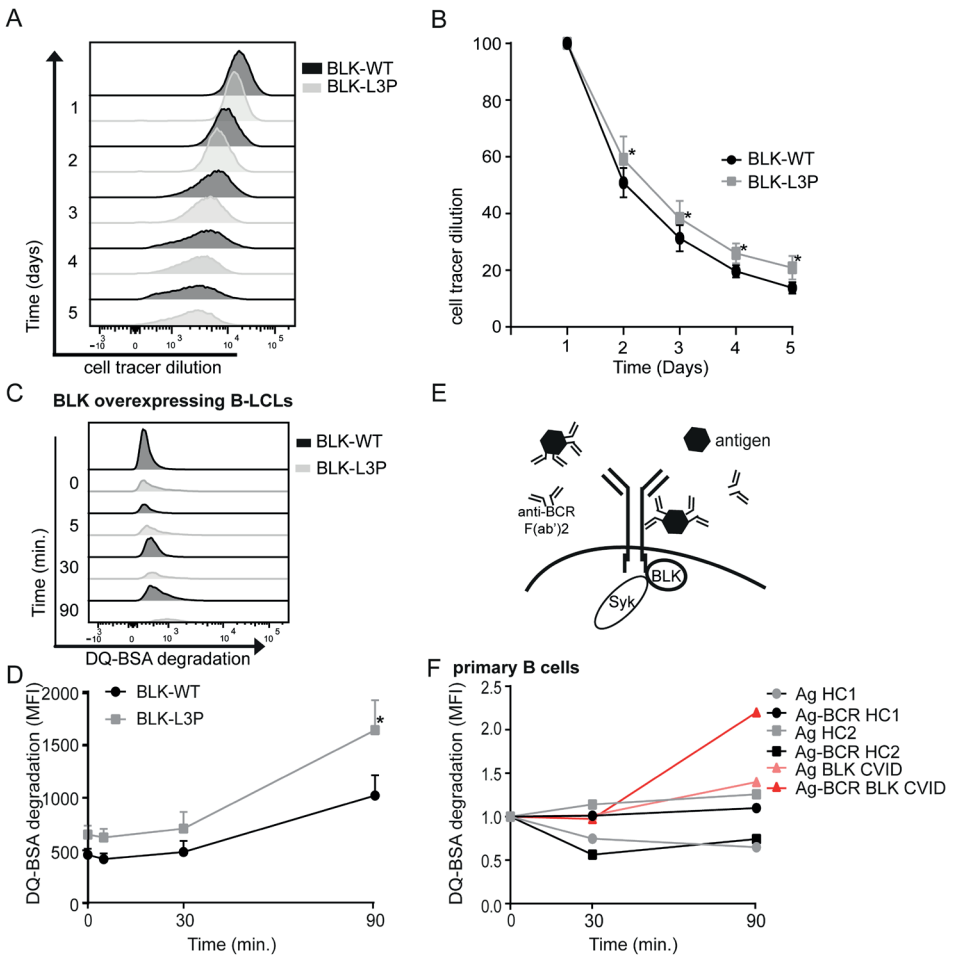


Figure 3

L3P-BLK has diminished ability to transmit tonic and ligand-induced B cell receptor signals.
A and B. B-LCLs overexpressing either L3P- or common BLK variant are stained with cell tracer violet and B cell proliferation was determined by dilution of cell tracer violet MFI measured by flow cytometry each day. Data of 3 independent experiments, represented as mean +/- SEM. C. DQ-BSA becomes fluorescent when cleaved. DQ-BSA degradation was measured by flow cytometry as MFI increase. D and E. Streptavid-modified DQ-BSA is complexed in 4:1 ratio with biotinylated anti-IgM and anti-IgG to target to BCR. These anti-BCR/DQ-BSA complexes are administered to B-LCLs overexpressing L3P- or common BLK variant, or F. in primary CD19+ B cells derived from healthy volunteers (squares and circles) or BLK-L3P carrying CVID patients (triangles). Representative (F) of Data (D) of 3 independent experiments, represented as mean +/- SEM. *P-value <0.05, **P-value <0.01, Two-tailed Wilcoxon-signed rank test.

B-cell receptor endosomal routing upon antigen binding is altered in B-LCLs that overexpress L3P-BLK compared to the common BLK variant

While superfluous in tonic BCR signaling, Syk kinase function is considered essential for the propagation of antigen-induced B-cell signaling (reviewed in [23]). To investigate whether the L3P-BLK defect to elicit Syk phosphorylation propagates to downstream defects, we determined the fate of BCR-targeted antigen upon endocytosis, using DQ-BSA complexed to anti-IgG F(ab')2 fragments (Figure 3E). DQ-BSA is a model antigen that becomes fluorescent upon proteolytic degradation in acidic late endosomal compartments. After BCR-mediated uptake of anti-IgG/DQ-BSA complexes in B-LCLs, we observed accelerated degradation of DQ-BSA in L3P- than common BLK expressing B-LCLs (Figure 3C and 3D). Similarly, our CVID patient B-cells showed faster degradation of specifically BCR-targeted DQ-BSA in comparison to non-targeted DQ-BSA or $CD19^+$ B-cells derived from healthy individuals (Figure 3F).

Internalization of BCR complexes upon crosslinking with anti-aIgG antibodies seems unaffected in B-LCLs expressing either BLK variant (supplemental figure 2), which corroborates with unaltered expression of B-cell (co)receptor complex components on B-cell surface (Figure 2F). All together, the L3P-BLK variant when seems to direct the sorting of BCR-internalized antigen/ IgG complexes towards proteolytic active, degradative endosomal compartments.

L3P-BLK variant obstructs B-cell receptor mediated antigen presentation to antigen-specific CD4+ T-cells

Could the rapid degradation of antigen-BCR complexes modulate the ability of L3P-BLK B-cells to function as antigen presenting cells? The generation of high-affinity IgG and IgA antibodies requires class II MHC-mediated antigen presentation by B-cells to invoke T-cell help and CD40 binding by CD40L upregulation on antigen-triggered T-cells [26]. Indeed, Syk signaling participates in BCR-mediated antigen processing and presentation of human B-cells [27, 28]. Considering both L3P-BLK CVID patients are hypogammaglobulinemic and have low post-vaccination titers elicited by T-cell-dependent antigens, we hypothesized that L3P-BLK protein may be less capable to facilitate class II MHC antigen presentation by B-cells, when compared to the common BLK protein. To address this question, we generated Tetanus Toxoid (TT)-IgM/G immune complexes by incubating biotinylated anti-IgM/anti-IgG F(ab')2 fragments to streptavidin-conjugated TT in 2:1 or 4:1 (*w:w*) ratio overnight (Figure 3B) [29]. We administered BCR-targeted TT-Ig complexes to paired B-LCL lines expressing either L3P-BLK or the common BLK for 4 hours (37°C), followed by overnight co-culture with TT- specific $CD4^+$ T-cells. We determined stimulation levels of TT-specific $CD4^+$ T-cells by measure of upregulated CD40L, using flow cytometry (Figure 4A). The fraction of $CD4^+$ T-cells expressing high levels of CD40L increases in antigen dose-dependent manner by both L3P- or common BLK variant expressing B-LCLs. However, antigen-

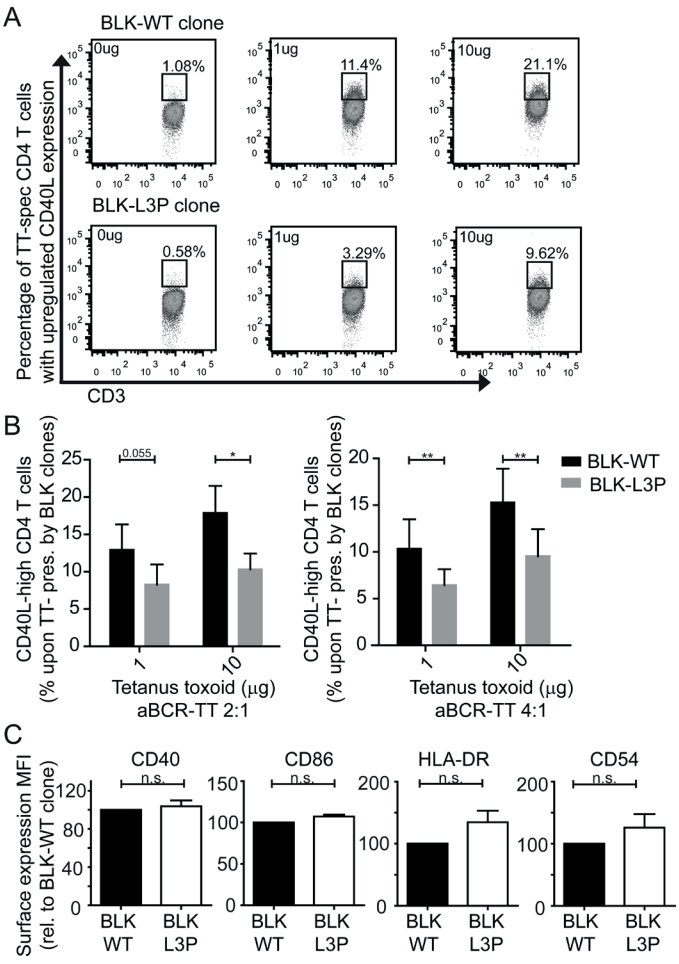


Figure 4

Decreased capacity of L3P-BLK to facilitate B cell receptor-mediated HLA-DR/peptide presentation to CD4+ T-cells.

Tetanus toxoid (TT) protein is complexed with anti-IgG F(ab')2 fragments in ratio 1:2 or 1:4 for 6 hours at 4C (using streptavidin/ biotin conjugation). Paired human B-LCLs expressing common or L3P-BLK variant are loaded (4hrs, 37°C) with 0, 1, or 10 μg anti- IgG-TT complexes. Next, human TT peptide/MHCII-specific CD4+ T-cells are added for co-culture with the B-LCLs (1:1 ratio, O/N, 37°C). B-LCL mediated activation of antigen-specific CD4+ T-cells was measured by analysis of induced CD154 (CD40L) expression. A. CD40L staining in absence of exogenous antigen is set as background levels for both B-LCLs expressing common BLK (upper panel) or L3P-BLK (lower panel). Representative of 3 independent experiments. B. L3P-BLK expressing B-LCL cells (grey) are less able to induce activation of antigen-specific CD4+ T-cells (CD40L high phenotype)

compared to common BLK-expressing B-LCL cells (black). Experiments performed on B-LCLs of three independent donors, in at least three independent experiments. Data represented as mean +/- SEM. C. Expression of L3P-BLK does not affect expression of class II MHC molecules (HLA-DR) and co-stimulatory molecules (CD40, CD86, and CD54) in B-LCLs. Data of 3 independent experiments, represented as mean +/- SEM. *P-value <0.05, **P-value <0.01, Two- tailed Wilcoxon-signed rank test.

driven activation of the TT-specific $CD4^+$ T-cells is significantly suppressed in co-cultures using B-LCLs expressing L3P- BLK compared to common BLK variant (Figure 4A, 4B). These data support that L3P-BLK obstructs antigen- specific B-cell mediated antigen presentation to $CD4^+$ T-cells. Considering that the recruitment of $CD4^+$ T-cell help in form of CD40-CD40L engagement is required to generate high affinity antibodies, these data clarify an unexpected role for B-cell expressed L3P-BLK in the elicitation of $CD4^+$ T-cell help, with relevance to CVID disease pathology. After 4 hours of incubation of TT-anti IgG F(ab')2 complexes with B-LCLs overexpressing L3P- or common BLK variant, we measured the surface expression levels of CD40, CD86, class II MHC (HLA-DR), and CD54 (ICAM-1). Surface expression of these markers was comparable, as determined by flow cytometry (Figure 4C). This unaltered display of HLA-DR and co-stimulatory molecules suggest that the L3P mutation in BLK does not affect overall antigen-induced BCR-crosslinking dependent B-cell activation. Thus, the L3P-BLK variant obstructs BCR-signaling and sorting of BCR-internalized antigen towards endosomal compartments conductive to Class II MHC-mediated antigen presentation. These data clarify a previously unrecognized role of human BLK through Syk signaling in the support of antigen processing and peptide/Class II MHC presentation by B-cells.

DISCUSSION

The selection and expansion of antigen-specific B-cells to become functional Ig-secreting plasma cells and generate memory B-cells requires BCR-signaling and antigen-specific $CD4^+$ T-cell help, most notably through CD40L-CD40 engagement [30–32]. Molecules involved in the Ig isotype class-switching and eventual production of large amounts of high affinity antibodies include RAG [33], AID [34], and UNG [35], and additionally the following B- and T-cell receptor signaling molecules are involved: CD20 [7, 36], CD21 [37], CD81 [8], ICOS [38], and CD40L [39]. Mutations affecting genes encoding these proteins are found in human patients that suffer from primary immunodeficiencies, including CVID [2]. We here report the functional analysis of a CVID-associated variant in the protein BLK, a Src-kinase family member that serves early downstream of the BCR. We describe a defect in BCR-triggering-induced Syk

phosphorylation by the CVID-associated L3P-BLK variant. Analysis of this CVID-related BLK mutant moreover allowed us to clarify a role for BLK in B-cell proliferation and BCR-mediated antigen presentation to elicit activation of antigen-specific $CD4^+$ T-cells in humans.

As member of the Src kinase family, BLK shares a conserved tyrosine kinase domain with other family members. While mouse-based experiments suggested that the catalytic activity of Src kinases is redundant [18–20], we found that in human B-cells this is not the case. Specificity is supported by tissue-specific expression and subcellular localization. Myristoylation, at the second amino acid of BLK, is necessary for its membrane localization and kinase activity. Exchange of the third amino acid of BLK for a cysteine has earlier been shown to abrogate its ability to phosphorylate Igα [17]. Hence, our identified mutation of the third amino acid of BLK into Proline may cause defective myristoylation and thereby affect localization of BLK to lipid bilayers. However, the retroviral construct we used to overexpress BLK and L3P-BLK in B-LCLs has incorporated an additional myristoylation-tag distal from the L3P mutation, thereby relieving the requirement of the myristoylation motif around the L3P mutation. Thus, while we did find a functional defect in B-LCLs overexpressing L3P-BLK, the defect in patient B-cells may be even more severe due to obstruction of myristoylation-mediated subcellular membrane localization. Of note, besides myristoylation, additional motifs support the anchorage of Src kinases to membranes, including palmitoylation [17, 40]. In Src, and probably BLK, several basic amino acids appear to interact with inner leaflet membrane phospholipids that are acidic [40]. Considering that electrostatic interactions are sensitive to disruption, supporting the possibility that a L3P mutation could have effects on localization and thereby function. Attempts to address this possibility directly, by visualization of the subcellular localization of BLK and L3P-BLK in primary B-cells from patients and healthy controls, were unfortunately unsuccessful due to the relative small cytosol volume present in primary B-cells.

In mice, functional redundancy between Src kinase family members is apparent as a single Src kinase member- knockout mice has no or subtle deficiencies [18–20]. Only when several Src family members are deleted, in double or triple knock outs, major defects are observed; SRC/ YES and SRC/FYN are lethal whereas HCK/FGR double knock-out mice are immune-compromized [18]. In the human, Src-kinase family members are less superfluous [41]. Especially BLK seems to be non-redundant in function, as BLK is the only Src kinase family member that is able to phosphorylate and subsequently associate with co-transfected Igα and Igβ chimeras *in vivo* [4]. We believe this to be the reason that the CVID-associated BLK mutation has functional consequences.

Diminished B-cell proliferation and T-cell help is associated with reduced numbers of class-switched memory B-cells and defective production of high affinity antibodies, as

showed for CD20 [2, 36], CD21 [37], CD81 [8], ICOS [11], and CD40L [42] deficient CVID patients. In addition, selective CVID patient T-cells have a reduced T-cell responses to tetanus toxoid, even though primary allo-stimulation of the same T-cells was normal in CVID patients [43]. Moreover, reduced $CD4^+$ T-cell numbers are reported in several CVID patients. All these data support that defective elicitation of $CD4^+$ T helper cell help may contribute or even cause pathology in a subset of CVID patients. In line with this, our CVID patients that also show reduced numbers of class-switched memory B-cells and defective production of high affinity antibodies carry a L3P-BLK variant that distort BCR signaling required for B-cell proliferation and recruitment of T-cell help. We propose that dysfunctional BLK variant underlies CVID disease pathology by perturbing B-cell proliferation and elicitation of antigen-specific $CD4^+$ T-cell help. Further research should be aimed to determine the proportion of CVID patients that harbor defects in BLK or other early B-cell activation-related signaling molecules, and how gene defects overall relate to distinct B-cell functions as antigen presenting cells and Ig-secreting plasma cells.

MATERIALS AND METHODS

Patients and healthy donors

The index patient, his parents, and his brother and sister were included in this study. Adult volunteers were healthy employees of the University Medical Center Utrecht. This study was approved by the institutional review board, and informed consent was obtained.

Targeted next-Generation Sequencing

The Next-Generation Sequencing is targeting 170 PID-related (IUIS[2]) and >350 putatively PID-related genes[9]. We used both targeted array-based and in-solution enrichment combined with a SOLiD sequencing platform and bioinformatics analysis, as described previously [12]. Subsequently, the selected variant was validated with Sanger sequencing. Amplicons were bidirectly sequenced with the Big Dye Terminator version 3.1 cycle sequencing kit and an ABI 3730 DNA Analyzer (Life Technologies). Sequences were compared with reference sequences by using Mutation Surveyor (SoftGenetics). The prevalence of the BLK gene variant was determined in the dbSNP and GoNL exome databases.

B-cells overexpressing B-Lymphoid tyrosine kinase variants

The CVID-associated mutation of BLK was inserted in pWZL-Neo-Myr Flag-BLK (Plasmid 20430, Addgene) by site-directed mutagenesis according to manufacturers protocol (Qiagen) using primers (Sigma-Aldrich): BLK Fwd1: CACCTGGATGAAGACAAGCA and BLK Rev1: CCTTCCGACCCTGTGATCTA. Packaging cells (Phoenix-Ampho) were

transfected with gag-pol (pHIT60), env (pCOLT-GALV), and pWZL-Neo-Myr Flag-BLK wildtype or disease-associated variant, using Fugene6 (Promega). The produced virus particles were applied to freshly thawed B-Lymphoblastoid Cell Lines from 4 different healthy donors. After 1 week of selection, B-LCLs were used in experiments.

Quantitative PCR

Freshly isolated PBMCs or cultured B-LCLs overexpressing BLK disease-associated or wildtype variant were lysed and total mRNA was isolated using Tripure isolation reagent (Roche Diagnostics) according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometer and equalized for all samples prior to reverse transcription using an iScript cDNA synthesis kit (Biorad). Primers were mixed with IQ SYBR green supermix (BioRad). The detection run started at 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60°C for 1 min. Assays were performed in duplicate or triplicate as 15µl reactions in 96well plates using C1000 Thermal Cycler (BioRad). Results were normalized to the endogenous GAPDH and Actin mRNA. The following primers were used: GAPDH Forward 5'-GTCGGAGTCAACGGATT-3'; GAPDH Reverse 5'-AAGCTTCCCGTTCTCAG-3'; Actin Forward 5'-CATGTACGTTGCTATCCAGGC-3'; Actin Reverse 5'-CTCCTTAATGTCACGCACGAT-3; BLK Forward 5'-CACCTGGATGGAAGACAAGCA-3'; BLK Reverse 5'-CCTTCCGACCCTGTGATCTA-3' (All Sigma- Aldrich).

Flow cytometry and functional assays

Isolate PBMCs by Ficol-plaque and let them rest for at least 2hours at 37C. Stimulate rested PBMCs or equal amount of B-LCLs (BLK-wt and L3P variant) for 0, 4, 10, 30, 90, 120, and 240 min. with goat anti-human IgM and IgG F(ab')2 fragments (5µg/ml). This is followed by fixation with 1.3% EM grade paraformaldehyde (Electron Microscopy Technologies) for 5 min. at RT. These cells were washed and taken up in FACS buffer (PBS complemented with 1% Bovine Serum Albumin (BSA, Roche) and 0.1% Sodium Azide). Extracellular CD20 (Pacific Blue, Biolegend) and IgM or IgG (PE, Fab fragments, Invitrogen) were stained (RT, 30 min.) prior to permeabilization with ice-cold methanol (4C, 5 min.). Cells are rehydrated and washed with PBS+ 1% BSA, and phosphorylated Syk was stained (PECy7, cl17A1P- ZAP70, BD Bioscience, RT, 20min.)

Goat anti-human IgG and IgM F(ab')2-fragments (Invitrogen) were conjugated to EZ-link Sulfo-NHS Biotin according to manufactures protocol (Thermo Scientific). Purified Tetanus Toxoid (RIVM, the Netherlands) or DQ-BSA (Life Technologies) was conjugated to Lightening-link streptavidin according to manufactures protocol (Novus Biologicals). Biotinylated anti-IgM or anti-IgG F(ab')2 fragments are complexed overnight to streptavadin-conjugated DQ-BSA or Tetanus Toxoid in 2:1 or 4:1 (*w:w*) ratio.

For $CD4^+$ T-cell activation assay, these complexes were incubated for 4 hours to PBMCs or B-LCLs. Followed by overnight incubation with tetanus toxoid- specific $CD4^+$ T-cell clones. Antigen-specific CD154 expression on $CD3^+CD4^+$ T-cells was determined by staining with CD154 (Pacific Blue, Biolegend), CD3 (APC, BD Bioscience), and CD4 (FITC, eBioscience).

For antigen degradation assay, anti-IgM/G DQ- BSA complexes are administered to B-LCLs expressing either L3P- or common BLK variant and put on ice at indicated timepoints. BCR-targeted antigen destruction was determined by Flow cytometric analysis of the MFI emitted by processed DQ-BSA per B-LCL.

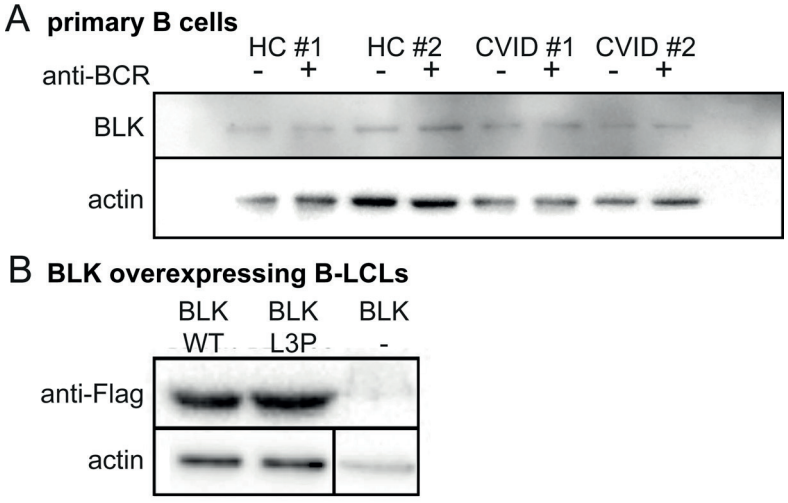
BCR complex molecules and co-stimulatory molecules were determined by flow cytometric analysis upon staining PBMCs or B-LCLs with following antibodies: CD19, CD21, CD81, CD40, and CD54 (all from BD Bioscience); CD20, CD86, HLA-DR, CD154, streptavadin-APC (all Biolegend).

B-cell proliferation assay

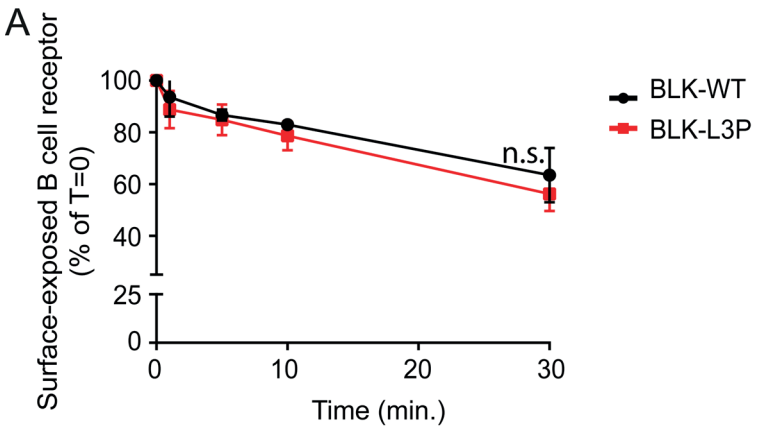
B-LCLs proliferation was determined by the dilution rate of Cell Tracer Violet (Invitrogen). To this end, 1x10^6 B-LCLs expressing either BLK variants are stained with 2μM Cell Tracer Violet for 10 minutes at 37C. Staining and excess Cell tracer Violet was removed by spinning B-cells down in 5x volume Fetal Calf Serum (FCS). Cells were maintained in a humidified incubator at 37 °C with 5% CO for 4 days in RPMI 1640 medium with 1% (v/v) PenStrep (Invitrogen), 1% (v/v) GlutaMAX (Invitrogen), and 10% (v/v) FCS. Mean Fluorescent Intensity was determined on the Flow cytometer at day 1, 2, 3, 4.

Statistical Analysis

Flow cytometry data were collected on FacsCanto II (Becton Dickinson) and analyzed with BD FACSDiva v6.1.3 and Flowjo 7.6 software (Treestar). All data were statistically analyzed and plotted with GraphPad Prism® 5 software (GraphPad Software, Inc, La Jolla, California).



Supplemental figure 1



Supplemental figure 2

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CHAPTER 3

CD2-signaling and immunological synapse stability in T-cells requires PSTPIP1, as identified in primary immunodeficiency patients

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Manuscript in preparation

ABSTRACT

PSTPIP1 is a cytosolic adaptor protein involved with T-cell activation, differentiation, and cell migration. Upon T-cell receptor (TCR) triggering, PSTPIP1 is recruited to surface-expressed CD2, where it regulates actin cytoskeletal support for immune synapses (IS) formation. From genetic screening of primary immunodeficiency patients, we identified two mutations in PSTPIP1 in patients with opposing T-cell differentiation defects, R228C and T274M. The R228C patient had increased T-cell numbers with mostly naive phenotype and few memory T-cells; the T274M patient had 75% reduction in CD4 T-cells that were predominantly of memory subset.

We observed f-actin polymerization defects in both PSTPIP1 patient T-cells, most notably in T274M. Unstimulated R228C PSTPIP1 patient T-cells showed reduced levels, and T274M significantly increased levels of polymerized f-actin. The capping of lipid micro-domains upon antibody binding at the cell surface is known to require f-actin polymerization. Capping of CD2-containing micro-domains was disrupted when analyzed in transfected HEK cells overexpressing T274M PSTPIP1. Analysis of IS formation using immortalized Jurkat T-cell transfectants revealed a reduction in f-actin accumulation at the APC contact area, again especially in T274M PSTPIP1 cells. TCR-triggering of PSTPIP1-T274M patient T-cells resulted in a significantly diminished calcium flux. Based on these findings, we propose that PSTPIP1 T-cell differentiation defects are caused by defective control of f-actin polymerization. A pre-activated polymerized f-actin status, as seen in PSTPIP1-T274M T-cells, appears particularly damaging. The consequential decrease in dynamic control of f-actin remodeling causes less stable cell adhesion, and eventually T-cell depletion.

INTRODUCTION

Common Variable Immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency in adults and children. Patients encounter recurrent viral and bacterial infections of the respiratory and gastrointestinal tract due to ineffective pathogen clearance through strongly diminished immunoglobulin levels(1–3)(4). Novel primary immunodeficiency (PID) associated genetic defects are now identified more rapidly through next generation sequencing (NGS) techniques. However in case of CVID, monogenetic causes of disease are not identified in the vast majority of patients(5,6). Next to B-cell differentiation and maturation disturbances (low class switched memory B-cell numbers) CVID patients may also exhibit aberrations within the T-cell compartment(4)(7)(8,9). Typically these patients display low circulating T-cells numbers, relative CD8 T-cell expansion due to diminished CD4 T-cell numbers and low numbers of CD4 naive T-cells. Several early T-cell signaling gene mutations have been described in PID patients, which can be (in)directly linked to f-actin remodeling, essential for immune synapse (IS) formation(10). Protein defects in Vav1 and ZAP70 are examples of T-cell early signaling genes that have been specifically associated with CVID disorders, where the hampered TCR activation and strongly diminished f-actin remodeling resulted in defective IS formation(9,11).

We identified two novel Proline Serine Threonine Phosphatase Interacting Protein 1 (PSTPIP1) mutations, a threonine to methionine substitution at aa274 (T274M) and arginine to cysteine at aa228 (R228C), in two patients with CVID and T-cell disturbances, the R228C patient with increased naive CD4 T-cell percentages and the T274M patient with low naive CD4 T-cell percentages. Upon T-cell activation via antigen-specific T-cell receptor (TCR) interaction with cognate peptide/HLA complexes, cytosolic PSTPIP1 protein is recruited to the surface-expressed TCR co-receptor and cell adhesion molecule CD2. PSTPIP1 thereafter acts as a scaffolding protein for signal transduction towards T-cell activation. Accordingly, PSTPIP1 is dephosphorylated by PTP-PEST in order to downregulate the CD2- mediated cell adhesion and on the other hand modulate f-actin polymerization through the coupling of PTP-PEST to WASP, optimally geared towards formation of a stable T-cell:APC immune synapse(12–17). Clinically, PSTPIP1 mutations are known to cause pyogenic arthritis, pyoderma gangrenosum and acne (PAPA)-syndrome and are associated with Familial Mediterranian Fever (FMF) syndromes(19–24).

In this study we aimed to clarify the role of PSTPIP1 in T-cell differentiation, by study of the two CVID patients in which the T274M and R228C PSTPIP1 variants were found. To this end, we performed functional experiments with primary patient T-cells and PSTPIP1 WT, T274M and R228C mutation transfected cell lines. We demonstrate in primary patient CD4 T-cells that the T274M PSTPIP1 variant derails activation-induced

f-actin polymerization, as contributed by increased steady-state binding of PSTPIP1 to CD2, leading to a pre-activated f-actin cellular state. Furthermore the T274M patient CD4 memory T-cells were reduced in responsiveness to TCR activation, as showed by reduced cytosolic calcium mobilization and diminished f-actin recruitment. Overall our findings support that PSTPIP1 mutations can underlie T-cell defects in CVID patients and should not only be considered in PAPA- or FMF-syndrome patients.

RESULTS

Two CVID patients with novel PSTPIP1 mutations

Patient 1 presented with recurrent respiratory tract infections complicated with bronchiectasis, severe enteropathy and an unclassified skin rash. Laboratory screening revealed low IgG, IgA and IgM levels (<-2SD), absent specific anti-pneumococcal serotype IgG responses and diminished memory B-cell percentages.

Patient 2 presented with recurrent viral and bacterial infections of the respiratory tract and learning disabilities. Again laboratory screening revealed low IgG and IgA levels (<-2SD) and absent specific responses to pneumococcal serotypes. Also memory B-cell percentages were strongly diminished (no detected IgA memory B-cells, IgG memory B-cells 0.2%). Furthermore, both patients exhibited severe T-cell abnormalities in opposite directions. Patient 1 presented with a high number of T-cells (2286/mm3 N 700-900) and high percentage of naïve CD4+ T-cells whereas patient 2 presented with low T-cell numbers (305 /mm3 N 700-900) and very low CD4 T-cell numbers (157/mm3 N 560-1067) and low naïve CD4+ T-cell percentages (<-2SD) (Fig1A), with a slight overrepresentation of central memory T cells (Fig 1B).

Based on the clinical and laboratory findings both patients were diagnosed as CVID and were treated with immunoglobulin substitution therapy (24). The clinical course of patient 1 was progressive with regard to the CVID-related enteropathy, where the patient only partially responded to immunosuppressive therapy, anti-TNFa therapy and tacrolimus. Furthermore the patient developed a gastric adenoma complicated by a pneumocystis pneumonia.

Through next generation sequencing(25), patient 1 was found to have a heterozygous point mutation c682C>T in the PSTPIP1 gene, resulting in a R228C amino-acid substitution in the coiled coil region of the PSTPIP1 protein. Patient 2 had a heterozygous point mutation at c821C>T resulting in a T274M amino-acid substitution (Fig 1C,D). Pathogenicity of both point mutations was predicted to be high (no expression in the normal population) and predicted to be disease causing.

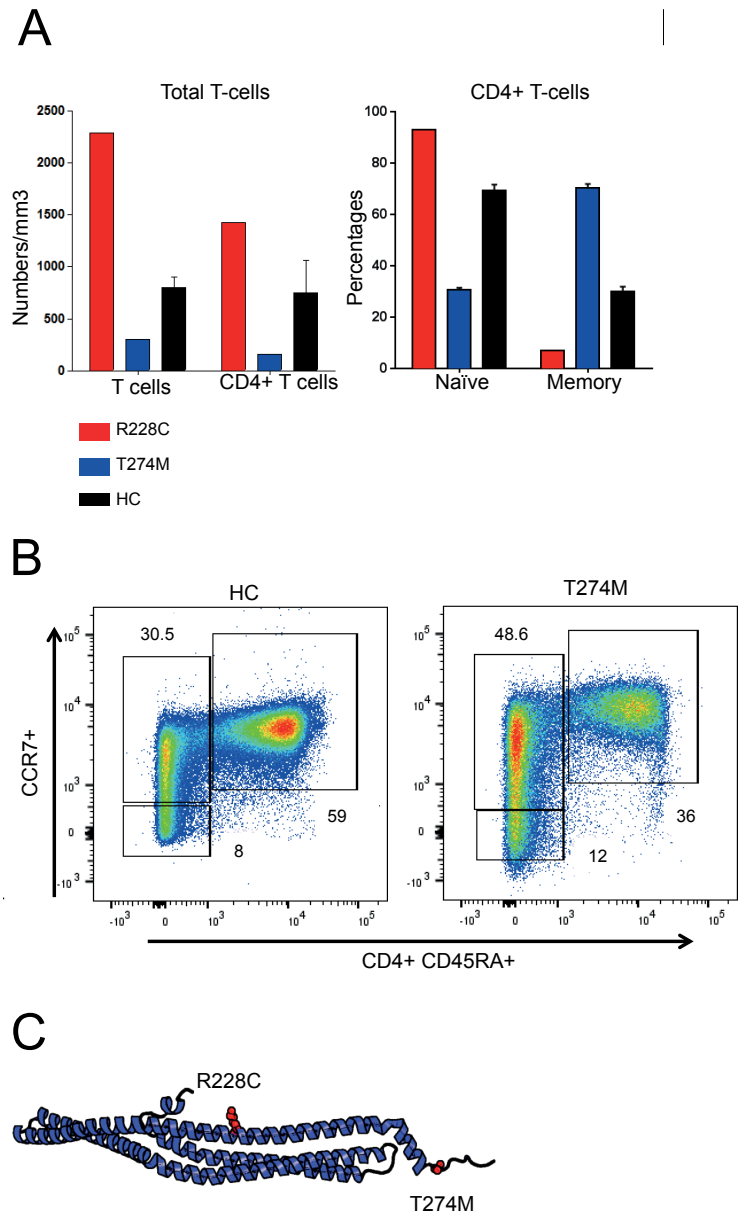


Figure 1 (A, B, C)

A) Total and CD4+ T-cell numbers of R228C and T274M PSTPIP1 patients as compared to healthy controls B) CD4+ memory T-cells of T274M and HC (representative from n=5), down left effector memory, up left central memory, up right naive CD4 T-cells C) in silico model of the PSTPIP1 coiled coil domain with identified mutations

HC: Healthy control; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells

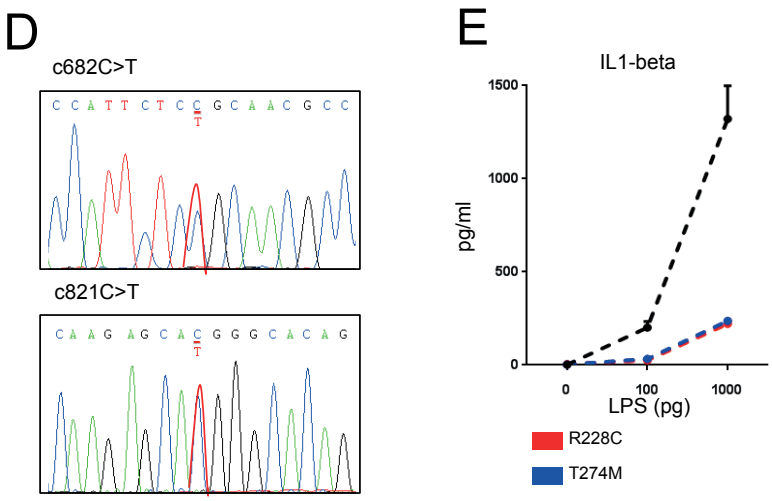


Figure 1 (D, E)

D) DNA sequencing of the R228C (up) and T274M (down) PSTPIP1 patients E) LPS stimulation of R228C and T274M PBMC in comparison to HC's (n=3)

HC: Healthy control; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells

PSTPIP1 mutations are shown to be causative of Pyogenic Arthritis, Pyoderma gangrenosum and Acne (PAPA)-syndrome(23,20). Our patients did not have any PAPA syndrome characteristics, even more, serum IL1-beta levels were not increased (data not shown), and no increased IL1-beta levels were detected after PBMC stimulation with LPS(19)(Fig 1E).

We thus concluded that the PSTPIP1 mutations as found in our patients did not result in a typical PAPA syndrome, but could possibly contribute to the disturbed T-cell compartments of the two CVID patients. To this end, we performed functional experiments in order to further investigate the role of PSTPIP1 in the altered T-cell differentiation.

Defective activation-induced f-actin polymerization of T274M PSTPIP1 patient CD4 T-cells

PSTPIP1 in T-cells contributes to the regulation of f-actin remodeling. Triggered by TCR and CD2 ligation, PSTPIP1 serves to scaffold the interaction between CD2, protein tyrosine phosphatase (PTP-PEST) and the Wiskott Aldrich Syndrome Protein (WASP), the latter protein driving f-actin polymerization. PTP-PEST downregulates TCR/CD2 signaling and WASP activation through dephosphorylation of PSTPIP1, thus keeping the f-actin modulation and immune synapse formation under tight control(14,16, 17, 23). We here addressed whether the disturbed PSTPIP1 patient T-cell phenotypes might result from aberrant PSTPIP1 functioning in regulating f-actin polymerization.

To this end, primary CD4 T-cells of both patients were stimulated through crosslinking of anti-CD2 and anti-CD3 antibodies with goat anti-mouse IgG fragments for 0, 2, 5 and 10 minutes. Subsequently f-actin polymerization was quantified through staining with phalloidin-FITC and measured by flow cytometry (12). We found increased levels of polymerized f-actin in the unstimulated T274M, when compared to healthy controls (Fig1A,B). The CD4 T-cells of the T274M patient seemed particularly unresponsive to anti-CD2 stimulation, as indicated by limited f-actin polymerization responsiveness (Fig2A,C) and a significantly lower area under the curve (AUC) when compared to healthy age matched controls (Fig2C). Stimulation of CD4+ T274M patient T-cells with anti-CD3 resulted in a steady increase of f-actin content over time (Fig2A and 2B), as opposed to the healthy controls and the R228C patient which showed a decrease of f-actin after 10 min. Thus, especially the CD4+ T274M T-cells show a pre-activated status of f-actin, which derails cytoskeletal remodeling, triggered by T-cell activation through anti-CD2 or anti-CD3.

Does the cytoskeletal remodeling defect in T274M patient T-cells have signaling consequences? We next assessed the cytosolic calcium mobilization of primary patient and healthy control CD4 memory and naive T-cells, as triggered by crosslinking of CD3 with anti-CD3 and goat anti-mouse IgG fragments. First of all, the baseline calcium levels were higher in the T274M patient CD4 T-cells than in CVID patients with normal or altered CD4 T-cell differentiation (Fig 1D), known to associate with a pre-activated state. Activation-induced calcium mobilization of T274M T-cells was low when compared to either healthy control T-cells or CVID patient T-cells with T-cell differentiation defects (low CD4 naïve T-cell percentages) (Fig 2E). Separate analysis of naïve and memory T-cell calcium fluxes (CD4CD45RA+ and CD4CD45RO+), revealed that especially the memory subset of CD4 T274M T-cells were less responsive than their cellular counterparts of CVID patients with altered T-cell differentiation (Fig 2D). The TCR CDR3 repertoire however did not differ significantly from healthy control in contrast to what has been described in other T-cell CVID patients(27,28) (our unpublished data).

Considering that controlled f-actin polymerization is pivotal for cell motility(29)(30), we investigated T-cell migration of primary CD4 T-cells of the T274M patient and healthy controls. CD4 T-cells were enriched from T274M patient (n=2) and healthy control (n=5) PBMC's. Thereafter, T-cell migration was observed in a collagen matrix by use of light microscopy in time-lapse manner (2 images per minute), for the duration of 4 hours. Indeed, the T274M CD4 T-cells displayed faster motility than the healthy controls at baseline without stimulation (Fig 2F) and this corresponded with the higher baseline f-actin content of the T274M CD4 T cells.

Taken together, the resting CD4 T cells of the PSTPIP1 T274M variant patient are already in a pre-activated state and are then unable to further respond to TCR and anti-CD2 triggering.

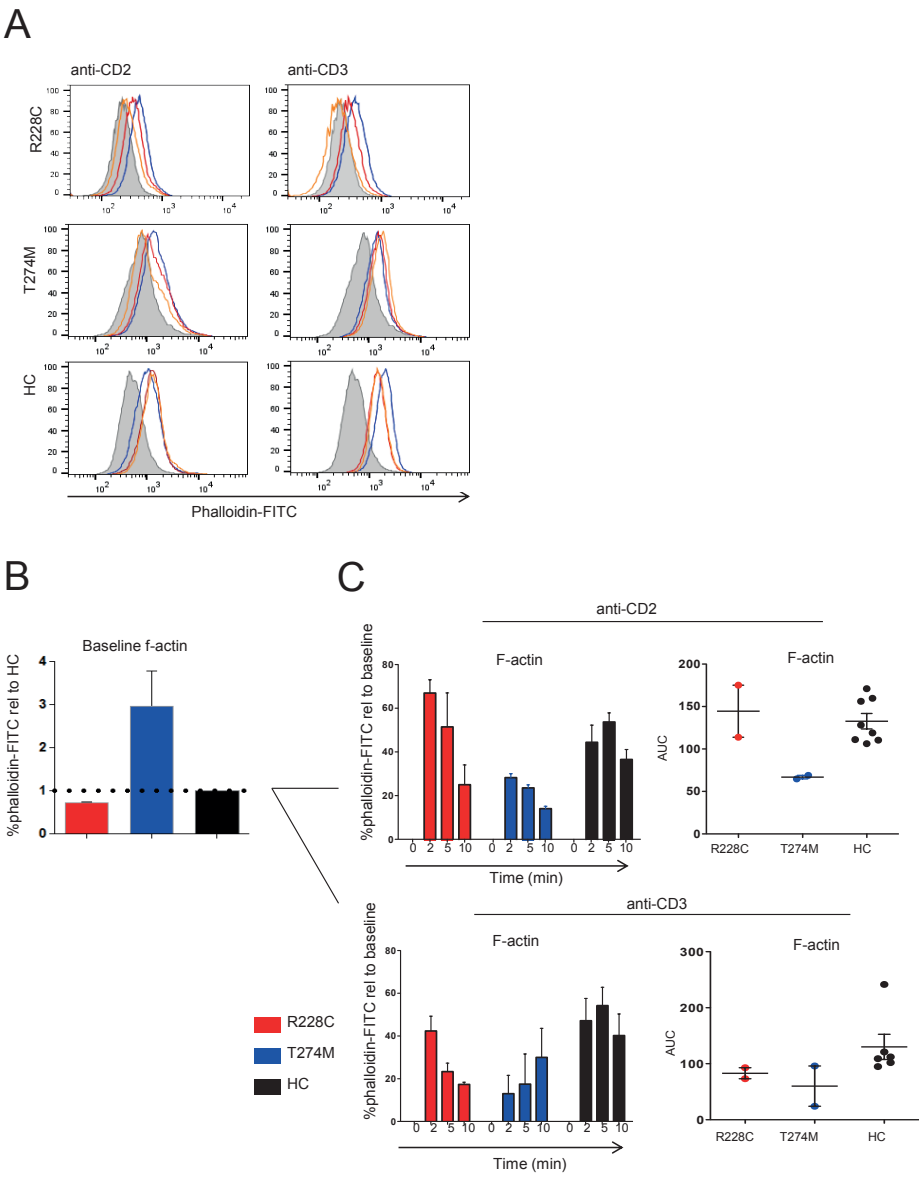
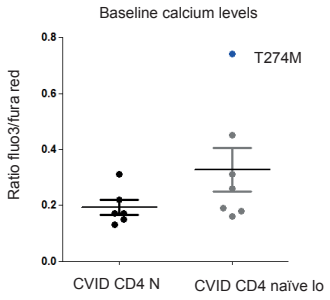


Figure 2

A) Representative histograms of f-actin MFI's (as stained with phalloidin-FITC) of R228C, T74M, and HC CD4+ T-cells stimulated with anti-CD2 and anti-CD3 antibodies B) Baseline f-actin percentages of R228C and T274M PSTPIP1 patients relative to HC baseline f-actin C) f-actin increase of R228C (n=2), T274M (n=2) and HC (n=8) upon anti-CD2 and anti-CD3 stimulation relative to the baseline f-actin levels (as depicted in 2B) of the individual patients and HC's D) Baseline cytosolic calcium levels of CVID patients with normal CD4+ T-cell compartments as compared to CVID patients with

D



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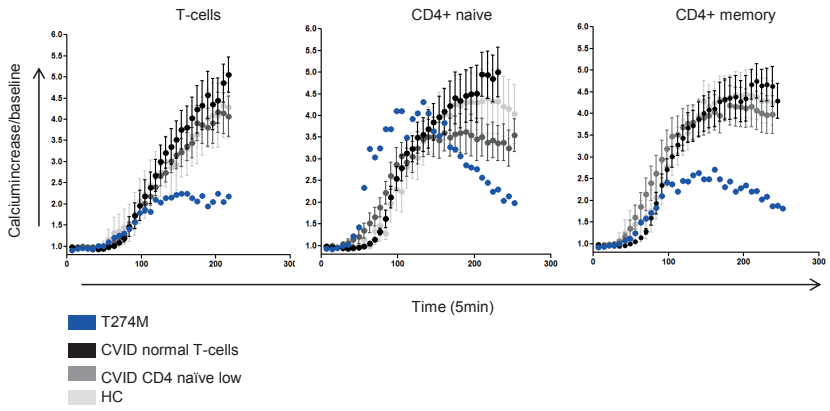

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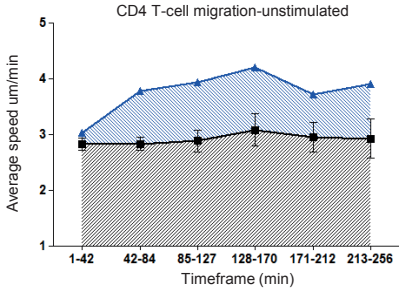

low CD4+ naïve T-cells E) Cytosolic calcium increase upon anti-CD3 stimulation of CD4+ T-cells, naïve CD4+CD45RA+ T-cells and memory CD4+CD45RA- T-cells of CVID patients with normal CD4+ T-cell compartments (n=6) as compared to CVID patients with low CD4+ naïve T-cells (n=6) and HC's (n=6) F) Unstimulated CD4+ T-cell migration of T274M CD4+ T-cells (n=2) and HC's (n=4) CVID: Common Variable Immunodeficiency; HC: Healthy control; MFI: mean fluorescence intensity

Loss of CD2 foci in activated T274M PSTPIP1 transfected HEK293T cells

PSTPIP1 in resting cells is mostly distributed in the cytosol, available to be recruited to surface-expressed CD2 upon triggering of the TCR and co-receptors(13). The differences in activation-induced f-actin polymerization between the patients and healthy control CD4 T-cells prompted us to address the binding between CD2 and PSTPIP1. We hypothesized that increased association of PSTPIP1 with CD2 might explain the pre-activated f-actin status of T274M T-cells, disabling the T cells to further respond to CD2 signaling upon activation.

We therefore transfected HEK293T cells with expression plasmids encoding HA-tagged versions of WT, R228C and T274M PSTPIP1 together with a flag-tagged CD2 expression plasmid (transfection efficiencies were always 50-80%). We generated cell lysates using 1% triton X-100 detergent, and from these lysates we performed pulldown of CD2-flag with anti-flag conjugated agarose beads. In the CD2-flag pulldown fractions, we measured the amount of CD2-associated PSTPIP1 variants, both under unstimulated and anti-CD2 stimulated (2 min) conditions. We found increased T274M PSTPIP1 recruitment to CD2 upon CD2 stimulation, when compared to WT and R228C PSTPIP1, at least in cell lysates (Fig3A). Thus, defects in PSTPIP1 association to surface-expressed CD2 relates to disrupted f-actin polymerization and early CD4 T-cell signaling. Relative increase in PSTPIP1 binding to CD2, as seen for T274M PSTPIP1, appears to result in derailed f-actin polymerization.

Does the PSTPIP1 defect in control of f-actin polymerization involve differential CD2 ligation? To address this point, we performed confocal microscopy analyses of HEK293T cells expressing CD2-flag and PSTPIP1-HA variants. We observed CD2 clustering in WT and R228C PSTPIP1 transfected cells upon CD2 stimulation, while in the T274M PSTPIP1 transfectant, CD2 remained distributed in scattered fashion throughout cellular membranes. We found WT PSTPIP1-HA recruitment around the CD2 clusters in WT PSTPIP1 cells, as has been shown before(13). In T274M PSTPIP1 cells, we observed a stronger PSTPIP1 signal surrounding CD2 at the cell surface, however not polarized to one focal area in the cell membrane as observed in WT PSTPIP1 (Fig3B). Cross-sectional analyses supports these notions, with higher PSTPIP1 signals colocalizing with the CD2 signal in the T274M PSTPIP1 variant (Fig3C). These data support a role for PSTPIP1 as scaffold protein that connects CD2 to f-actin polymerization, as mediated by WASP(31)(12).

Defective immune-synapse formation in T274M PSTPIP1 T-cells

Formation of a stable IS upon cognate peptide/MHC interaction is essential for T-cell proliferation and differentiation. Since the T274M patient showed less f-actin polymerization upon CD2 stimulation and seemed less responsive to TCR activation, especially of the memory T-cell compartment as shown by diminished calciumfluxes, we hypothesized that f-actin-dependent IS formation would also be disrupted. We therefore

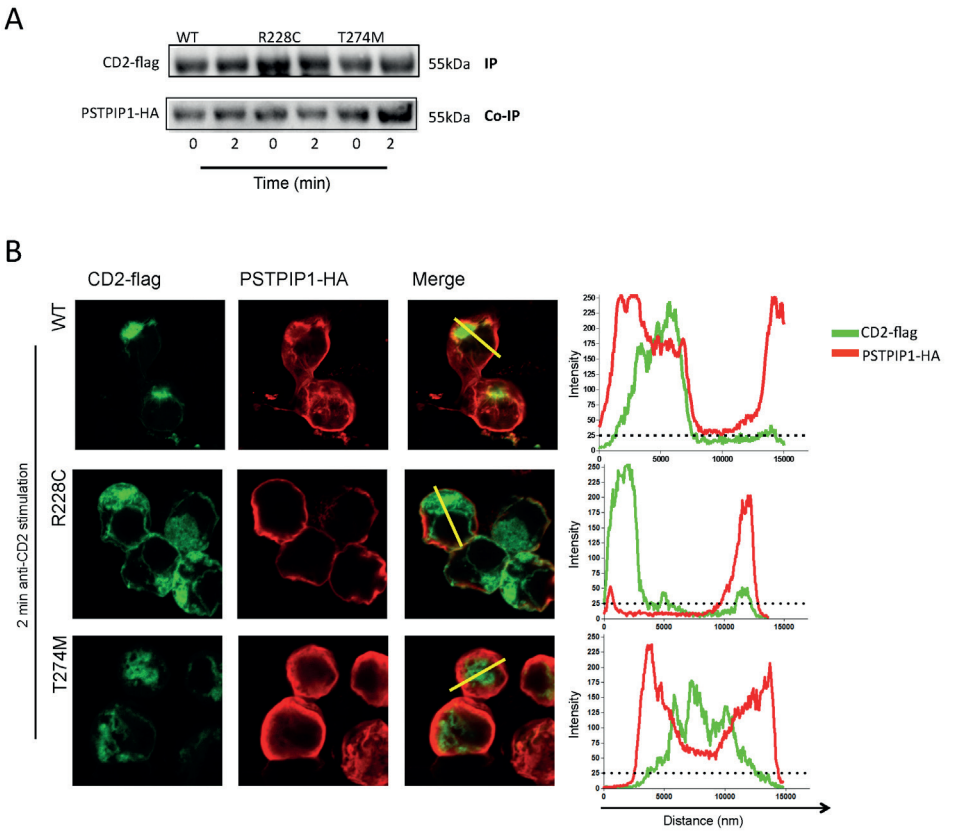

Figure 3

A) Representative western blot of CD2-flag pull down lysates upon anti-CD2 stimulation (2 min) of HEK293T cells transfected with WT, R228C and T274M PSTPIP1-HA and CD2-flag (n=3) B) Localization of CD2-flag and WT, R228C and T274M PSTPIP1-HA PSTPIP1-HA transfected HEK293T cells (2 min anti-CD2 stimulation) as imaged through confocal imaging. Right: histograms of anti-flag FITC and anti-HA AF647 intensities

WT: wild type

next performed f-actin staining upon anti-CD3/CD28 bead contact. Immortalized Jurkat T-cells were transfected with plasmids encoding the WT, T274M and R228C PSTPIP1 variants, incubated with anti-CD3/CD28 beads (10 min) and subsequently fixed, permeabilized and stained with phalloidin-FITC. Confirming the elevated baseline f-actin content of the patient, without stimulation, the T274M PSTPIP1 transfected Jurkat cells already contained a brighter phalloidin-FITC signal (Fig 4A,B). We next visualized the specific recruitment of f-actin towards the bead contact site, and quantified images using ImageJ. Jurkat T-cells overexpressing the R228C variant showed intermediately

reduced f-actin recruitment to the anti-CD3/CD28 bead, while the T274M PSTPIP1-overexpressing Jurkat T-cells much reduced f-actin recruited to the beads (Fig4B,C). Thus, PSTPIP1 controls IS formation, which already had been shown to be an f-actin-dependent process(32,33). Considering that the two patients expressing R228C and T274M PSTPIP1 demonstrated T-cell immunodeficiency, the f-actin polymerization defects observed in CD4 T-cells may contribute to the clinical phenotypes observed.

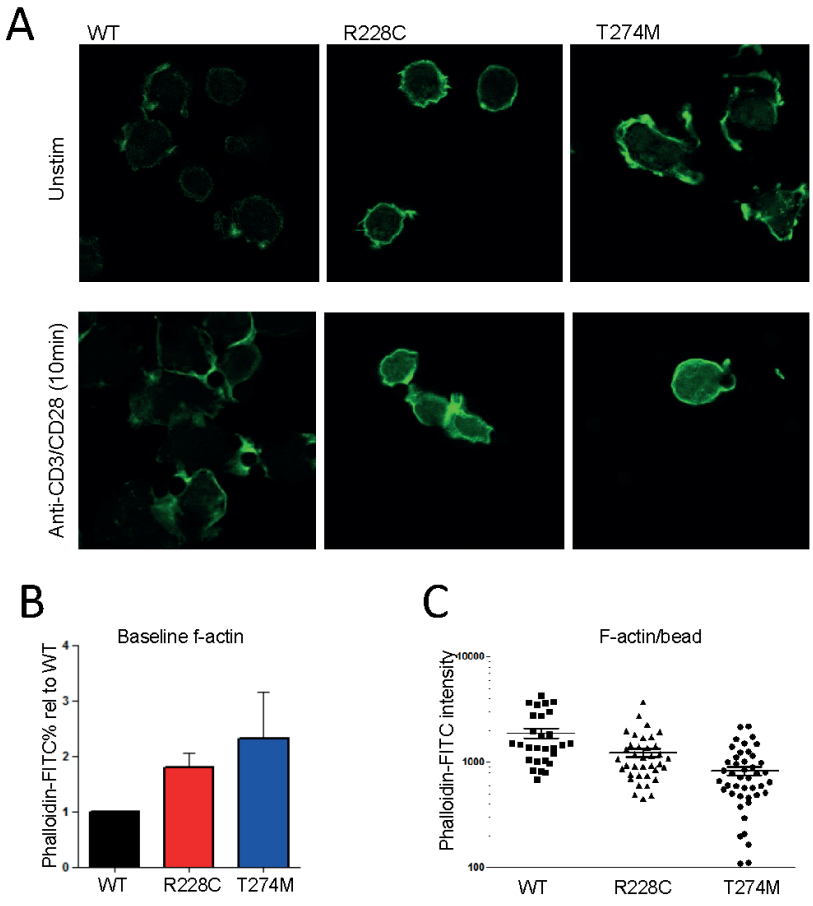

Figure 4

A) Representative confocal images of f-actin recruitment in unstimulated and anti-CD3/CD28 bead stimulated (10 min) WT, R228C and T274M PSTPIP1-HA transfected Jurkat cells B) Baseline f-actin MFI's of R228C PSTPIP1-HA and T274M PSTPIP1-HA relative to WT PSTPIP1-HA transfected Jurkat cells (n=5) as quantified with flow cytometry C) Quantification of f-actin recruitment around anti-CD3/CD28 beads as visualized by confocal imaging

MFI: mean fluorescence intensity; WT: wild type

DISCUSSION

We here describe two novel PSTPIP1 mutations, R228C and T274M in two CVID patients presenting with aberrant T-cell compartments, but with no classical PAPA or Familiar Mediterranean Fever syndrome characteristics. Specifically the T274M patient resting CD4 T-cells, we found were in a pre-activated state and subsequently less responsive to anti-CD2 stimulation particularly. The latter we argue may relate to the observed increase in PSTPIP1-CD2 association, thereby enabling WASP mediated f-actin polymerization.

In this study we primarily focused on the T-cell compartments of the patients, elaborating onto earlier T-cell model-based PSTPIP1 research (12,13). To our knowledge, we are the first to report how PSTPIP1 mutations may contribute to human immunodeficiency disorders, by showing how PSTPIP1 mutations modulate the early signaling events in primary CD4 T-cells. CVID patients with T-cell defects usually present with insufficiency of T-cell proliferation, low CD4 T-cell counts and low CD4 naïve T-cell numbers, findings we confirmed for the T274M patient. Mechanistically, we noted that resting T274M CD4 T-cells exhibit a relative increase in f-actin polymers, implying a pre-activated state. Accordingly, resting T-cells showed defects in stimulation-induced f-actin polymerization, and consequential failure to create sustained TCR signaling. T-cell unresponsiveness was especially evident in CD4 memory T-cells, displaying diminished cytosolic calcium mobilization upon TCR triggering, onward from an elevated baseline calcium level. Of note, in healthy conditions, naive T-cells mobilize relatively more calcium upon TCR specific triggering than memory T-cells (34)(and own unpublished data). The severe reduction in calcium mobilization of CD4 memory T-cells of the T274M patient might relate to the altered composition of the T-cell memory compartment as consequence to lymphopenia–induced homeostatic memory cell proliferation, although further investigation falls beyond the scope of this study.

We next looked into cell migration of the primary CD4 T-cells from patient and healthy donor controls, without prior stimulation. CD4 T-cells of healthy controls (n=5) migrated at a median velocity of 3 μm/min, as earlier reported for similar collagen densities (1.7 mg/ml)(35). T274M patient T-cells velocity approached 4 μm/min instead. Since T-cells rely heavily on intact actin dynamics for their motility, the increase in f-actin polymers in resting T274M patient T-cells likely contributes to increased motility. In contrary, increase in f-actin polymers in T274M PSTPIP1 expressing cells was associated with defective immune synapse formation. Thus the higher f-actin content at baseline facilitates enhanced T-cell motility but probably ineffective adhesion once an antigen would be encountered, since CD2 mediated f-actin polymerization is hampered.

A limitation of our study is the lack of functional experiments focusing on the B-cell compartment. Both patients were incapable to generate normal memory class switched B-cell numbers (combined <2% of total B-cell population) and had absent anti-pneumococcal serotype responses. We propose that the aberrant f-action polymerization and signaling of CD4 T-cells in the T274M PSTPIP1 patient may similarly cause unstable immune synapses between CD4 T-follicular helper cells and B-cells, for abolishment of T-cell help as needed for B-cell differentiation and memory class switching. PSTPIP1 gene variants might also cause intrinsic B-cell defects, as it is expressed in B-cells albeit less than in T-cells(13). Lack of sufficient patient B-cells, absence of pre-existing research on PSTPIP1-associated surface proteins in B-cells, and a good model to study the PSTPIP1 functioning in B-cells however precluded PSTPIP1 research in B-cells.

Considering that T274M patient CD4 T-cells had a distorted equilibrium of f-actin polymerization both in the steady-state and upon anti-CD2 stimulation, we investigated the CD2-PSTPIP1 association. We found that T274M PSTPIP1 associated more strongly to CD2 than the common PSTPIP1 variant, using immune-precipitation experiments in HEK293T cells. In support, we visualized that CD2 triggering caused increased recruitment of PSTPIP1 around CD2, however corroborating less f-actin-mediated CD2 membrane clustering, by confocal imaging. In vivo the coiled coil region of the PSTPIP1 protein is constituted as a dimer(37)(38) and both the R228C and T274M PSTPIP1 mutations are located in the coiled coil region. The substitution of the polar threonine to non-reactive methionine might destabilize the PSTPIP1 multimerization process, enforcing a more monomer-biased PSTPIP1-T274M conformation. Of note, the control of f-actin polymerization by PSTPIP1 might still involve other cytosolic proteins including the tyrosine phosphatase PTP-PEST. However, PTP-PEST was shown to dephosphorylate PSTPIP1 at residue 344 (PMID 11711533), making its involvement in T274M PSTPIP-mediated f-actin polymerization less likely.

In conclusion, our data collectively support a role for PSTPIP1 mutations in T-cells, other than described for inflammasome-mediated disorders such as PAPA syndrome. We here show a role for PSTPIP1 that revealed itself in CVID patients, by contribution to T-cell deficiency through altered f-actin polymerization. Further studies on PSTPIP1 to elucidate the mechanisms of PSTPIP1 in antibody deficiencies are warranted.

METHODS

Patient samples

Patients gave written informed consent to participate in this study, which was approved by the Medical Ethical Committee of the UMC Utrecht. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using a standardized ficol procedure. CD4+ T cells were enriched by use of a CD4+ T lymphocyte enrichment cocktail (BD) and streptavadin beads, according to manufacturers protocol.

Flow Cytometry

For f-actin quantification, primary CD4 T-cells were fixed and permeabilized (FixPerm BD) at several time points (0,2,5, 10 min) after anti-CD2 stimulation with CD2 TII2 and TII3 antibodies 1:100 dilution (kindly provided by dr E. Reinherz Dana Farber Institute Boston) and anti-CD3 stimulation (OKT3 clone eBioscience). Upon fixation cells were stained with Phalloidin-FITC 50ug/ml dilution (Enzo Life sciences) in permeabilization buffer (BD) for 20 min 4C, washed and immediately acquired by flow cytometry (Canto II BD).

Cytosolic calcium mobilization

PBMC of the patient were stained with anti-CD3, anti-CD4 and anti-CD45RA/RO antibodies in RPMI 1640 medium without phenol red. To visualize cytosolic calcium increase Fluo-3 am (5umol/l) and Fura-Red (10umol/l))(Thermo Fisher Scientific) were used as calcium indicators. Calcium dyes were incubated at 37 C degrees for 30 min the last 10 minutes specific antibodies were added. Cells were washed and resuspended in calcium containing Hanks Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) with 10% FCS added. Calcium mobilization was acquired with flow cytometry, to induce calcium fluxes anti-CD3 was crosslinked with a goat anti-mouse IgG antibody 20 mg/ml (Jackson Immunoresearch).

Site-directed mutagenesis

Mutated PSTPIP1 was generated by site-specific mutagenesis of wild type PSTPIP1 The vector EX-T2966-M68 (GeneCopoeia) was grown in DH5a in order to obtain methylated DNA and cDNA was purified by maxiprep according to manufacturer's instruction (Macherey-nagel). PSTPIP1 variants were entered by use of PCR reaction according to the guidelines of Phusion DNA polymerase (Finnzymes, F-5302S) using primers. Template (methylated) DNA was degraded by adding 1 µL DpnI (NEB, R0176s) and incubated overnight at 37 °C. Wild type and mutant vector were transformed in commercial DH5a (NEB, C2987I), and cDNA was purified. The PSTPIP1 variants were confirmed by sequencing.

Transfection

Jurkat cells were electroporated at 250 V and 975 mF (Genepulser II from Bio-Rad Laboratories, Hercules, Calif) with an EX-T2966-M68 vector (GeneCopoeia) containing

human HA tagged (N-terminus) WT-PSTPIP1, T274M- or R228C- mutated PSTPIP1 and flag tagged CD2 (C terminus). HEK293T cells were transfected with the same vectors, by use of FuGENE HD Transfection Reagent (Promega), according to manufacturer's instructions.

Immunoprecipitation

HEK293T cells transfected with WT-,or T274M,or R228C PSTPIP1-HA and CD2-flag and cultured in DMEM supplemented with 10% FCS. Approximately one and a half days after transfection cells were harvested, resuspended in PBS and stimulated with anti-CD2 antibodies and kept on ice directly after 2 min stimulation. Subsequently cells were lysed in a standardized immunprecipitation buffer with 1% Triton and 1% protease inhibitor (HALT Thermo Fisher Scientific) and CD2-flag pulldown was performed with FLAG conjugated beads (Sigma). Lysates were subjected to SDS-PAGE and Western blotting for CD2-flag (monoclonal anti-FLAG M2-peroxidase Sigma Aldrich) and PSTPIP1-HA (mouse IgG2bk anti-HA 12CA5) and Donkey anti-Mouse IgG light chain specific antibody-HRP conjugated (Jackson Immunoresearch).

Confocal imaging

Jurkat cells were transfected WT-,or T274M,or R228C PSTPIP1-HA and kept in RPMI 1640 medium supplemented with 10% FCS. After two days cells were adhered to poly-l-lysine coated confocal chambers and stimulated with anti-CD3/CD28 beads for 10 minutes. Upon stimulation cells were kept on ice and fixed/permeabilzed with fix/perm (BD) for 30 min, after fixation cells were stained with phalloidin-FITC 5mg/ml (1:200 dilution) (Enzo Life Sciences) and washed with permeabilization buffer (BD).

For imaging of HEK 293T cells transfected with WT-, or T274M,or R228C PSTPIP1-HA and CD2-flag, cells were permeabilized in triton 0.1%, blocked with 5% BSA and washed in PBS with Tween 0.5%. Cells were stained with monoclonal FLAG M2-FITC (1:200) and mouse IgG2bk anti-HA 12CA5 (both Sigma Aldrich) and after block with goat serum with goat anti-mouse Alexa Fluor 647. Acquisition was performed on a Zeiss LSM710 confocal microscope. Images were obtained with a 1.3× optical zoom using Plan-Apochromat 63× 1.40 oil differential interference contrast M27 objective (Zeiss) and processed using Zen 2009 software (Zeiss Enhanced Navigation)

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CHAPTER 4

A novel human STAT3 mutation presents with enhanced B cell maturation and immunoglobulin production via elevated IL-17

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Published in: Oncotarget 2015

ABSTRACT

Mutations in STAT3 have recently been shown to cause autoimmune diseases through increased lymphoproliferation. We describe a novel Pro471Arg STAT3 mutation in a patient with multiple autoimmune diseases, causing hyperactivation of the Th17 pathway. We show that IL-17 production by primary T cells was enhanced and could not be further increased by IL-6, while IL-10 reduced Th17 cell numbers. Moreover, specific inhibition of STAT3 activation resulted in diminished IL-17 production. We show that the Pro471Arg STAT3 mutation yields both increased levels of IgA and IgG, probably due to high IL-21 levels. When remission was reached through medical intervention, IL-17 levels normalized and the clinical symptoms improved, supporting the idea that STAT3 gain-of-function mutations can cause hyperactivation of the Th17 pathway and thereby contribute to auto-immunity.

INTRODUCTION

Three recently published studies show that STAT3 mutations can underlie autoimmune pathology [1-3]. Although these studies provide pathophysiological explanations as causes for the disease phenotype for several STAT3 mutations, the disease mechanisms caused by STAT3 mutations are not yet fully understood. STAT3 has a role in the development of Th17 cells, which have been associated with several autoimmune diseases [4]. We here provide experimental immunological support in an exploratory study that increased Th17 activity can contribute to autoimmune pathology in patients with STAT3 mutations.

RESULTS

A 17 year old female was referred for immune-diagnostics because of multi-organ autoimmune disease that was present since early childhood. Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) or IPEX-like syndrome were excluded by showing normal regulatory T cell (Treg) number and function. Screening of primary immunodeficiency related genes [5] showed a *de novo* heterozygous mutation in STAT3 (c.1412C>G p.(Pro471Arg), NM_139276.2) with potential pathological consequences (see Figure 1A&B). The patient and her parents gave written informed consent for this study and blood was sampled during active disease and remission on Rapamycin treatment.

Patient History

17 year old female born full term as a first child to non-consanguineous parents presented with autoimmune symptoms in the first months of life, starting with alopecia (Table 1&2). At the age of 8 months she presented first signs of autoimmune enteropathy (positive anti-enterocyte antibodies) for which she was treated with parenteral nutrition and tacrolimus. She was also diagnosed with celiac disease based on antibodies and histology (Marsh 3b/c). In the following years the enteropathy was controlled with gluten free diet and azathioprine.

At the age of 10 years she developed autoimmune hypothyroidism. At age 14 she received a liver transplant due to acute liver failure as a result of autoimmunehepatitis (ANA+, anti-smooth muscle intermediate).

After transplantation and under the immunosuppressive treatment of prednisone, tacrolimus and azathioprine, her autoimmune disease remained stable for several years. However, recently, she had a relapse of her autoimmune enteropathy, making it necessary to increase prednisone and tacrolimus dosage and eventually to add Rapamycin (Table 3).

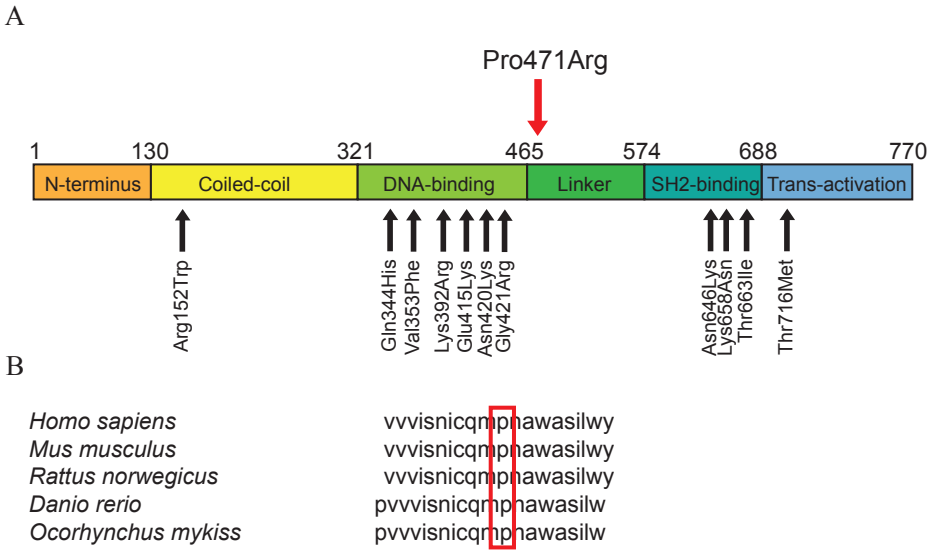


Figure 1

Described mutations in and evolutionary conservation of the STAT3 gene.

(A) Location of the Pro471Arg mutation in the STAT3 gene (red arrow) and already described gain-of-function mutations (black arrows) and (B) conservation of the amino acid order throughout species

The blood sampling for this study was performed prior to the addition of Rapamycin, unless otherwise indicated.

Considering that IL-6 mediated STAT3 activation drives Th17 cell development [6] we tested components of this pathway in blood plasma and peripheral blood mononuclear cells. First, we explored circulating cytokines involved in the Th17 pathway by multiplex analysis of plasma obtained during active disease. IL-6, IL-17 and IL-21 were elevated in the patient compared to healthy donors (Fig 2A). In accordance with this, PMA/ionomycin activated PBMC of the patient showed increased numbers of IL-17 producing cells and increased RORγt expression within the $CD4^+$ T cell compartment (Fig 2B). In contrast, IL-21 and IL-6 production by PBMC was reduced, possibly as a compensatory mechanism for already high plasma levels (Fig 2C). To further assess the nature of this elevated Th17 cell activity we cultured PBMC with increasing concentrations of IL-6. The number of Th17 cells was already markedly elevated in the patient cell culture, irrespective of IL-6 stimulation (Fig 2D). Addition of IL-6 did not result in more Th17 cells, but showed a dose-dependent increase of IL-17 in the culture supernatants of both the healthy donors and patient. In contrast, STAT3 activating cytokine IL-23 did not have

Table 1

Symptoms and age of onset.

Organ system	Symptoms	Age of Onset
Skin	Alopecia	6m
	Eczema	6m
Gastrointestinal	Auto-immune Enteropathy	8m
	Celiac Disease	8y
	Hepatitis	14y
Endocrine	Hypothyreosis	10y
Pulmonary	Lower Diffusion Capacity	16y
Cardiovascular	No symptoms	-
Renal & Urinary	No symptoms	-
Hematological	No symptoms	-

Table 2

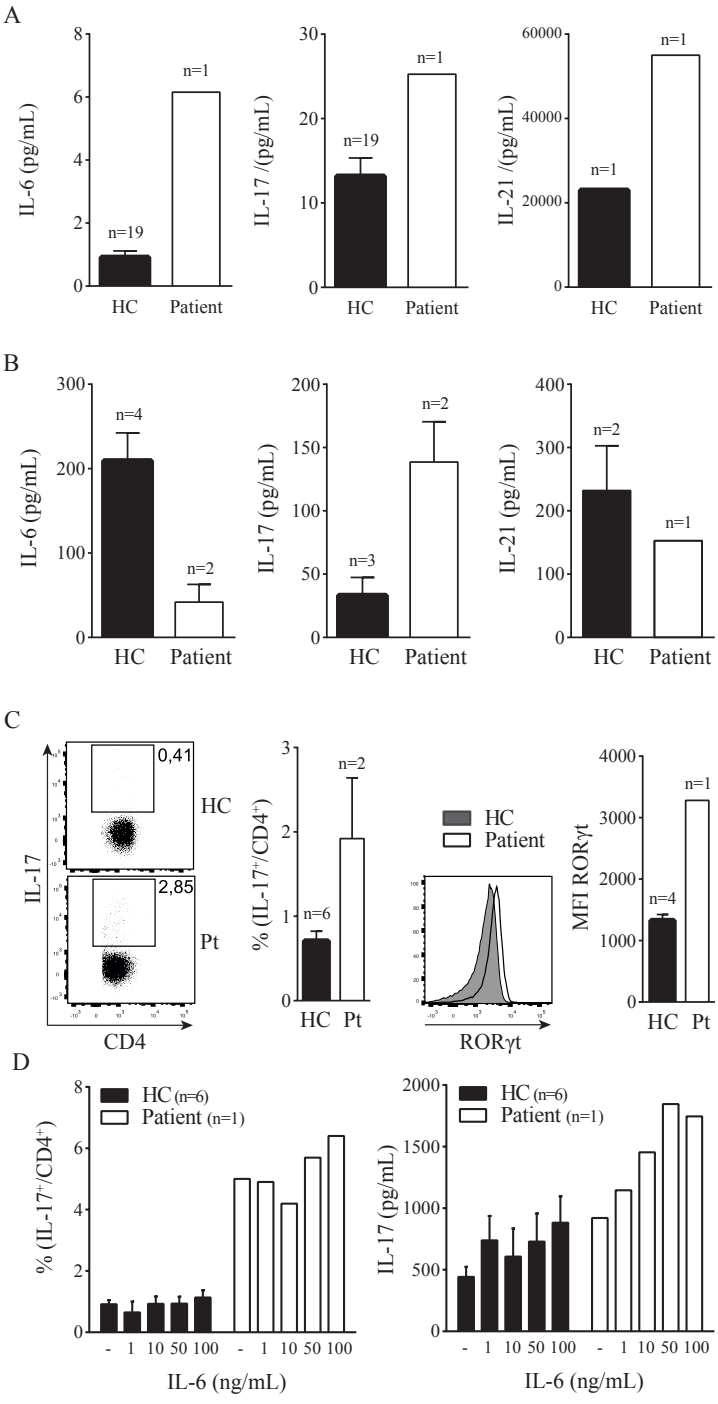
Clinical Characteristics

Patient characteristic	Clinical data
STAT3 mutation	c.1412C>G p.(Pro471Arg)
Sex	Female
Age	17 y
Birthweight (SD)	-1.5
Growth (SD)	-2.5
Puberty	Normal
Infection Susceptibility	Recurrent *Herpes zoster* infections under immunosuppression

Table 3

Rapamycin treatment intervals

Aug 2013	Feb 2014	May 2014	Nov 2014	Jan 2015
Start	Non compliance, undetectable blood levels	Sufficient blood levels	Stop	Restart


A
IL-6 (pg/mL)
n=19
n=1
HC
Patient
IL-17 /(pg/mL)
n=19
n=1
IL-21 /(pg/mL)
n=1
n=1
B
IL-6 (pg/mL)
n=4
n=2
IL-17 (pg/mL)
n=3
n=2
IL-21 (pg/mL)
n=2
n=1
C
IL-17
CD4
0,41
2,85
HC
Pt
% (IL-17+/CD4+)
n=6
n=2
HC
Patient
RORγt
MFI RORγt
n=4
n=1
D
% (IL-17+/CD4+)
HC (n=6)
Patient (n=1)
IL-6 (ng/mL)
IL-17 (pg/mL)
- 1 10 50 100

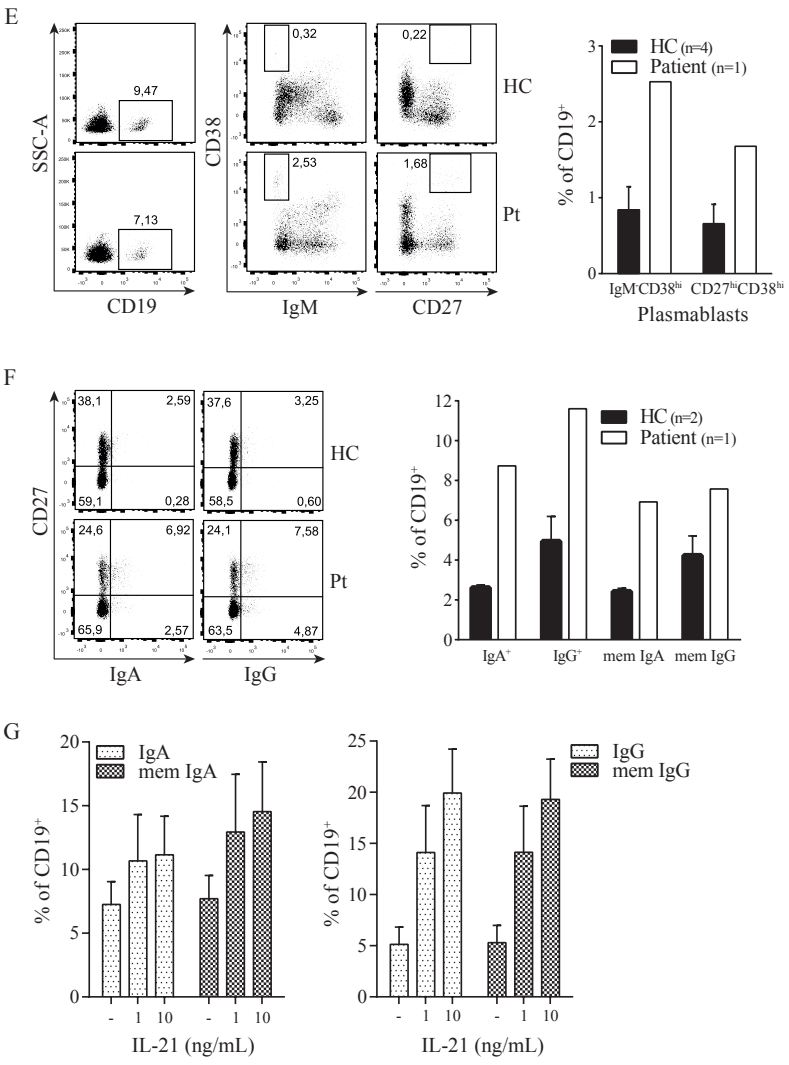


Figure 2

STAT3 Pro471Arg mutation causes hyperactive Th17 pathway and aberrant B cell phenotype. (A) Blood plasma levels of IL-6, IL-17 and IL-21. (B) IL-6, IL-17 and IL-21 production by ex vivo PMA/Ionomycin activated PBMC. (C) Percentage of circulating IL-17+ CD4+ T cells. (D) Increasing concentrations of IL-6 were added to PBMC cultures with plate bound anti-CD3 ('-'); the intracellular expression of IL-17 and RORγt in CD4 T cells was measured by flow cytometry (left) and the amount of IL-17 produced was determined in the supernatant after four days of culture (right). (E+F) B cell phenotype; percentage of circulating plasmablasts (E) and IgA and IgG expression (F). (G) Increasing concentrations of IL-21 were added to PBMC cultures with plate bound anti-CD3 ('-'); surface expression of IgA (left) and IgG (right) was measured by flow cytometry (n=3). *Error bars represent SEM.*

an IL-17 inducing effect on patient cells (not shown). These results show a contribution of the Pro471Arg STAT3 mutation to a persistent hyperactivation of the Th17 response independent of IL-6 signaling.

Next to the aberrant T cell phenotype we observed B cell abnormalities in the patient, characterized by an increased presence of plasmablasts and elevated IgA and IgG surface expression *ex vivo* (Fig 2E-F). This observation correlated with elevated concentrations of IgA and IgG in patient plasma as compared to healthy donor values (see Fig 3). It is well known that B cell class switching, plasma cell formation and immunoglobulin production depend amongst others on IL-21 [7] which is elevated in our patient. To investigate whether Th17 cells effector cytokines could account for the observed B cell abnormalities, we cultured healthy donor PBMC with IL-17 and IL-21. IL-21 caused a dose-dependent increase of class switched memory B cells and surface IgA and IgG expression (Fig 2G), as has been shown before [8]. IL17 had no effect on B cell maturation and immunoglobulin expression (data not shown). Thus the STAT3 dependent high IL-21 levels observed in the patient during active disease may contribute to the hypergammaglobulinemia phenotype of the patient.

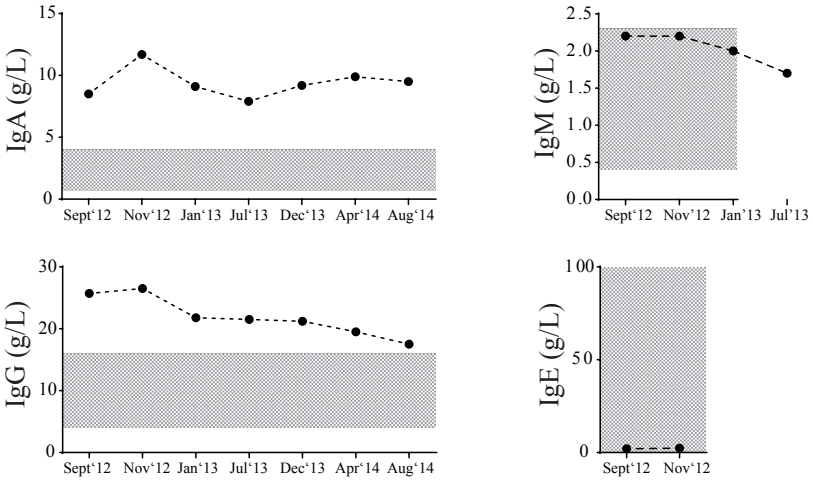


Figure 3

Immunoglobulin levels in patient blood plasma.

Levels measured at several time-points, compared to standard healthy donor values (shaded area)

To investigate whether the increased Th17 activity in the patient could be influenced by agents that are known to modulate the STAT3-Th17 pathway, we cultured PBMC with IL-10, IL-6 receptor blocking antibody tocilizumab and STAT3 inhibitor S3i-201. Increasing concentrations of IL-10 did not clearly affect healthy donor Th17 cells, but in patient cell cultures the number of Th17 cells and the amount of IL-17 produced were decreased (Fig 4A). Tocilizumab reduced the Th17 activity in healthy donors and the patient (Fig 4B), whereas S3i-201 did not have an effect on Th17 numbers, but only reduced the IL-17 production (Fig 4C). We tested the Th17 response again when the patient showed reduced disease activity during treatment with Rapamycin. Indeed, concomitant with an increase in Treg numbers (not shown), Rapamycin treatment caused a reduction in the number of IL-17 producing T cells after PMA/Ionomycin stimulation (Fig 4D). These results indicate that even though Th17 activity is markedly increased in the patient, it can be dampened by (in)direct inhibition of the STAT3-Th17 axis, *in vitro* as well as *in vivo*.

To formally address whether an altered phosphorylation state of the STAT3 protein may contribute to the patient's phenotype, we analyzed the phosphorylation of the tyrosine residue 705 (pY705) of STAT3 in fresh cells and upon culture with anti-CD3 with or without IL-10, tocilizumab or S3i-201. The Y705 phosphorylation status was comparable between the patient and healthy donor cells (Fig 4E). After 4-day culture, STAT3 Y705 phosphorylation was even lower in patient cells. Addition of tocilizumab or S3i-201 did reduce the phosphorylation in both healthy donor and patient cells. (Fig 4F). Thus, constitutive phosphorylation of the STAT3 Y705 protein does not explain the observed phenotype.

DISCUSSION

In summary, we here show in primary patient material that the Pro471Arg STAT3 mutation correlates with a highly activated Th17 pathway and contributes to autoimmune pathology and activated B cells. The elevated IL-21 levels (which could be produced by Th17 cells but also by NKT and T follicular helper cells) as found in the patient plasma likely supports class switching and could thereby contribute to the observed hypergammaglobulinemia. The clinical characteristics of the patient described here and the patients described elsewhere [1-3] clearly diverge from hyper-IgE syndromes that are associated with STAT3 mutations [9]. The diversity in clinical manifestations of STAT3 mutations suggests that mutations in this gene might underlie more autoimmune disorders, specifically when disease onset occurs at early age. While we concede that the Th17 pathway may not account for all these phenotypes and most data are obtained from a limited number of samples, the immunological data provided are suggestive of the Th17 pathway contributing to more autoimmune disorders than currently recognized. We therefore suggest that sequencing of STAT3 should be considered in more patients with

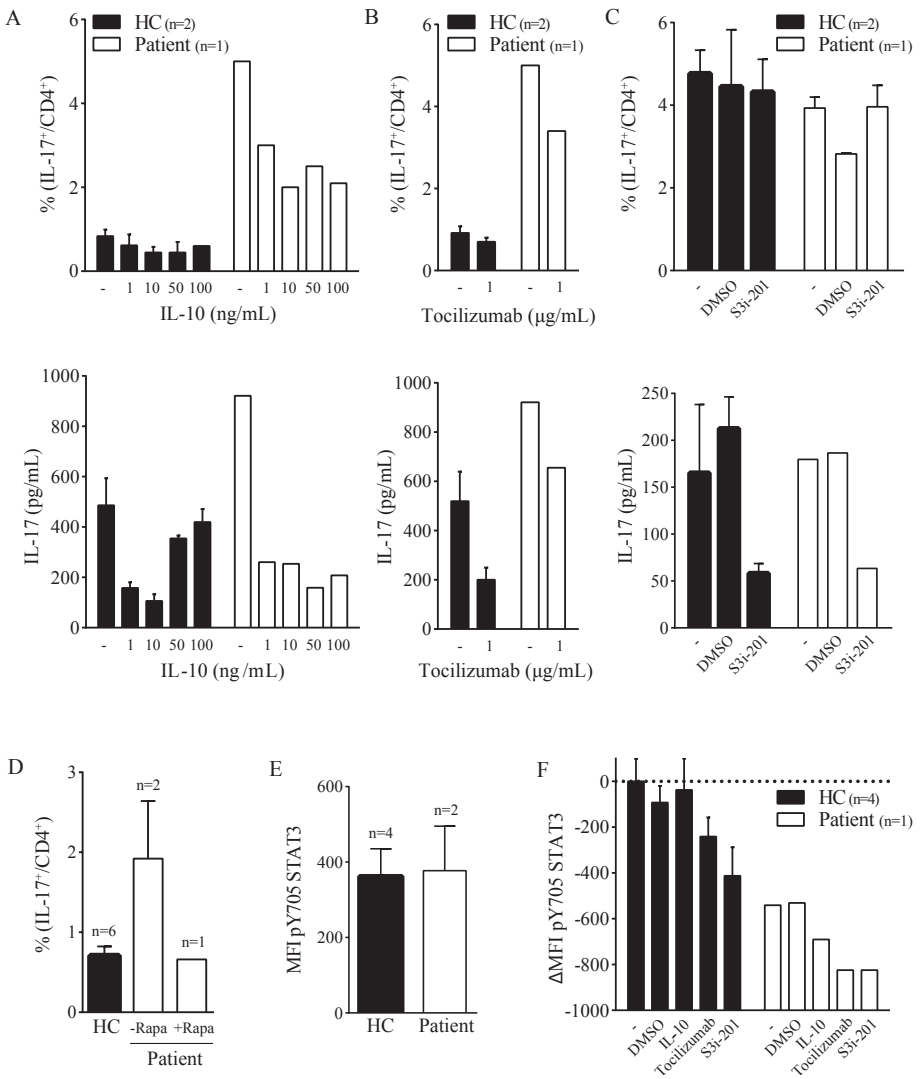


Figure 4

Th17 hyperactivation can be dampened.

PBMC were cultured with plate bound anti-CD3 ('-') with or without IL-10 (A), tocilizumab (B) or S3i-201 (C) for 4 days; the percentage of IL-17+ CD4+ T cells was measured by flow cytometry (upper panel) and the amount of IL-17 produced in the supernatant was determined by multiplex immunoassay (lower panel). (D) Percentage of IL-17+ CD4+ T cells in the patient during and after treatment with Rapamycin (HC and -Rapa same data as Fig 1C) E) MFI of ex vivo pY705 STAT3 in total T cells. (F) ΔMFI (relative to HC with anti-CD3 only) of pY705 STAT3 after 4 day culture as described above (IL-10: 10 µg/mL). Negative values indicate decrease in MFI. *Error bars represent SEM.*

severe autoimmunity, and specified treatment options focusing on the Th17 pathway like Tocilizumab should be further explored for these patients.

METHODS

Patient material was collected once during active disease and once during disease remission on Rapamycin treatment. Plasma and peripheral blood mononuclear cells (PBMC) of patient and healthy controls were stored at -150°C for later use in experiments. Cytokine concentrations were measured in plasma and culture supernatants by multiplex immunoassay as described elsewhere [10] PBMC were activated with 20ng/ml phorbol 12-myristate 13-acetate (PMA) and 1µg/ml ionomycin for 4 hours to measure cytokine production in supernatant or by 1µg/ml anti-CD3 (okt3, eBioscience, San Diego, USA) and cultured for four days with or without recombinant IL-6 (Miltenyi, Bergisch Gladbach, Germany) or IL-10 (BD Bioscience, San Jose, USA). Monensin (Golgistop, BD Bioscience) was added during the last 3.5h PMA/Ionomycin stimulation for flow cytometric analysis of intracellular cytokine expression. STAT3 activity was inhibited by addition of 50µM S3I-201 (Santa Cruz Biotechnology, Heidelberg, Germany) and phosphorylation investigated with anti-pY705 STAT3 (Clone 4/P-STAT3 BD Bioscience). For analysis of B cell differentiation, PBMC were cultured for 5 days with plate bound anti-CD3 and rhIL-21 (1 and 10 ng/ml; Immunotools) or rhIL-17 (1 and 10 ng/ml; Immunotools). Suppression assays were conducted with Treg inspector beads (Miltenyi) and $CD4^{+}CD25^{+}CD127^{-}$ sorted Treg.

Funding
Willemijn Janssen was funded by an unrestricted educational grant from Baxter, the Netherlands

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4

CHAPTER 5

F-actin remodeling defects as revealed in primary immunodeficiency disorders

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Published in: Clinical Immunology 2016

ABSTRACT

Primary immunodeficiencies (PIDs) are a heterogeneous group of immune-related diseases. PIDs develop due to defects in gene-products that have consequences to immune cell function. A number of PID-proteins is involved in the remodeling of filamentous actin (f-actin) to support the generation of a contact zone between the antigen-specific T cell and antigen presenting cell (APC): the immunological synapse (IS). IS formation is the first step towards T-cell activation and essential for clonal expansion and acquisition of effector function. We here evaluated PIDs in which aberrant f-actin-driven IS formation may contribute to the PID disease phenotypes as seen in patients. We review examples of such contributions to PID phenotypes from literature, and highlight cases in which PID-proteins were evaluated for a role in f-actin polymerization and IS formation. We conclude with the proposition that patient groups might benefit from stratifying them in distinct functional groups in regard to their f-actin remodeling phenotypes in lymphocytes.

INTRODUCTION

Currently, more than 250 human primary immunodeficiencies (PIDs) have been reported[1, 2]. PIDs represent a heterogeneous group of diseases caused by genetic defects that culminate in malfunction of the immune system[3]. These defects can obstruct a variety of processes, including the initiation of the adaptive immune system branch, as required for high affinity antigen-specific immunity and the induction of recall immune responses. Adaptive immune activation commences with the presentation of antigenic peptide/major histocompatibility complexes (MHC) to antigen-specific T cells. The process of antigen-specific T cell activation starts by the productive binding of a cognate T cell receptor (TCR), which triggers the assembly of a molecular structure at the T cell surface, the immunological synapse (IS)[4, 5] (Figure 1). Considering the importance of stable IS assembly to the generation of effective adaptive immune responses, for selected PIDs, defects in IS assembly may contribute to the disease phenotype observed.

When cell surface molecules are triggered, associated signaling molecules transmit signals that induce f-actin remodeling. Here, f-actin monomers rearrange to form polymers, which helps to recruit additional cell surface molecules to the IS, and mediate T cell activation[7, 8]. Considering the importance of signaling molecules in early T cell signaling, we explored a selective group of PID-associated signaling molecules for contribution to IS formation defects and the clinical PID phenotype (Fig 1 and 2). First, we discuss upstream signaling molecules that transmit the antigen ligation-induced signal from the TCR inward. Second, we describe downstream signaling molecules that promote stable IS formation via f-actin polymerization and adhesion molecules. Third, we consider downstream actin-regulatory molecules, which induce tightly regulated f-actin remodeling.

1. Upstream signaling molecules

Lymphocyte-specific protein tyrosine kinase (LCK), ZAP70, class IA phosphatidylinositol 4,5-bisphosphate 3-kinases (PI3K), and interleukin-2-inducible T cell kinase (ITK) are PID-related kinases that are involved in f-actin remodeling and IS formation. In the next sections we discuss these upstream PID-related signaling molecules in more detail (as depicted in fig. 2).

Upstream signaling molecules and PID phenotype

LCK and UNC119

Currently, three PID patients with reduced LCK expression have been reported[10, 15, 16]. However, in only one of these patients a mutation in the LCK gene was determined[10]. On a molecular level, this patient displayed reduced phosphorylation of early signaling molecules, including CD3ζ, ZAP70, LAT, and 1-phosphatidylinositol 4,5-bisphosphate

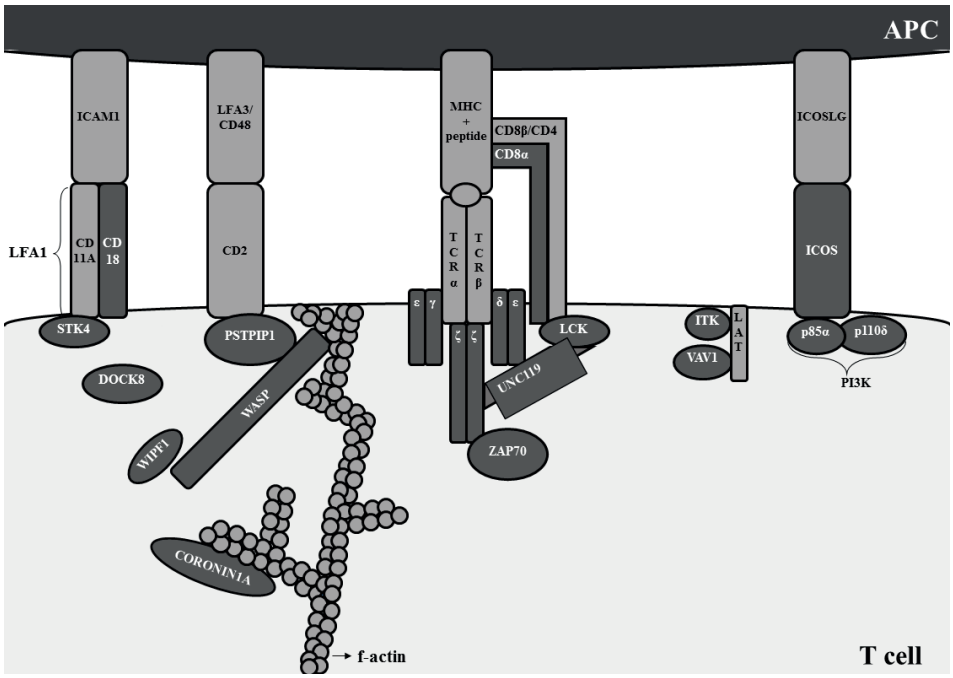

Figure 1

Overview of the immunological synapse between a CD4+ or CD8+ T cell and an APC.
Examples of PID-proteins that are linked to IS formation and discussed in this article are depicted in dark –grey

phosphodiesterase (PLC)γ1[10]. PLCγ1 is important for calcium mobilization, which is required for f-actin remodeling and thereby mature IS formation[17]. Consequently, LCK-mutant cells show no calcium fluxes[10], impaired f-actin remodeling[18], and reduced T cell/APC synapse formation[18]. Furthermore, LCK serves as upstream mediator of integrin recruitment and activation, processes that are also dependent on f-actin remodeling[18-20]. In line with the crucial role of LCK in early T cell signaling, the LCK mutation patient displayed a severe phenotype including $CD4^+$ T cell lymphopenia, recurrent infections, autoimmunity, autoinflammation, and T cells originating from a small number of clones and showing little surface expression of CD4 and CD8[10]. Thus, in LCK-deficient cells or cells expressing mutant LCK, PID disease may involve a failure to direct integrin alpha-L/beta-2 (LFA1) to the IS, thereby disturbing T cell adhesion to APCs[18].

Uncoordinated 119 homolog A (UNC119) is required for LCK activation and localization to the plasma membrane[9, 21]. Since UNC119 is expressed at low levels in murine thymocytes, PID disease in UNC119-gene variant patients may not be related to LCK

activation during thymic development of T cells[21]. Instead, in peripheral T cells, in which UNC119 expression is increased and essential to target LCK in an f-actin-dependent manner to the developing IS, UNC119 may contribute to early activation of mature T cells[9]. In support, a mutation in UNC119 hampers the protein's association with LCK, thereby causing impaired LCK activation and localization in peripheral T cells. Because T cell development in UNC119-gene variants is likely intact, clinical PID manifestations in UNC119 variants exhibits $CD4^+$ T cell lymphopenia and a variety of bacterial, viral, and fungal infections[21]. Taken together, f-actin remodeling and IS formation may be impaired in LCK and UNC119-gene variant PID patients and thus contribute to the clinical and immunological phenotypes observed in both gene variants, namely $CD4^+$ T cell lymphopenia and recurrent infections.

ZAP70

Similar to LCK, ZAP70 is an important upstream signaling molecule that drives T cell activation upon TCR ligation and involve f-actin remodeling and functional IS formation[22-24]. ZAP70 facilitates f-actin rearrangement and functional IS formation by recruitment and/or activation of other signaling molecules[23, 24], although the amount of T cell/APC conjugates is comparable irrespective of ZAP70 presence in T cells[18, 24]. PID patients harboring ZAP70-gene variants exhibit decreased levels of peripheral $CD8^+$ T cells and dysfunctional $CD4^+$ T cells. Their clinical phenotype is heterogeneous, although all patients display recurrent infections[22, 25-27]. The strongly diminished peripheral $CD8^+$ T cell count might be explained by requirement for ZAP70 in the development of these cells. The peripheral defect in $CD4^+$ T cells indicates redundancy for ZAP70 during their development, but not during their activation[27]. Experiments in patients' T cells that address f-actin remodeling and IS formation are not yet reported, although *in vitro* work supports a role for ZAP70 in f-actin rearrangement and functional IS formation[23, 24].

PI3K

Besides LCK and ZAP70, class IA PI3Ks are important upstream signaling molecules. Class IA PI3Ks comprise a regulatory and a catalytic subunit (P85α, P55α, P50α, P85β, or P55γ associating with P110α, P110β, or P110δ, respectively). All regulatory subunits can associate with all catalytic subunits and when associated, regulatory subunits regulate the activity, localization, and stability of catalytic subunits[13, 28]. Following T cell/APC conjugate formation, PI3K is recruited to receptor and adaptor protein-expressed motifs (phosphotyrosine – any amino acid – any amino acid – methionine), to which the regulatory subunits bind[28, 29]. Accordingly, inhibition of the catalytic subunit is released, allowing conversion of PIP_2 to PIP_3[13, 29]. PIP_3 can bind several actin linked proteins[14, 30, 31], one of which, ITK, will be discussed in the following section. In addition, PI3K is required to activate cofilin1, a protein involved in f-actin remodeling and IS formation[32].

PI3K mutations can contribute to PIDs, as shown by gene mutations affecting P85α, P55α, and P50α (that are transcribed from the same gene and C-terminally identical), which can result in decreased protein stability and reduce inhibitory interaction with P110δ[13, 33]. As a consequence, P110δ is rendered constitutively active[33], comparable to gain-of-function mutations in P110δ[13, 33]. Although the clinical presentation is heterogeneous, features of both defects include recurrent sinopulmonary infections, dysregulated antibody production, lymphadenopathy, decreased amounts of naive $CD4^+$ and $CD8^+$ T cells, and an increase in transitional B cells. Besides, activation-induced cell death is more frequently observed in patient T cells, who simultaneously have an increased risk of lymphoproliferation and perpetuating malignancies. T cell lymphopenia is hitherto only observed in patients with P110δ mutations[13, 28, 33-38].
In conclusion, attenuated PI3K signaling does not necessarily impair actin remodeling and IS formation. Currently reported PID-related mutations in P85α, P55α, P50α, and P110δ result in elevated PI3K signaling[13, 28, 33, 34] and it is still unclear if f-actin also plays a role in the onset of these PID diseases. Tight regulation of PI3K signaling is undoubtedly instrumental for proper f-actin rearrangement and IS formation. Thus, elevated PI3K signaling could disturb conjugate formation between T cells and APCs and thereby contribute to the PID phenotype.

ITK

ITK is recruited to the membrane-associated molecule PIP_3, which is generated by PI3K activity, as described in the previous section. After membrane targeting, ITK also binds to LAT residues that were phosphorylated by ZAP70[39], and ITK is activated through phosphorylation by LCK and subsequent autophosphorylation[11]. Thereafter, ITK activates PLCγ1[11], indicating that LCK and ZAP70 mediate calcium mobilization via ITK. [14].

Furthermore, ITK is required for f-actin rearrangement at the IS[11, 40] and T cell/APC conjugate formation[41]. Correlating with ITK's role in f-actin remodeling, ITK is involved in recruitment of the guanine exchange factor (GEF) proto-oncogene vav (VAV1) to the IS[40, 41]. ITK also phosphorylates LAT, creating binding sites for VAV1[42]. Since expression of a membrane-targeted VAV1 construct in ITK-depleted cells restored actin polymerization[40], ITK can regulate f-actin polymerization and T cell/APC conjugate formation via recruitment of VAV1 to the IS, at least in this experimental setup.

The PID phenotype of ITK-gene variant patients can be divided in Epstein-Barr virus (EBV)-associated and EBV-independent features. Since T cells of ITK PID patients are defective at controlling EBV, patients encounter EBV-associated characteristics including lymphadenopathy, hepatosplenomegaly, large pulmonary interstitial nodules, a progressively reducing antibody production[1, 43], B cell lymphoproliferation, and a higher risk of EBV-related malignancies. EBV-independent features are other infections,

a progressive decline in $CD4^+$ T cells, a loss of naive T cells[44], and a low level or absence of invariant natural killer T (iNKT) cells[14, 45-49]. In conclusion, ITK contributes to the regulation of f-actin and IS formation through other associated proteins such as VAV1 and PLCg1. However, the exact role of ITK-mediated f-actin remodeling and T cell/APC conjugate formation in the phenotype of ITK-deficient patients is not yet fully understood.

2. Downstream signaling molecules - towards f-actin polymerization and integrin activation

Upstream molecule activation as elicited by TCR ligation, induces at least two distinct downstream pathways that lead to f-actin polymerization and integrin activation. We will discuss both pathways and focus our narrative on three PID-related molecules: VAV1, dedicator of cytokinesis 8 (DOCK8), and serine/threonine protein kinase 4 (STK4, also called MST1).

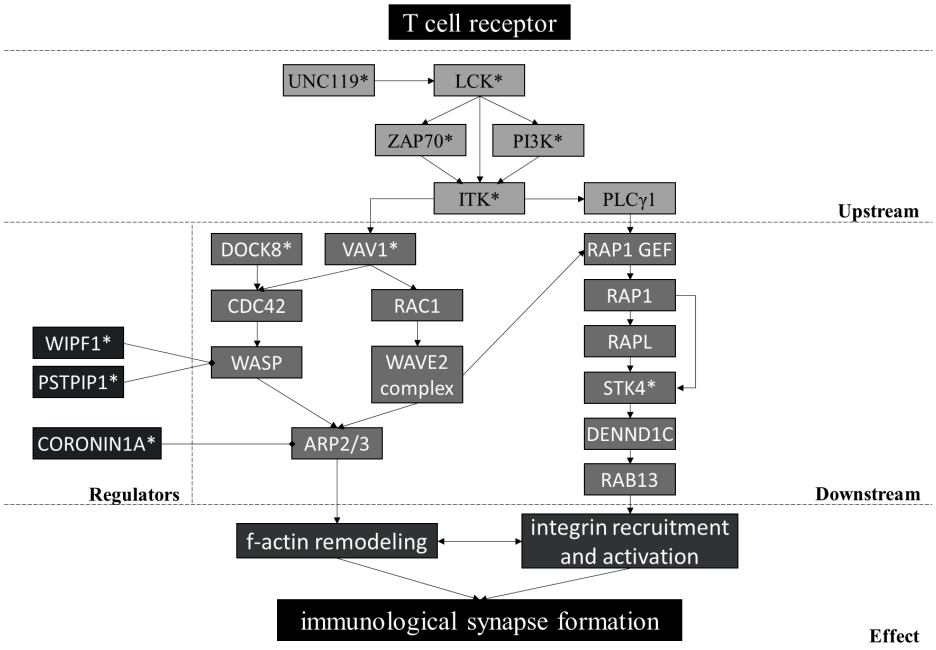

Figure 2

Overview of pathways induced upon TCR activation that are involved in IS formation.

Depicted are the pathways and proteins discussed in the present review. The three distinct clusters discussed in paragraphs 1, 2, and 3 (upstream, downstream, and regulators, respectively) in the current review are indicated. PID-proteins that are discussed in detail are marked with an asterisk (*).

Downstream signaling molecules and PID phenotype

VAV1

The recruitment of the guanine exchange factor VAV1 to the IS requires ITK[40, 41]. VAV1 thereby activates the GTPase molecules CDC42 (cell division control protein 42 homolog) and RAC1 (Ras-related C3 botulinum toxin substrate 1). Subsequently, CDC42 triggers WASP (Wiskott-Aldrich syndrome protein), while RAC1 activates the WAVE2 complex[50]. Both WASP and the WAVE2 complex bind and activate the ARP2/3 complex[8, 51], which is required for actin polymerization[8]. WASP is an extensively described protein[52-56] whose relation to actin, the IS, and PID has been recently reviewed[8]. The WAVE2 complex is involved in calcium mobilization, actin rearrangement, integrin activation, and consequently T cell/APC conjugate formation[6,51].

VAV1 deficiency is observed in T cells from patients with common variable immunodeficiency (CVID), a subgroup of PIDs characterized by antibody deficiency. In a subgroup of CVID patients IgG/IgA antibody deficiency arises as a consequence of defects in T cells, considering that $CD4^+$ T cell help is required for B cells to undergo antigen receptor class-switching and selection prior to development into IgG or IgA-secreting plasma cells[57]. In one VAV1-deficient patient, an extensive deletion in one VAV1 allele was detected, while the genetic origin of VAV1 deficiency in other patients remained unclear. The patient harboring the mutation showed recurrent infections, infection-triggered bronchiectasis, strongly reduced antibody levels, osteoporosis, and colon diverticulitis[58]. In accordance with the function of VAV1, T cells from VAV1-deficient patients displayed reduced calcium fluxes and diminished f-actin remodeling[57]. Thus, VAV1 plays a pivotal role in f-actin rearrangement and IS formation. VAV1 deficiency impedes T cell provided B cell help[59], contributing to the IgG/IgA deficiency and recurrent infections as observed in the VAV1 PID patient.

DOCK8

Another guanine exchange factor for CDC42 is DOCK8[60]. In $CD8^+$ T cells, DOCK8 deficiency may disturb f-actin organization at the IS, thereby impairing recruitment of LFA1 to the synapse to generate a prolonged T cell/APC interaction. Such long-lasting contacts are necessary for the formation of properly functioning $CD8^+$ memory T cells[61]. Patients with autosomal recessive DOCK8 deficiency owing to mutations in the DOCK8 gene develop a PID characterized by atopic dermatitis, cutaneous infections, recurrent respiratory tract infections, eosinophilia, and low IgM levels and high IgE levels[62, 63]. These patients exhibit reduced numbers of naive and memory $CD8^+$ T cells, whereas they show an increased presence of senescent $CD8^+$ T cells[61]. Furthermore, DOCK8 plays an important role in maintenance of the lymphocyte shape during T-cell migration. Without normal DOCK8, T-cells are submissive to a new form of cell death called cytothripsis, where lymphocytes undergo nuclear deformation and cell elongation during prolonged migration through tissue. As a consequence the cell cannot

endure the mechanical forces of the surrounding tissue and the plasma membrane breaks. Thus DOCK8 deficiency patients experience severe cutaneous viral infections also through the inability of cytotoxic T cells to migrate to the infected site[108]

In conclusion, the impaired IS formation and consequently arising $CD8^+$ memory T cell defect in DOCK8-deficient patients may contribute to the frequently observed occurrence of viral infections. In turn, repetitive viral infections may induce senescence of $CD8^+$ T cells[61].

STK4

The phenotype of DOCK8 deficiency partially corresponds to that of STK4 deficiency[64]. Such similarity is not unexpected, since STK4 indirectly recruits and activates DOCK8 in murine thymocytes, in which DOCK8 recruitment and activation is suggested to be a necessity for f-actin rearrangement and T cell exit from the thymus[65]. Upstream from STK4, PLCγ1 induces the activation of GEFs for the small GTPase RAS-related protein RAP1 (RAP1)[66]. Following its activation by these GEFs, RAP1 associates with RAPL (regulator for cell adhesion and polarization enriched in lymphoid tissues)[67]. RAPL subsequently binds STK4 and in murine cells active RAP1 facilitates RAPL's binding to STK4. Together, RAP1 and RAPL activate STK4, whereupon STK4 induces the adhesive activity of LFA1, thereby facilitating conjugate formation between T cells and APCs[68]. Furthermore, the active conformation of LFA1 induces f-actin reorganization, which also stimulates adhesion[69].

More recently, a pathway downstream of STK4 was elucidated, again in murine T cells[70]. DENN domain-containing protein 1C (DENND1C) is a protein downstream of STK4, which can be phosphorylated in an STK4-dependent manner. Subsequently, DENND1C acts as a GEF to activate the RAS-related protein RAB13 (RAB13). RAB13 is proposed to be involved in transport of LFA1 to the IS and subsequent IS formation. Therefore, a complex of RAPL, STK4, DENND1C, and RAB13 may play an important role in the recruitment and activation of LFA1[70], which is essential for proper T cell/APC conjugate formation. In STK4-gene variant PID patients, symptoms are recurrent viral, bacterial, and fungal infections, predominantly of the skin and respiratory tract; autoimmunity; decreased numbers of $CD4^+$ T cells, naive T cells, and T cells that recently left the thymus; and elevated IgG, IgA, and IgE levels[64, 71-73].

To our best knowledge, f-actin polymerization and IS formation have not been examined in STK4-deficient patients so far. Furthermore, the relation between DOCK8 and STK4 in peripheral T cells remains to be elucidated. However, the hampered conjugate formation in STK4 deficiency may, similar to DOCK8 deficiency, give rise to defective $CD8^+$ memory T cells and consequently increase the susceptibility to recurrent infections.

Table 1

PID protein mutations and clinical phenotype

PID protein	Mutation (DNA and/or protein level)	Immunological phenotype	Clinical phenotype
LCK	c.1022T>C /p.L341P	$CD4^+$ T cell lymphopenia	Recurrent infections, autoimmunity, autoinflammation
UNC119	c.136G>T /p.G22V	$CD4^+$ lymphopenia	Recurrent bacterial, viral, and fungal infections
ZAP70	Non-coding intron G-to-A substitution and as listed in Hauck et al [27]	Diminished $CD8^+$ T cells, dysfunctional $CD4^+$ T cells, high IgE.	Recurrent infections, SCID-like phenotype.
PI3K	*PIK3R1* (P85α, P55α, P50α): splice site mutations, deletion exon 10. *PIK3CD* (P110δ): c.3061G>A/p.E1021K; c.1246T>C/p.C416R; c.1002C>A/p.N334K; c.1573G>A/p.E525K	T-cell lymphopenia, SmB-, hypogammaglobulinaemia, high IgM levels.	Recurrent respiratory infections, bronchiectasis; autoimmunity; chronic EBV and CMV infection, B-cell lymphoma, malignant lymphproliferative syndromes.
ITK	c.1003C>T/p.R335W; c.1764C>G/p.Y588X; c.86G>A/p.R29H; c.1497delT/p.S499SfsX4; c.468delT/p.P156PfsX109; c.49C>T/p.Q17X	$CD4^+$ lymphopenia, loss of naive T cells, EBV- associated B- cell lymphoproliferation, reduced antibody production, Decreased numbers of iNKT- cells	EBV infections, Hodgkin lymphoma, hepatosplenomegaly, lymphadenopathy, EBV-related malignancies.

STK4	c.58-61delATAG/p.E22X; c.750G>A/p.W250X; c.349C>T/p.R117X; c.1103delT/p.M368RfsX1; c.343C>T/p.R115X	$CD4^+$ lymphopenia, EBV- associated B-cell lymphoproliferation, neutropenia	Recurrent viral, bacterial, and fungal infections of the skin and respiratory tract, cutaneous warts, skin abscesses, autoimmune phenomena
VAV1	Deletion exon 2-27	Hypogammaglobulinemia, T cell dysfunctioning	Recurrent infections, bronchiectasis, osteoporosis, colon diverticulitis
DOCK8	Point mutations, splice site mutations, creation of cryptic splice site, premature termination, (exon) deletions	Eosinophilia, low IgM, levels, high IgE levels, reduced naive and memory $CD8^+$ T cell numbers.	Recurrent upper- and lower respiratory tract infections, skin abscesses, recurrent viral infections (HSV, molluscum contagiosum), candidiasis, atopic dermatitis
CORONIN1A	chr. 16p11.2 deletion; c.248-249delCT/p.P83RfsX10; c.1077delC/p.Q360RfsX44; c.400G>A/p.V134M; c.35G>T/p.R12L	EBV associated B cell lymphoproliferation; decreased lymphocyte numbers; T cell lymphopenia, SmB-	Early-onset recurrent infection
WIPF1	c.1301C>G/p.S434X	Impaired NK cell function and T cell lymphopenia.	Recurrent infections; eczema; thrombocytopenia. WAS-like phenotype

Summary of immunological and clinical phenotype of the primary immunodeficiency proteins as described in the review. EBV: Epstein-Barr Virus; HSV: herpes simplex virus; iNKT: invariant natural killer T; NK: natural killer; SCID: severe combined immunodeficiency; SmB-: decreased/absent switched memory B cells; WAS: Wiskott-Aldrich Syndrome.

3. Downstream regulators of actin polymerization

Uncontrolled actin polymerization is likely to produce far-reaching cellular consequences, as exemplified by activating mutations in WASP that cause X-linked neutropenia[8]. Actin polymerization is therefore tightly regulated in the normal situation. Older actin filaments need to be severed to recycle actin monomers and stimulate dynamic f-actin rearrangement. Actin regulators serve the function to guide such dynamic remodeling. We describe three PID-related regulators, namely Coronin 1A, WAS/WASL-interacting protein family member 1 (WIPF1, also called WIP), and proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1, also called CD2BP1).

Regulatory molecules and PID phenotype

Coronin 1A

Coronin 1A binds to f-actin and to the ARP2/3 complex[74]. In yeast, the Arp2/3 complex functions in initiation of actin filament polymerization towards either activation or inhibition by the local concentration of Coronin1A protein[75, 76]. Coronin1A also interplays with Cofilin, an f-actin severing and depolymerizing protein; both compete for binding to newly polymerized actin filaments. Coronin1A binds in a distinct manner to older actin filaments and enhances severing of these filaments by Cofilin[76, 77], giving Corinin1A an important role in f-actin turnover[77].

In human T cells, TCR signaling induces actin filament polymerization, upon which Coronin 1A is recruited to the IS [78-80]. The further presence of Coronin 1A at the IS remains disputed; on one hand, it has been shown that Coronin 1A partially disappears from the IS within 30 minutes after T cell activation [79]. Contrastingly, in another study Coronin 1A was observed to be predominantly excluded from the IS[81].

Features observed in patients harboring CORO1A mutations are recurrent infections, EBV-associated lymphoproliferative disorders, T cell lymphopenia, severe naive T cell lymphopenia, diminished (memory) B cell numbers, a small TCR repertoire breadth, and absence or diminished presence of antigen-specific antibodies. As expected, the PID phenotype depends on the nature of the CORO1A gene mutation[74, 82-85].

Considering that the role of Coronin 1A in f-actin regulation and IS formation is not yet fully clarified, distinct explanations exist for the phenotype of Coronin 1A deficiency. The observed severity of naive T cell lymphopenia may relate to augmented baseline levels

of f-actin in Coronin 1A-deficient T cells, thereby culminating in apoptosis as mediated by a reduction in mitochondrial membrane potential [86]. Apoptosis can however not be induced by an increase in baseline f-actin levels alone[80, 87]. Alternatively, decreased survival of naive T cells might not be related to f-actin levels, but be a result of reduced calcium mobilization ability, a feature that is required for the production of survival signals[87, 88]. A third explanation is that Coronin 1A deficiency induces hyperstable f-actin and ISs, which may result in attenuated calcium mobilization and therefore increased apoptosis[89]. Thus, hyperstability of the IS may relate to diminished survival of naive T cells[89], thereby contributing to T cell lymphopenia, a restrained TCR repertoire[74], diminished specific antibody titers, diminished memory B cell numbers, and recurrence of infections.

WIPF1

Our second discussed example of a PID-related f-actin regulator is WIPF1. WIPF1 binds and stabilizes f-actin *in vitro*[90]. Furthermore, WIPF1 associates with several actin-linked proteins including cytoplasmic protein NCK1 (NCK1, also called NCK), profilin, and SRC substrate cortactin (cortactin), which binding is suggested to promote actin filament polymerization[91, 92].

Moreover, WIPF1 binds to WASP and CRK-like protein (CRKL)[23]. The association with WASP is required to protect WASP from ubiquitination and subsequent degradation by the proteasome, and calpain-mediated degradation[93, 94]. WIPF1's binding to CRKL regulates the recruitment of WASP to the developing IS. CRKL itself is recruited to ZAP70 after CD3 stimulation. Accordingly, TCR triggering induces recruitment of the CRKL-WIPF1-WASP complex to ZAP70 and the IS. WIPF1 and WASP eventually dissociate due to phosphorylation of WIPF1's WASP-binding domain by protein kinase C (PKC) isoform θ and subsequently an increased accumulation of f-actin occurs in the established IS [23]. By analysis of stimulated murine T cells, it was shown that WIPF1 is required to increase the f-actin content of the cell and augment the density of the cortical f-actin network. Furthermore, WIPF1 is involved in cell spreading and T cell/APC conjugate formation, although not through LFA1 recruitment[95]. Binding of WIPF1 to actin is indispensable to cell spreading and formation of a proper cortical f-actin network[96].

In support of the notion that WIPF1 is essential to WASP stability, WASP deficiency was also observed in a WIPF1-deficient patient and WIPF1 transfection of the patient's T cells ameliorated the amount of WASP[97]. In addition, WIPF1 deficiency results in similar features as WASP deficiency, comprising eczema, recurrent infections, thrombocytopenia, T cell lymphopenia (especially of $CD8^+$ T cells), and an elevated amount of natural killer cells that are, however, dysfunctional. Nevertheless, some features of WIPF1 deficiency are more severe compared to WASP deficiency[97].

Because of the close interrelationship between WASP and WIPF1, similar to WASP-deficient patients, WIPF1-deficient patients may show aberrant f-actin rearrangement with consequential unstable IS formation and impaired T cell activation[53]. T cells that have undergone a suboptimal activation program are suggested to differentiate into interleukin (IL)-4 producing T helper-2 cells instead of interferon (IFN)-γ producing T helper-1 cells[99]. Since IFN-γ is required for killing of intracellular pathogens and IL-4 is related to IgE production and eczema, the above-proposed mechanism may contribute to infections with intracellular pathogens, elevated IgE levels, and eczema observed in the WIPF1-deficient patient[97, 99]. Since the number of T cell/APC conjugates is unaffected in WASP deficiency[53], the WASP-independent function of WIPF1 in f-actin polymerization through binding to f-actin, NCK1, profilin, and cortactin may reduce the amount of T cell/APC conjugates in case of WIPF1 deficiency. Thus, disturbed WASP-independent functions of WIPF1 may result in a more pronounced proliferation defect[97], resulting in a more severe phenotype.

PSTPIP1

Mutations in PSTPIP1, our third example of an f-actin regulatory protein, are associated with the pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome. Currently, the pathways leading from PSTPIP1 mutations in PID patients to excessive IL-1β production in monocytes/macrophages have been principally elucidated. However, PSTPIP1 plays a distinct role in T cells, which is not completely understood[100]. Known however is that PSTPIP1 associates with CD2, amongst others [101]. CD2 is a T cell surface receptor that binds to lymphocyte function-associated antigen 3 (LFA3) or CD48 on APCs. PSTPIP1 can also indirectly bind to CD2, using CD2 associated protein (CD2AP) as a bridging molecule. Furthermore, the SH3 domain of PSTPIP1 binds to WASP. Due to such adaptor function, PSTPIP1 is important to recruit a complex of CD2, CD2AP, PSTPIP1, and WASP at the IS, which is required for actin polymerization and proper conjugate formation between murine T cells and APCs[101].

Another binding protein of PSTPIP1 is tyrosine-protein phosphatase non-receptor type 12 (PTPN12, also called PTP-PEST). PSTPIP1 binds with its coiled-coil domain to PTPN12, thus indirectly linking PTPN12 to WASP that binds to the SH3 domain of PSTPIP1. PTPN12 dephosphorylates WASP, thereby inhibiting actin polymerization and synapse formation of murine T cells and APCs. Therefore, PSTPIP1 in complex with PTPN12 might play an important role in the delicate regulation of WASP-mediated actin polymerization[102].

Dependent on the location and/or type of a mutation in human PSTPIP1, a more pronounced T cell defect may arise in patients, for example owing to aberrant f-actin polymerization and/or IS formation. The underlying genetic cause of such defect may be mutations that affect both PSTPIP1's function in regulating IL-1β production and binding of PSTPIP1 to CD2, CD2AP, WASP, and/or PTPN12.

Perspective

In recent years, next generation sequencing has transformed PID research by better enabling the identification of gene variants that cause PID. Current PID work often combines the discovery of such mutations with description of the clinical and general immunological features of the PID patient in which the mutation was found. A next frontier in PID research is to stratify patients according to common genetic pathways (perhaps the reminiscent of the role of adaptor proteins in TLR signaling [109]) rather than clinical features, to benefit the development of new targeted treatments.

While single case reports aim at explaining the disease features from functions of the mutant PID-protein, the observed f-actin and IS-related function of the protein and the possible contribution of malfunction to disease might be overlooked. We here reviewed available data that connects f-actin and IS-related function of PID-associated proteins to the PID phenotype by exploring the contribution of these functions to the phenotype. Our main finding is that aberrant f-actin remodeling and IS formation indeed can affect disease features observed in selected PID patients. However, we found that the mechanism of contribution often remains elusive, as not for every protein variant detailed studies can be performed. Furthermore, we based the majority of our conclusions on observations in cell lines, (modified) cells from healthy donors, and murine cells. PID mouse models obviously do not fully correspond with the phenotype of PID patients[14, 27] and phenotypes may vary depending on genetic background[48]. Therefore, we recommend that case reports of mutations in proteins related to f-actin and the IS also investigate the status of f-actin and T cell/APC conjugate formation in patient cells. Using such an approach, we should gain more insight into the importance of f-actin driven IS formation and the underlying mechanisms by which it contributes to PIDs.

The focus of this review article was on IS formation in T cells, while besides in IS formation, the discussed actin-linked proteins are usually involved in other actin-related and unrelated processes. Examples of these are development, migration, and regulation of apoptosis (STK4)[71], or serine/threonine-protein kinase mTOR (mTOR) signaling (PI3K)[28, 33]. Therefore, we acknowledge that defects in these processes might also influence or dominate the PID phenotype. Another note of caution is that other immune cells besides T cells, and non-immune cells can also be affected by the discussed protein defects. For instance, Coronin 1A and WIPF1 deficiency hamper natural killer (NK) cell cytotoxicity[103, 104]. Furthermore, DOCK8 deficiency impedes NK cell cytotoxicity and f-actin driven cytotoxic synapse formation[105], impairs the development and functioning of natural killer T (NKT) cells[106], and reduces antibody production in B cells by disturbing intrinsic signaling pathways[107]. As a final example, STK4 is involved in the antiproliferative capacity of keratinocytes, making STK4-deficient keratinocytes more prone to viral infections[73]. Therefore, although properly

functioning T cells are indispensable for adequate immunity, defects in other cell types may aggravate or dominate the phenotype.

In summary, the current review has shed light on PID-related signaling proteins in the T cell contact zone with APCs and connected their function in f-actin remodeling and IS formation to the phenotype observed in PID patients. We illustrated the relevance of examining the status of f-actin and T cell/APC conjugate formation in patient cells defective in such proteins. Clarification of the relative involvement of PID-proteins in f-actin-mediated cytoskeletal remodeling in T cells should help to stratify the otherwise diverse cluster of PID patients.

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CHAPTER 6

Measurement of pneumococcal polysaccharide vaccine responses for diagnosis of common variable immune deficiency: a comparison of luminex and ELISA-based methods

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Published in: Journal of Clinical Immunology 2014

ABSTRACT

Two distinct methods for the quantification of PnPS vaccination responses are now in use for immunodeficiency diagnostics, those that involve IgG measurement of separate serotypes by a luminex method, and total IgG measurement of the Pn-PS vaccine specific serotypes by combined ELISA. We here compare the usefulness of the combined PnPS IgG response measurement with specific serotype IgG response measurement in a defined cohort of immunodeficiency patients and control patients.
Patients analysed were CVID or specific antibody deficient (SAD), as controls we used children with recurrent infections without underlying immunodeficiency. The combined IgG responses and specific serotype IgG responses after PnPS vaccination were measured respectively by ELISA and Luminex.
Our data show that combined PnPS IgG response measurement can mask the presence of immunodeficiency in up to 42 % of patients. The specific serotype method is superior in detection of Pn-PS vaccination responses and thereby for immunodeficiency diagnostics.

INTRODUCTION

Common variable immunodeficiency (CVID) is a heterogeneous primary immunodeficiency characterized by defective antibody production[1]. One hallmark in the diagnosis is a poor response to vaccination[2], which is often gauged by measuring IgG responses to 23-valent pneumococcal polysaccharide (PnPS) vaccine. The majority of childhood vaccination programs now include vaccination against Pneumococcal disease using a 7-, 10- or 13- valent conjugated Pneumococcal vaccine, that has been shown to provide protection against invasive Pneumococcal disease by the serotypes in these vaccines[3]. However, vaccination using a conjugate Pneumococcal (Pn-C) vaccine may interfere with the diagnostic use of the 23-valent polysaccharide vaccine[4].

Two different methods for the quantification of the Pn-PS vaccination response are available and in use by diagnostic laboratories which are IgG measurement of separate serotypes by luminex[5], and a commercially available combined Pn-PS IgG measurement by ELISA. We here compare the usefulness of the combined PnPS IgG measurement with specific serotype measurement in a defined cohort of immunodeficiency patients and control patients. Our data show that the ELISA-based combined measurement of IgG Pn-PS responses can mask the presence of immunodeficiency in up to 42 % of patients. We conclude that the luminex-based serotype specific method is superior in detection of Pn-PS vaccination responses and thereby for immunodeficiency diagnostics.

METHODS

Subjects:
We included CVID patients (n=11) and specific antibody deficient (SAD) patients (normal IgG, IgM and IgA, with disturbed specific antibody production; n=13), diagnosed according to the ESID criteria[6], in a retrospective cohort study at the University Medical Centre Utrecht from 2010-2011. As control group, we enrolled children referred to our hospital with recurrent infections (infections only group; IO; n=22), who proved to lack a diagnosis of primary immunodeficiency and that were otherwise healthy (see Table I). Patients were vaccinated with 23-valent Pn-PS vaccine Pneumovax®. All vaccinations were part of our institutional work up scheme for evaluation of recurrent infections.

Luminex and ELISA:
Measurement of specific serotype IgG responses by luminex and combined IgG responses by ELISA were performed according to standard operation procedures 4 (3-6) weeks after vaccination and data were analysed using SPSS (Mann Whitney-test, Chi square for statistics). For luminex assays, we measured the IgG response of 11 serotypes: 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F[5]. Luminex reagents were purchased

from Jackson Immuno Research, Valley Biomedical, Merck, and Statens Serum Institut Denmark. For the ELISA method, we measured the combined IgG response to the 23 serotypes present in the Pneumovax® vaccine. The Binding Site Group Ltd kindly donated ELISA kits used in this study.

RESULTS AND DISCUSSION

Patients enrolled in this study were divided in two groups; immunodeficient (CVID and SAD combined) or infections only (IO). The diagnosis of CVID/SAD was based on luminex test results by the percentage of serotypes found responsive (IgG>1 mg/ml) after diagnostic vaccination with Pneumovax®[7]. In patients previously vaccinated with a Pn-C vaccine, the luminex results of the serotypes not present in the Pn-C vaccine (serotypes 1, 3, 5 and 7F) were evaluated. We further made use of the following benchmarks to correct for young age: for ages 4-6 years, an abnormal Pn-PS result was defined as <50% of serotypes evaluated reaching a post vaccination IgG titer of > 1 mg / ml. For age ≥ 6 years, an abnormal result was defined as < 75% of serotypes evaluated reaching a post vaccination IgG titer of ≥ 1 mg / ml (Table I). For the combined IgG ELISA, we used an IgG reference value of 40 mg/L[8].

We asked how the combined IgG response measurement by ELISA compares to the serotype specific IgG response measurement by luminex. To this end, we first assessed the vaccination responses by luminex and categorised these according to their readings from combined serotype ELISA data: below 40 mg/L (Figure 1A) or above (Figure 1B).

We then found that 10 out of 24 (42%) patients (by luminex) had a sufficient response by ELISA (sensitivity 58%). There were no false positive test results (<40 mg/L) for the healthy subjects (Table II).

We next focused our study on patient samples that were tested by ELISA as sufficient (>=40mg/L), (Figure 1B). We observed that individual immunodeficiency patients exhibit diverse presentations in serotype-specific IgG responses as described previously[9]. Most Pn-PS IgG responses were dominated by only few serotype-specific IgG that varied amongst individuals. Moreover, 3/10 patients showed an insufficient response on all 11 serotypes measured by luminex, while being tested as sufficient response by ELISA, probably through high IgG titers on the remaining serotypes not measured by luminex. Thus, false negative results from the ELISA method are caused by selective responsiveness to pneumococcal serotypes that conceal defective immune responsiveness to a majority of pneumococcal serotypes.

Table 1
Baseline characteristics

	CVID and SAD patients (N=24)	Infection Only (N=22)	
Male (n)	14	5	P<0.08
Female (n)	10	17	
Age (means, range)	12y (4-67)	11 y (3-35)	P<0.106
Number of patients pre-vaccinated with 7-valent Pn-C vaccine (n)	3	5	P<0.887
Specific serotype IgG response (mg/ml): amount of serotypes sufficient/total			
CVID patient 1 (prevnar)	1/4	IO 1 (prevnar)	3/4
CVID patient 2	6/11	IO 2	10/11
CVID patient 3	5/11	IO 3	6/11
CVID patient 4	3/11	IO 4	9/11
CVID patient 5	3/11	IO 5 (prevnar)	4/4
CVID patient 6	1/11	IO 6	11/11
CVID patient 7	0/11	IO 7	9/11
CVID patient 8	0/11	IO 8	11/11
CVID patient 9	0/11	IO 9	11/11
CVID patient 10	0/11	IO 10	10/11
CVID patient 11	0/11	IO 11	10/11
SAD patient 1 (prevnar)	0/4	IO 12 (prevnar)	4/4
SAD patient 2	7/11	IO 13 (prevnar)	4/4
SAD patient 3	7/11	IO 14	8/11
SAD patient 4	6/11	IO 15	7/11
SAD patient 5	5/11	IO 16	10/11
SAD patient 6	5/11	IO 17	9/11
SAD patient 7	4/11	IO 18	10/11
SAD patient 8	4/11	IO 19 (prevnar)	2/4
SAD patient 9	3/11	IO 20	11/11
SAD patient 10	2/11	IO 21	6/11
SAD patient 11	1/11	IO 22	10/11
SAD patient 12	0/11		
SAD patient 13 (prevnar)	0/4		

Baseline characteristics of patient- and control group. The patient group consists of CVID (N=11) and SAD (N=13). The control group consists of patients with recurrent infections, but a normal evaluation for immune deficiency. Prevnar: pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F individually conjugated to diphtheria CRM197 protein. Specific serotype IgG response: amount of serotypes found responsive (IgG>1 mg/ml) after diagnostic vaccination with Pneumovax®.

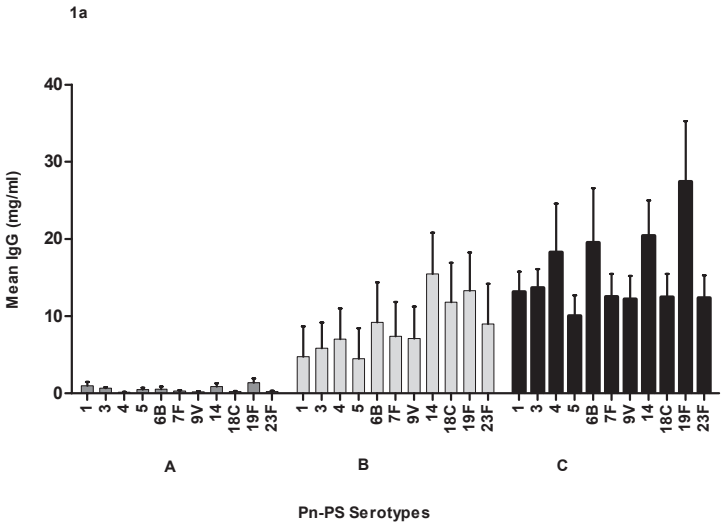

Figure 1A

Mean Pn-PS vaccination response in CVID and SAD patients and infection only group

Mean IgG (mg/ml) responses per serotype as measured by luminex. A: CVID and SAD patients with insufficient response by combined ELISA (<40 mg/ml; N=14), B: CVID and SAD patients with sufficient response by combined ELISA (≥40 mg/ml; N=10), C: infections only patients (N=22). Pn-PS: pneumococcal polysaccharide.

We finally hypothesized that pre-vaccination with Pn-C vaccine (e.g. Prevnar®), before vaccination with Pneumovax®, may additionally contribute to false negative ELISA test results, through protein-conjugate mediated priming of the IgG response[10]. Patients that had been pre-vaccinated with Prevnar indeed routinely had increased Pn-PS responses to the serotypes present in Prevnar when compared non-pre-vaccinated patients (Figure 2).

The use of a combined Pn-PS ELISA for diagnosis of CVID/SAD patients is especially troublesome in Pn-C pre-vaccinated individuals, even more for future testing since a majority of childhood vaccination programmes now include Pn-C vaccination. The luminex method tests serotypes separately and allows evaluation of serotypes not present in Pn-C vaccines. For CVID / SAD patients, we observed a 42% false-negative rate when the combined Pn-PS ELISA is used. We therefore advocate the use of a Pn-PS luminex method that allows measuring serotypes not present in any of the Pneumococcal conjugate vaccines for diagnostic purposes of antibody deficiency patients.

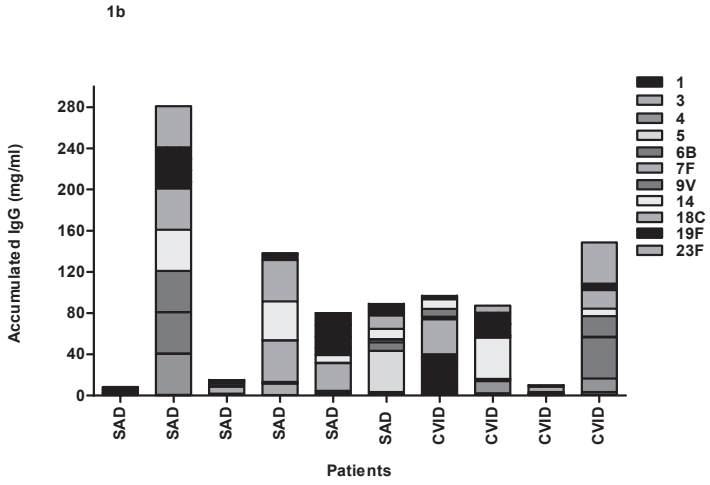

Figure 1B

Pneumococcal polysaccharide antibody responses in CVID and SAD patients with normal combined ELISA results

Specific serotype responses as measured by luminex for patients (N=10) with sufficient response by ELISA (≥40 mg/L). Accumulated bars indicate the absolute value of IgG response (mg/ml). Pn-PS: pneumococcal polysaccharide. Patients <6 y reached a post vaccination IgG titer of > 1 mg / ml for <50% of serotypes. Patients ≥ 6 reached a post vaccination IgG titer of ≥ 1 mg / ml for < 75% of serotypes

Table 2

Cross table of specific serotype and combined IgG response measurement

	Patient (N=24)	Infection Only (N=22)	
ELISA IgG response >=40mg/L (n)	10/24 (42%)	22/22 (100%)	P<0.00
ELISA IgG response < 40 mg/L (n)	14/24 (58%)	0 (0%)	

Crosstable of CVID/SAD patients and Infection Only group as determined by luminex-based specific serotype IgG response measurement versus ELISA-based combined IgG response measurement with cut off 40 mg/L. Sensitivity 58%, specificity 100%.

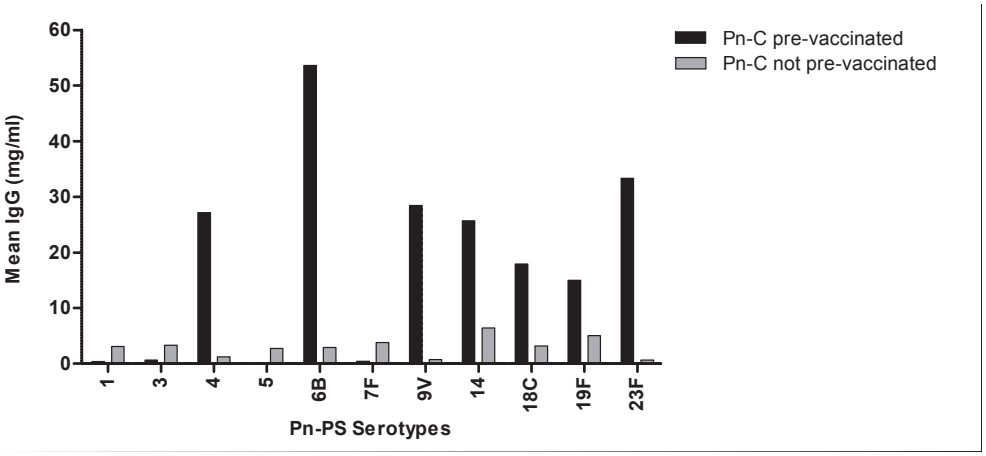

Figure 2

Mean Pneumococcal polysaccharide antibody response per serotype in CVID and SAD patients, with and without Prevnar pre-vaccination

Mean IgG (mg/ml) responses upon Pn-PS vaccination per serotype as measured by luminex, for patients with and without pneumococcal conjugate pre-vaccination (Prevnar) (N=3 and N=21, respectively). Pn-PS: pneumococcal polysaccharide

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CHAPTER 7

Antigen-specific IgA titers after 23-valent Pneumococcal vaccine differentiate transient from persistent antibody deficiency disease

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Published in: Vaccine 2015

ABSTRACT

Pediatric patients with antibody deficiency may either be delayed in development of humoral immunity or may be persistently deficient in antibody production. To differentiate between these entities, we examined the 23-valent pneumococcal polysaccharide (PnPS) vaccine-induced IgM-, IgG- and IgA antibody responses in a cohort of 66 children with recurrent respiratory tract infections. Individual serum titres against 11 pneumococcal serotypes were measured by Luminex. The cohort contained 33 antibody deficiency patients, 17 transient antibody deficiency patients and 16 patients without antibody deficiency diagnosis (control group). Transient antibody deficiency patients produced consistently higher levels of PnPS-specific IgA responses than antibody deficiency patients. Decreased IgA responses to serotypes 1, 5, 7F and 18C were most discriminative to stratify transient antibody deficiency patients from antibody deficiency patients with persistent disease. We conclude that measuring PnPS-specific IgA responses may predict the disease course in young children diagnosed with antibody deficiency and suggest confirmation of these data in a prospective setting.

INTRODUCTION

Common Variable Immunodeficiency Disorder (CVID) is one of the most prevalent primary immunodeficiency in children and adults [1, 2]. Diagnosis is based on a combination of decreased (< -2SD) serum IgG and IgA / IgM levels, absent isohemagglutinins and/or insufficient vaccination responses against tetanus and/ or pneumococcal polysaccharide vaccines [3]. In pediatric patients, impaired immunoglobulin levels and defective responses against these vaccinations bring forward antibody-deficient children who may either be delayed in the development of the humoral branch of their immune system, or alternatively be persistently deficient in antibody production[4, 5], predicting a life-long disease prospect which implicates more frequent and intense immune monitoring and additional prophylactic and therapeutic measures [6].

Recent studies indicate that the assessment of anti-PnPS IgA- and IgM responses can be of additional value in immunodeficiency diagnostics[7-12]. For example, adult CVID patients with low memory IgM B-cell percentages and combined insufficient anti-PnPS IgM- and IgA responses were described to be at increased risk of developing pulmonary complications such as bronchiectasis[8, 11]. In the elderly, decreased anti-PnPS IgA- and IgM responses seem to be indicative for immunosenescence, more than the specific anti-PnPS IgG responses[9]. The anti-PnPS IgA response of elderly shows delayed maturation through which it only reaches an anti-PnPS IgA response level similar to that in young adults after 28 days.

In this study, we hypothesized that children diagnosed with antibody deficiency disease based on insufficient anti-PnPS IgG responses but with partially conserved anti-PnPS IgA- and IgM responses will display delayed maturation of the immune response with spontaneous recovery. We therefore investigated a cohort of 66 children with recurrent respiratory infections, and analysed their vaccination-induced IgM-, IgG- and IgA anti-pneumococcal polysaccharide (PnPS) responses. We show that measuring PnPS-specific IgA responses may indicate the antibody deficiency disease course in young children, and provide cut-off values for PnPS serotypes 1, 5, 7F, 18C that may be of diagnostic use with 84% accuracy.

METHODS

Subjects

We conducted a retrospective cohort study in 96 children (age range: 3-17 years) who underwent protocolled diagnostic- and follow up evaluations for suspected antibody deficiency at the Wilhelmina Children's Hospital /University Medical Centre Utrecht between 2008 and 2014. From the 96 patient records studied, 66 suspected antibody

deficiency patients had a complete diagnostic and follow up (after 3-5 years) evaluation of laboratory and clinical values as specified below. These patients were included for analysis. For laboratory measurements initial samples were used, as obtained when the subjects were first seen at the patient outward clinic. Informed consent to analyse the data obtained from the medical charts was waived by the Institutional Review Board.

Laboratory measurements

The standardized diagnostic evaluation included a full blood count and differentiation, measurement of serum IgM, IgG and IgA titers, IgG subclass titers and measurement of anti-pneumococcal IgM, IgG and IgA antibodies 4-6 weeks after 23-valent pneumococcal polysaccharide (PnPS) vaccine (Pneumovax®, Merck, The Netherlands). Follow up evaluations included evaluation of IgM, IgG, IgG subclass and IgA levels and a repeat test of specific antibody responses to PnPS vaccine. Patient serum samples were stored pre- and 4-6 weeks post vaccination with 23-valent PnPS vaccine at -80C. Anti-PnPS IgM, IgG and IgA responses to pneumococcal serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F were determined by Luminex as described previously [13, 14].

Criteria for a sufficient anti-PnPS IgG response were age dependent: in children <6 years, a normal response was defined as serotype specific IgG responses of >1 ug/ml in at least 6/11 (50%) of serotypes measured, in children >6 years a normal response was defined as IgG responses of >1ug/ml in at least 8/11 (75%) of serotypes measured[15]. Four patients were pre-vaccinated with a 7-valent pneumococcal conjugate vaccine (PnC, Prevnar®, Pfizer, The Netherlands) and of these patients only PnPS serotypes 1, 3, 4 and 7F (which are not present in the 7-valent PnC vaccine) were analysed.

Definition of patient groups:

The 66 subjects were divided in the following three groups:

1. Antibody deficiency patients:

Patients diagnosed with CVID or anti-polysaccharide antibody deficiency (APAD) based on the following criteria.

CVID: 1. recurrent infections (respiratory and/or gastrointestinal), 2. insufficient anti-PnPS IgG responses (see above) 3.IgG and IgA and/or IgM levels below -2SD, and 4. exclusion of known causes of hypogammaglobulinemia.

APAD: 1. recurrent infections (respiratory and/or gastrointestinal), 2. insufficient anti-PnPS IgG responses (see above) 3. normal values for IgG, IgA and IgM.

All patients in the group of persistent antibody deficiency had no change in laboratory or clinical diagnosis after 3-5 years of follow up.

2. Transient antibody deficiency of childhood (TAD).

Patients with a diagnosis of transient antibody deficiency of childhood had an initial diagnosis of CVID or anti-polysaccharide antibody deficiency, however they showed normalization of laboratory and clinical symptoms in the period 3-5 years following initial diagnosis. Because patients included in this study were >3 years old and had insufficient anti-PnPS IgG responses, they did not classify for diagnosis of transient hypogammaglobulinemia of the infancy (THI) ([6, 16]).

3. Control group

The control group consisted of subjects evaluated for recurrent infections and who proved to have normal levels of IgG, IgA and IgM, and intact vaccination responses, and thus did not fulfil criteria for antibody deficiency disease.

Statistical Analysis

All statistical analyses were performed with SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL, U.S.A). To compare continuous data between two groups, Mann-Whitney *U* test was used for non-parametric data,. Categorical data were tested with Pearson's chi-square tests. Receiver operator characteristics curves were composed through SPSS and area under the curves calculated. Tests were performed two-tailed, and p-values ≤ 0.05 were considered significant.

RESULTS

We included 33 antibody deficiency disease patients, 17 were transient antibody deficiency patients and 16 patients who had no antibody deficiency diagnosis and served as control group onward (see Table 1). The median age of the antibody deficiency patients was 9 years, 6 years for TAD patients and 10 years for the control group. All patients had recurrent respiratory infections and one antibody deficiency patient developed bronchiectasis, there were no autoimmune manifestations in our patient group. Maturation of the humoral immune system of the TAD patients occurred up to 16 years of age (see Table 1). In our cohort IgG, IgA and IgM levels did not stratify antibody deficiency patients from transient antibody deficiency (TAD) patients. Also memory IgM, IgG and IgA B-cell percentages of CVID patients were not statistically different between both groups (Supplemental Table 2). Within the antibody deficiency patient group 45% was treated with immunoglobulin replacement therapy, in comparison to 30% temporarily in the transient antibody deficiency children group.

We first compared the anti-PnPS IgM, IgG and IgA responses of the whole antibody deficiency patient group (n=50) to the control group (n=16). Pre-vaccination IgM-, IgG- and IgA anti-PnPS serum titers were not statistically different between patients and

Table 1
Baseline characteristics

	Patients	Control group
Number	50 (75%)	16 (25%)
Antibody deficiency patients	33 (75%)	
TAD patients	17 (25%)	
Age at vaccination response measurement (years, median)	8 (3-17)	10 (5-17)
TAD patients	6 (3-17)	
Antibody deficiency patients	9 (3-17)	
Gender (% male)	54%	31%
TAD patients	59%	
Antibody deficiency patients	52%	
Number of TAD patients per age group (n)		
- 3-6 years	8	
- 6-10 years	2	
- 10-17 years	7	

Baseline characteristics of patient- and control group. TAD: transient antibody deficiency.

controls for all 3 isotypes (data not shown). Post-vaccination anti-PnPS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F IgM-, IgG- and IgA titers were significantly higher in the control group when compared to antibody deficient patients (Fig 1A). Median anti-PnPS IgM-, IgG- and IgA titers with ranges and p-values for patients and controls per serotype are shown in supplemental Table 1A.

Next we compared the PnPS-vaccination induced IgM-, IgG- and IgA responses of antibody deficiency patients and TAD patients in detail. Transient antibody deficiency patients produced significantly higher anti-PnPS IgA levels for all 11 separate serotypes evaluated when compared to antibody deficiency patients (Fig 1B). Also, median anti-PnPS IgG responses were significantly higher in TAD patients when compared to the antibody deficiency patients, but only for selective PnPS serotypes 1, 4, 5, 7F, 9V, 14, 18C, 19F. Considering IgM responses, the anti-PnPS IgM was only significantly higher in TAD patients for PnPS serotypes 9V and 23F. Median (with ranges) anti-PnPS IgM, IgG and IgA responses and p-values of antibody deficiency patients versus TAD patients are shown in supplemental Table 1B. Overall, TAD patients produced higher PnPS-specific antibody responses than antibody deficiency patients, with PnPS-specific IgA levels being most distinctive to stratify both groups.

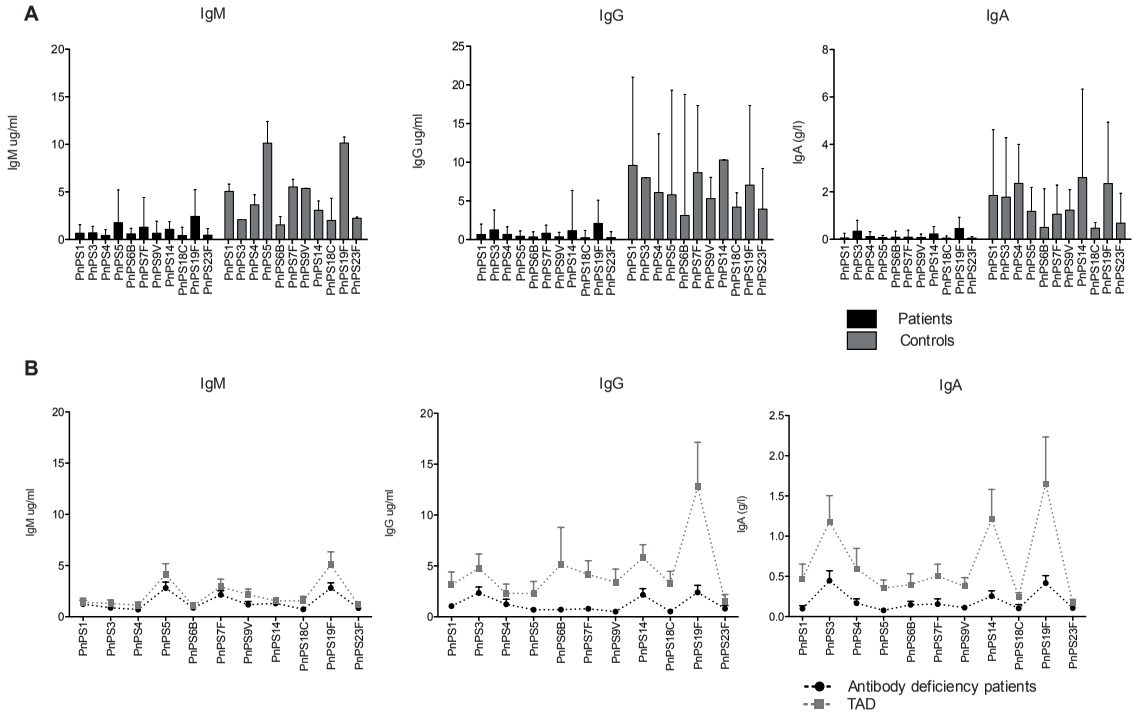

Figure 1

A) Anti-PnPS IgM, IgG and IgA response titers (median with interquartile range) of the total antibody deficient patient group versus control group.

B) Anti-PnPS IgM, IgG and IgA response titers (median with interquartile range) of TAD patients versus antibody deficiency patients.

Depicted are the medians with interquartile ranges of 11 PnPS serotypes measured: 1,3,4,5,6B,7F,9V,14,18C,19F,23F. PnPS response titers were measured 4-6 weeks after 23-valent PnPS vaccination.

PnPS: pneumococcal polysaccharide,

To investigate to what extend our TAD patient group differed from the control group, we assessed the differences in specific IgM-, IgA- and IgG anti-PnPS titers between TAD patients and controls (Fig 2A). The anti-PnPS IgA responses of the TAD patients were significantly diminished on all anti-PnPS IgA serotypes measured when compared to the control group, while 3 anti-PnPS IgG responses of TAD patients were similar to median anti-PnPS IgG responses of the control group for 5 out of 11 serotypes (3, 9V, 14, 18C and 19F) (Fig 2A). Still, the anti-PnPS IgA responses were significantly higher than the anti-PnPS IgA responses of the antibody deficiency patients. These data again support that PnPS-specific IgA levels may be better suited to identify TAD patients as a separate entity, different from healthy controls and from antibody deficiency patients.

Transient hypogammaglobulinemia of the infancy (THI) classically occurs in children < 3 years of age, however this entity has also been described in children older than 3 years [17, 18, 6]. To assess if a specific age range at which time the predictive value of PnPS-specific IgM, IgG and IgA responses could be most distinctive also existed in our cohort, we subdivided the TAD patients into groups with age <6 years (n=8) and age 6-17 years old (n=9). Transient antibody deficiency patients seemed better stratified from antibody deficiency patients in the group of 6 to 17 years for all PnPS-specific IgM-, IgG- and IgA responses (Fig 2B). Again, anti-PnPS IgA responses were most distinctive, showing significant differences between persistent and transient antibody deficiency disease patients for serotype 1, 3, 4, 5, 6B, 7F, 18C, 19F. The anti-PnPS IgG responses differed significantly for serotypes 1, 5, 7F, 14, 18C and 19F. The anti-PnPS IgM responses differed for none of serotypes.

Finally, we sought to provide a clear cut-off value to allow easier separation of TAD patients from antibody deficiency patients. To this end, we calculated optimal cut-off values through ROC curves, for the anti-PnPS IgA responses for the whole patient group (TAD + antibody deficiency patients) versus the control group and for TAD patients versus antibody deficiency patients (see Table 2). When comparing the whole patient group with control patients, the area under the curves (AUC) of the ROC curves using serotype specific IgA responses were all at least 85 %. The anti-PnPS IgA ROC curves for responses on serotypes 1, 5, 7F and 18C proved most distinctive between TAD patients and antibody deficiency patients with AUC's of 84-86%, with the lowest AUC of 70% for serotypes 6B and 23F. In comparison, the ROC curves for anti-PnPS IgG responses showed AUC's between 60%-75%. We therefore defined cut off values for anti-PnPS IgA responses on serotypes 1, 5, 7F and 18C, and suggest cut-off levels of 0.11 ug/ml for serotypes 1, 5, and 18C and 0.30 for serotype 7F as most distinctive to stratify TAD patients from antibody deficiency patients.

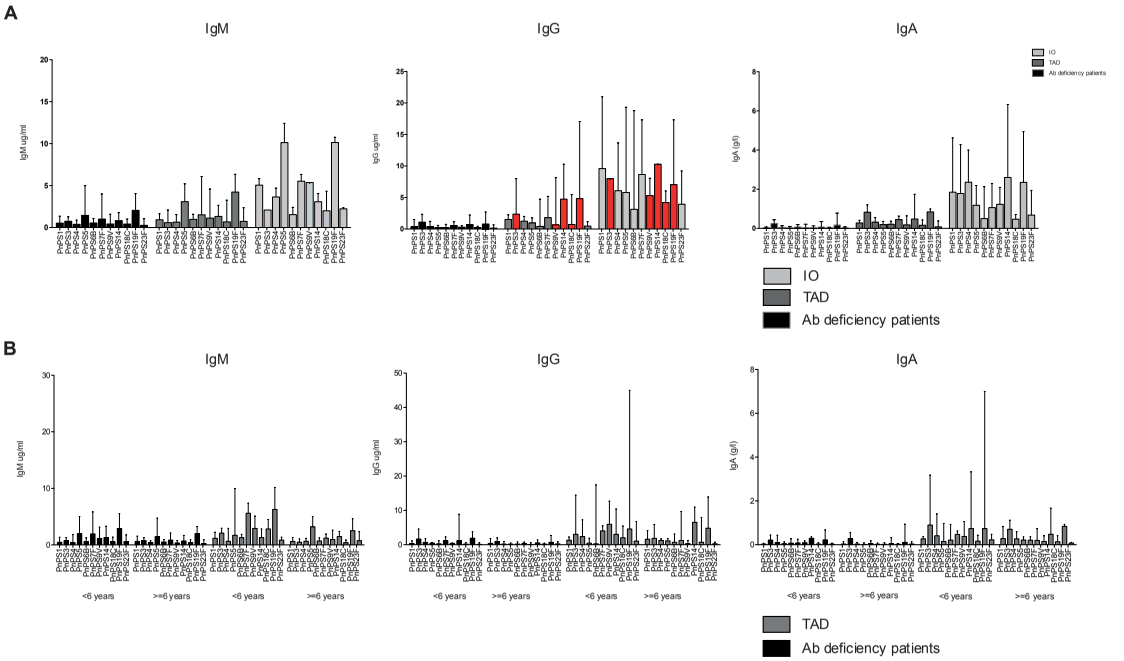

Figure 2

A) Anti-PnPS IgM, IgG and IgA response titers on individual PnPS serotypes of TAD and antibody deficiency patients <6 and >=6 years old.

B) Anti-PnPS IgM, IgG and IgA response titers on individual PnPS serotypes of TAD patients and control group. Overlap between anti-PnPS IgG response titers of TAD patients and control group are depicted in red.

Depicted are medians with interquartile ranges on 11 serotypes measured: 1,3,4,5,6B,7F,9V,14,18C,19F,23F. PnPS response titers were measured 4-6 weeks after 23-valent PnPS vaccination.

PnPS: pneumococcal polysaccharide.

Table 2

Cut off values for Pneumococcal polysaccharide anti-IgG and IgA responses

A Controls versus all patients (antibody deficiency patients+TAD)

IgA responses

	AUC (95% CI)	Optimal cut off value	Sensitivity	Specificity	LR+	LR-
PnPS1	0.95 (.91-1.0)	0.42	87%	93%	13	0.13
PnPS3	0.86 (.76-.96)	1.1	86%	86%	6.4	0.16
PnPS4	0.92 (.86-.99)	0.44	85%	80%	4.3	0.17
PnPS5	0.95 (.91-1.0)	0.5	89%	80%	4.5	0.12
PnPS6B	0.80 (.67-.93)	0.29	68%	70%	2.3	0.4
PnPS7F	0.88 (.80-.97)	0.43	80%	87%	6	0.23
PnPS9V	0.93 (.86-.99)	0.42	86%	80%	4.3	0.17
PnPS14	0.85 (.76-.95)	0.72	80%	74%	3	0.27
PnPS18C	0.90 (.787-.95)	0.24	82%	80%	4	0.23
PnPS19F	0.93 (.87-1.0)	1.31	87%	93%	13	0.13
PnPS23F	0.89 (.80-.99)	0.27	78%	86%	6	0.25

IgG responses

	AUC (95% CI)	Optimal cut off value	Sensitivity	Specificity	LR+	LR-
PnPS1	0.93 (.87-.99)	2.4	81%	93%	12	0.2
PnPS3	0.76 (.62-.90)	3.9	77%	80%	3.8	0.28
PnPS4	0.90 (.81-.99)	1.89	78%	93%	11.7	0.23
PnPS5	0.92 (.85-.98)	2	87%	86%	6.5	0.14
PnPS6B	0.79 (.64-.94)	1.34	80%	86%	6	0.22
PnPS7F	0.87 (.77-.96)	2	77%	80%	3.8	0.28
PnPS9V	0.85 (.75-.94)	1	76%	86%	5.7	0.27
PnPS14	0.71 (.56-.86)	3	65%	73%	2.4	0.47
PnPS18C	0.87 (.78-.95)	1.2	78%	86%	5.8	0.25
PnPS19F	0.79 (.67-.90)	5	76%	73%	2.8	0.32
PnPS23F	0.82 (.69-.95)	0.56	71%	86%	5.3	0.32

B Antibody deficiency patients versus transient antibody deficiency patients

IgA responses

	AUC (95% CI)	Optimal cut off value	Sensitivity	Specificity	LR+	LR-
PnPS1	**.85 (.73-.97)**	**0.11**	**75%**	**80%**	**3.8**	**0.3**
PnPS3	.78 (.65-.92)	0.53	78%	68%	2.5	0.3
PnPS4	.78 (.63-.93)	0.20	84%	75%	3.3	0.2
PnPS5	**.86 (.74-.97)**	**0.12**	**81%**	**80%**	**4**	**0.2**
PnPS6B	.70 (.54-.86)	0.22	78%	50%	1.5	0.4
PnPS7F	**.84 (.73-.95)**	**0.30**	**87%**	**60%**	**2.1**	**0.2**
PnPS9V	.77 (.62-.92)	0.20	87%	43%	1.5	0.3
PnPS14	.77 (.61-.92)	0.35	78%	68%	2.5	0.3
PnPS18C	**.86 (.74-.97)**	**0.13**	**73%**	**84%**	**3**	**0.2**
PnPS19F	.77 (.64-.91)	0.69	71%	68%	2.3	0.4
PnPS23F	.70 (.53-.84)	0.09	78%	50%	1.5	0.4

IgG responses

	AUC (95% CI)	Optimal cut off value	Sensitivity	Specificity	LR+	LR-
PnPS1	.74 (.60-.89)	1.2	71%	62%	1.9	0.45
PnPS3	.66 (.49-.84)	1.2	60%	68%	1.9	0.6
PnPS4	.70 (.55-.86)	0.83	72%	60%	1.8	0.45
PnPS5	.72 (.57-.87)	0.55	68%	56%	1.6	0.55
PnPS6B	.63 (.46-81)	0.29	51%	66%	1.5	0.72
PnPS7F	.83 (.71-.95)	1.3	81%	62%	2.1	0.3
PnPS9V	.72 (.56-.88)	0.47	69%	66%	2	0.46
PnPS14	.72 (.55-.89)	1.5	72%	66%	2.1	0.41
PnPS18C	.75 (.60-.90)	0.3	72%	66%	2.1	0.41
PnPS19F	.76 (.61-.91)	2.6	69%	66%	2	0.46
PnPS23F	.62 (.45-.79)	0.45	65%	53%	1.4	0.64

AUC: Area under the curve, CI: Confidence interval, LR: likelihood ratio, PnPS: pneumococcal polysaccharide.

DISCUSSION

We here show data that anti-PnPS IgA responses can be used to stratify transient antibody deficiency children from antibody deficiency disease patients. With the measurement of partially preserved anti-PnPS IgA titers after 23-valent PnPS vaccination, the group of antibody deficient patients with a transient disease course can now be recognized as a separate group. Moreover, with our definition of possible cut off levels for anti-PnPS IgA responses in serotype 1, 5, 7F and 18C (AUC 84%-86% and specificity 80%), the patients with recognized persistent antibody deficiency disease can be identified earlier for long term monitoring, considering their increased risk to develop antibody deficiency disease complications in the future.

We found that specifically the IgA anti-PnPS responses stratified TAD patients from antibody deficiency patients, while anti-PnPS IgG responses predicted disease course to a lesser extend. As has been shown by Schutz *et al.,* the IgG responses can be highly variable per serotype, more so than the anti-PnPS IgA and IgM responses[7]. We confirmed that in our cohort the anti-PnPS IgG titers were highly variable, especially in the young age groups. Moreover, the median anti-PnPS IgG responses of several serotypes of the transient disease group were comparable to the median anti-PnPS IgG responses of the control group. We thus conclude that IgG class anti-PnPS responses are important for the diagnosis of antibody deficiency disease, while the prognosis of future disease course can best be measured by IgA class anti-PnPS responses.

Other studies have suggested the importance of impaired memory anti-PnPS IgM responses and diminished IgM B-cell percentages in relation to infectious pulmonary complications [8, 11]. In our cohort, 32% (6 out of 19) of CVID patients had diminished IgM memory cells and low anti-PnPS IgM responses, whereas only one patient (>16 years) had developed bronchiectasis. However previously published studies on the relation between impaired anti-PnPS IgM responses and pulmonary complications were performed in adult CVID patients, as opposed to a median age in our antibody deficient patient group of 8 years. We thus suggest careful follow up of the paediatric patient group with low anti-PnPS IgM responses to confirm whether they are at increased risk to develop a form of pulmonary disease during adult life.

Transiency of humoral immunodeficiency is a recognized phenomenon, for example in transient hypogammaglobulinemia of infancy (THI). THI is diagnosed retrospectively in infants with recurrent infections and immunoglobulin levels <-2SD, which recover to normal values before 3 years of age [19-21]. Several studies described predictors for this disease entity. More specifically, IgM memory B-cells, immunoglobulin levels and sex were found to serve as predictors for persistent disease versus THI [22, 6, 21]. These parameters lacked predictive value in our cohort, however our current study differs from

the studies describing THI in the fact that patients of 3 to 17 years were included and that patients were selected on basis of abnormal vaccination responses (as opposed to hypogammaglobulinemia in the THI studies). Our study does confirm however that maturation of the humoral immune response may occur up to the age of 16 years.

A prospective study investigating the predictive value of anti-PnPS IgA, IgM and IgG responses, B- and T lymphocyte dynamics and disease course would provide further insight into the developmental maturation pattern of anti-PnPS IgA responses [23-27]. Future studies may also include the quantification of mucosal IgA and IgM class antibody production in antibody deficient patients, as IgA and IgM are most abundantly present in the upper respiratory and bronchial airways, where they exert important contributions for the defence against encapsulated bacteria. The relation between serum and salivary anti-PnPS IgA and IgG responses has been explored in healthy children [13], where salivary anti-PnPS IgA response titers were found to be higher than serum anti-PnPS IgA titers for certain PnPS serotypes. We hypothesize that the distinction between transient and persistent disease courses will be stronger on a mucosal level and that anti-PnPS IgA responses of transient patients are more similar to those measured in controls. Finally, some CVID patients still develop infectious pulmonary complications despite immunoglobulin (Ig) substitution [28-30]. Through measurement of local mucosal anti-PnPS IgA and IgM responses in these patients, the amount of dysfunction of the local mucosa can be assessed and related to disease severity and subsequent risks on infectious pulmonary complications. Moreover this would give a different perspective on the effect of antibiotic and Ig substitution therapies on the local mucosa and local defence.

In conclusion, we here show that diagnosis of conserved serum IgA production against pneumococcal polysaccharide vaccine predicts transient antibody deficiency in children, while absent PnPS IgA responses confirm a chronic disease course. Assessment of anti-PnPS IgA responses, using specific cutoff values, can thus be of use in immunodeficiency diagnostics. We further show that resolution of delayed maturation of the humoral immune response may occur up to the age of 16 years. Future studies are needed to clarify the underlying mechanisms of this delayed maturation of specific antibody production.

SUPPLEMENTARY TABLES

Table 1A

Anti-PnPS IgM, IgG, and IgA response titers of antibody deficiency patients and controls.

Anti-PnPS IgM response titers

Serotype	Patients (median, range)	Controls (median, range)	p-value
PnPS 1	0.66 (0-10.64)	5.06 (1.56-5.83)	0.000
PnPS 3	0.71 (0-5.45)	2.09 (1.65-2.09)	0.000
PnPS 4	0.43 (0-4.70)	3.65 (0.53-4.70)	0.000
PnPS 5	1.77 (0-15.69)	10.13 (2.39-14.24))	0.000
PnPS 6B	0.59 (0-4.37)	1.54 (0.37-3.32)	0.002
PnPS 7F	1.29 (0-9.29)	5.52 (0-6.34)	0.005
PnPS 9V	0.67 (0-6)	5.37 (1.01-5.37)	0.000
PnPS 14	1.08 (0-4.51)	3.07 (1.18-4.05)	0.000
PnPS 18C	0.43 (0-5)	2 (0.83-4.33)	0.000
PnPS 19F	2.43 (0-19.84)	10.14 (2-10.77)	0.001
PnPS 23F	0.46 (0-6.31)	2.23 (0.30-2.36)	0.001

Anti-PnPS IgG response titers

Serotype	Patients (median)	Controls (median)	p-value
PnPS 1	0.65 (0.02-21)	9.58 (1.80-21)	0.000
PnPS 3	1.24 (0.03-19.64)	8.00 (0.53-8)	0.001
PnPS 4	0.68 (0-14)	6.08 (0.66-13.01)	0.000
PnPS 5	0.43 (0-19.33)	5.79 (1.09-19.33)	0.000
PnPS 6B	0.35 (0-55.55)	3.12 (0.03-18.75)	0.001
PnPS 7F	0.83 (0.01-17.33)	8.66 (1.23-17.33)	0.000
PnPS 9V	0.37 (0-13.57)	5.30 (0.44-10.90)	0.000
PnPS 14	1.15 (0-12.74)	10.30 (0.09-10.30)	0.014
PnPS 18C	0.23 (0-15)	4.20 (0.84-14.16)	0.000
PnPS 19F	2.1 (0.01-45)	7.04 (0.85-43.33)	0.001
PnPS 23F	0.26 (0-12.98)	3.94 (0.03-27)	0.000

Anti-PnPS IgA response titers

Serotype	Patients (median)	Controls (median)	p-value
PnPS 1	0.06 (0-2.97)	1.85 (0.29-4.67)	0.00
PnPS 3	0.34 (0-5.02)	1.78 (0.31-14.33)	0.00
PnPS 4	0.11 (0-4)	2.36 (0.25-3.75)	0.00
PnPS 5	0.07 (0-1.41)	1.17 (0.25-3.75)	0.00
PnPS 6B	0.09 (0-1.61)	0.5 (0.03-4.97)	0.00
PnPS 7F	0.09 (0-2.49)	1.05 (0.20-3.46)	0.00
PnPS 9V	0.08 (0-1.49)	1.22 (0.13-4.22)	0.00
PnPS 14	0.23 (0-4.45)	2.60 (0.21-6.12)	0.00
PnPS 18C	0.05 (0-1.34)	0.46 (0.15-1.34)	0.00
PnPS 19F	0.45 (0-7)	2.34 (1.12-5.55)	0.00
PnPS 23F	0.04 (0-0.8)	0.67 (0.02-4.31)	0.00

PnPS: Pneumococcal polysaccharide. Depicted are medians with minimum to maximum values. Statistical differences were calculated with Mann Whitney U tests.

Table 1B

Anti-PnPS IgM, IgG and IgA response titers of transient antibody deficiency patients and antibody deficiency patients

Anti-PnPS IgM response titers

Serotype	Antibody deficiency patients (median)	TAD (median)	p-value
PnPS 1	0.53 (0-10.64)	0.92 (0-5.28)	ns
PnPS 3	0.74 (0-3.08)	0.59 (0-5.45)	ns
PnPS 4	0.39 (0-2.52)	0.65 (0.19-4.51)	ns
PnPS 5	1.45 (0-15.49)	3.09 (0.24-15.45)	ns
PnPS 6B	0.55 (0-4.37)	0.98 (0.04-2.78)	ns
PnPS 7F	1 (0-7.26)	1.52 (0-9.29)	ns
PnPS 9V	0.43 (0-6)	1.13 (0.07-5.93)	0.027
PnPS 14	0.82 (0-4.51)	1.32 (0-3.21)	ns
PnPS 18C	0.42 (0.01-3.73)	0.68 (0-5)	ns
PnPS 19F	2.05 (0-10.77)	4.23 (0.44-19.40)	ns
PnPS 23F	0.27 (0-6.31)	0.74 (0.04-2.32)	0.034

Anti-PnPS IgG response titers

Serotype	Antibody deficiency patients (median)	TAD (median)	p-value
PnPS 1	0.37 (0.02-5.21)	1.51 (0.42-20.58)	0.004
PnPS 3	1.08 (0.03-15.46)	2.35 (0.4-19.24)	ns
PnPS 4	0.39 (0-13.67)	1.24 (0.09-13.91)	0.026
PnPS 5	0.24 (0-5.07)	0.96 (0.21-19.12)	0.006
PnPS 6B	0.22 (0-4.5)	0.41 (0.02-55.54)	ns
PnPS 7F	0.55 (0.01-2.8)	1.8 (0.37-16.96)	0.000
PnPS 9V	0.29 (0-2.93)	0.66 (0.03-13.54)	0.015
PnPS 14	0.71 (0-10.30)	4.75 (0-12.74)	0.016
PnPS 18C	0.22 (0-3.41)	0.73 (0.1-14.90)	0.006
PnPS 19F	0.8 (0.01-18.08)	4.8 (0.09-44.91)	0.004
PnPS 23F	0.14 (0-8.64)	0.46 (0.04-10.11)	ns

Anti-PnPS IgA response titers

Serotype	Antibody deficiency patients (median)	TAD (median)	p-value
PnPS 1	0.03 (0-0.97)	0.27 (0.02-2.95)	0.000
PnPS 3	0.22 (0-3.01)	0.82 (0.09-4.93)	0.001
PnPS 4	0.04 (0-1.19)	0.31 (0.01-3.99)	0.002
PnPS 5	0.02 (0.0-0.83)	0.19 (0.01-1.4)	0.000
PnPS 6B	0.06 (0-1.09)	0.21 (0.01-1.6)	0.028
PnPS 7F	0.03 (0-1.89)	0.44 (0.07-2.42)	0.000
PnPS 9V	0.03 (0-0.9)	0.18 (0.01-1.49)	0.003
PnPS 14	0.07 (0-1.31)	0.47 (0-4.45)	0.004
PnPS 18C	0.03 (0-1.34)	0.15 (0.04-0.73)	0.000
PnPS 19F	0.17 (0-1.72)	0.83 (0.25-6.75)	0.003
PnPS 23F	0.03 (0-0.8)	0.07 (0.01-0.53	0.039

PnPS: Pneumococcal polysaccharide, TAD: transient antibody deficiency. Depicted are medians with minimum to maximum values. Statistical differences were calculated with Mann Whitney U tests.

Table 2

Immunoglobulin levels and memory B-cell percentages of antibody deficiency and TAD patients

	Ab deficiency patients	TAD
Immunoglobulin levels at initial diagnosis (percentage of patients)		
IgM low	3/33 (10%)	3/17 (17%)
IgA low	15/33 (45%)	6/17 (35%)
IgG low	15/33 (45%)	5/17 (30%)
Memory B-cells at initial diagnosis (percentage of patients)		
memIgM low	7/17 (41%)	3/11 (27%)
memIgA low	5/17 (29%)	2/11 (18%)
memIgG low	7/17 (41%)	3/11 (27%)

Immunoglobulin levels and memory B-cell percentages were not statistically different between groups (Mann Whithey U- test). TAD: transient antibody deficiency.

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CHAPTER 8

Increased prevalence of gastrointestinal viruses and diminished secretory immunoglobulin A levels in antibody deficiencies

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Published in: Journal of Clinical Immunology 2014
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ABSTRACT

Gastrointestinal disease occurs frequently in antibody deficiencies. This study aims to explore the relation between gastrointestinal infections and mucosal homeostasis in patients with antibody deficiencies. We performed an observational study including 54 pediatric antibody deficient patients (48% CVID, 41% CVID-like, 11% XLA) and 66 healthy controls. Clinical symptom scores and stool samples were collected prospectively. Stool samples were evaluated for bacteria, parasites, viruses, secretory IgA- and for calprotectin levels. Results were compared between patients and controls. Twenty-four percent of antibody deficient patients versus 9% of healthy controls tested positive for gastrointestinal viruses (p=0.028). Fecal calprotectin levels were significantly higher in virus positive patients compared to virus negative patients (p=0.002). However, in controls, fecal calprotectin levels were similar between virus positive and virus negative controls. Moreover, gastrointestinal virus positive patients had low serum IgA levels in 13/14 cases (94%) versus 40/62 (62%) patients in the virus negative patient group (p=0.04). The virus positive patient group also displayed significantly lower secretory IgA levels in stool (median 13 ug/ml) than patients without gastrointestinal viruses detected or healthy controls (median 155 ug/ml) (p=0.046).

We here report an increased prevalence of gastrointestinal viruses and gastrointestinal complaints in antibody deficient patients. Patients that tested positive for gastrointestinal viruses showed diminished serum- and secretory IgA levels, and only in patients, virus positivity was associated with signs of mucosal inflammation. These findings suggest that particularly patients with low IgA are at risk for longstanding replication of gastrointestinal viruses, which may eventually result in CVID-related enteropathy.

INTRODUCTION

Common variable immunodeficiency (CVID) is the most prevalent symptomatic primary immunodeficiency (PID) affecting 1:25.000-50:000 Caucasians, and is characterized by decreased immunoglobulin (Ig) levels and recurrent infections[1-3]. Gastrointestinal disease affects an estimated 20-60% of CVID patients and can be divided into four major forms, consisting of infectious-, autoimmune-, malignancy- and inflammatory disease[4-6].

Inflammatory enteropathy occurs in up to 9% of CVID patients and has, with a relative risk of 4.0, the highest mortality rate of non-malignant complications in CVID [6,7]. CVID-related enteropathy mimics other inflammatory gastrointestinal diseases such as Crohn's disease, ulcerative colitis or celiac disease [8,9]. The main histological differences with classical inflammatory bowel disease (IBD) are the absence of plasma cells, and the frequently observed follicular lymphoid hyperplasia in mucosal biopsies of CVID patients [8,10,11]

The pathogenesis of CVID-related enteropathy remains largely unknown. Polymorphisms in *NOD2*, a disease-modifying gene associated with Crohn's disease, were more prevalent in CVID patients with enteropathy[12] and anti-enterocyte antibodies have been detected [13,14] Other types of immune dysregulation in CVID include autoimmune cytopenias and granulomatous disease that may also affect lungs, spleen and kidneys[7,15-18]. The etiology of these various disorders is poorly understood. The ameliorating effect of immunosuppressive drugs in CVID-related enteropathy suggests a component of excessive immune activation upon unknown or recognized triggers, at least in the perpetuation of the enteropathy [5].

Secretory IgA is abundantly present at mucosal sites and is important for neutralization and clearance of pathogens. The bound IgA – pathogen complex causes steric hindrance and thus blocks the uptake of antigens through intestinal receptors. Secretory IgA is also important for intestinal immune homeostasis and helps to shape the intestinal microbial flora[27,28] The mechanisms by which secretory IgA influences the microbial flora and controls inflammation of the intestine are only recently starting to be revealed[27,28,30]. Selective IgA deficiency is common and usually asymptomatic; however, progression to CVID has been reported [32,33].

We recently reported on a CVID patient with severe protein-losing enteropathy due to CVID-related inflammation of the gut[19]. This patient shedded high titers of parechovirus and norovirus in his stool for more than two years prior to developing auto immune enteropathy. The clearance of several viruses in the gut relies on specific antibody production and we thus hypothesized that the continuous replication of gastrointestinal viruses, together with an incessant suboptimal immune response due to CVID, eventually

disturbs mucosal immune homeostasis and initiates enteropathy. For this reason, we performed a prospective longitudinal study assessing the association between mucosal IgA production, viral persistence and mucosal inflammation in pediatric antibody deficiency patients and compared patients with healthy controls.

METHODS

Patients and controls

Patients and controls (aged 4-18 years) were prospectively included between April 2010 and December 2012. CVID was defined by using criteria of the European Society of Immunodeficiencies (ESID)[2]. Briefly, this includes a marked decrease of IgG and at least one of the isotypes IgM or IgA (at least 2 standard deviations below the mean for age), in combination with an age of onset > 2 years, poor response to vaccines and exclusion of other defined causes of hypogammaglobulinemia. Patients with defective antibody production and decreased IgG or IgA (but lacking the criterium of a second decreased Ig class necessary for a complete CVID diagnosis) were referred to as 'CVID-like disease'[2]. The diagnosis of X-linked agammaglobulinemia (XLA) was based on the finding of (near) absence of B cells in combination with a pathogenic mutation in the *BTK* gene. All patients were on Ig replacement therapy with intravenous or subcutaneous immunoglobulins. Healthy age matched children were recruited as a control group and were non-relatives of the participating antibody deficiency patients. Geographic distribution of controls was similar to that of patients, as well as seasonal distribution of collection time points. The healthy controls were recruited from the community and did not visit the outpatient clinic for medical reasons. In most cases, only one child per family participated. In the patient group 5 families with 2 siblings in each family participated; these siblings were all diagnosed with CVID. In the healthy control group 4 healthy sibling pairs were included. If healthy or antibody deficient siblings were included, stool samples were collected at different time points to minimize bias due to contamination. Stool samples were immediately stored at -80 C. Exclusion criteria in controls were the presence or strong suspicion of immune-mediated diseases (immunodeficiencies, autoimmune disease) or inflammatory bowel diseases (Crohn's disease, ulcerative colitis or celiac disease). The study was approved by the Local Institutional Review Board of the University Medical Center Utrecht and written informed consent was obtained from both parents/legal representatives for all participants.

Study design

The study was conducted as a prospective observational study in patients and controls. Stool samples were collected at T=0 and T=6 months.These samples were used for detection of viruses, bacteria, parasites and levels of calprotectin, a cytosolic protein of neutrophils that can be used as a surrogate marker for gastrointestinal inflammatory

conditions. If a virus was detected in the stool at T=0 and/or T=6 months, additional samples were collected 3 months later to identify longstanding viral replication (defined as PCR positivity for the same virus ≥3 months). To evaluate gastrointestinal complaints, all participants filled out questionnaires during the week preceding sample collection addressing defecation frequency, stool consistency and gastrointestinal complaints including gastrointestinal pain or cramps.

Enteric virus, bacteria and parasite detection

RNA was extracted from 200 mg stool specimens using 1 mL stool transport and recovery buffer (S.T.A.R.; Roche Diagnostics Corporation, Indianapolis, IN) and 100 µL chloroform. Mixtures were centrifuged and supernatants were diluted with phosphate buffered saline. To monitor for effective extraction and amplification, all samples were spiked with internal control viruses (phocine herpes virus for DNA and murine encephalomyocarditis for RNA). Nucleic acids were extracted with the MagNa Pure LC nucleic isolation system using the total Nucleic Acid Isolation protocol. RNA was reverse transcribed into coding DNA with Multiscribe RT and random hexamer primers (Applied Biosystems). Samples were analyzed by real-time polymerase chain reaction (RT-PCR) in duplicate using PCR Mastermix (Applied Biosystems), and primers and probes specific for the viruses; adenovirus, enterovirus, parechovirus, astrovirus and norovirus (available upon request). Cycling in a Taqman 7500 or 7900 involved the following steps; 2'50°C, 10' 95°C, 45 cycles 15''95°C and 1'60°C; annealing temperature for astrovirus was 53°C. Stool samples were tested for antigens of rotavirus by ELISA using the Oxoid ProSpecT rotavirus microplate assay.

With a similar real time PCR protocol fecal samples were tested for the presence of common parasites *Blastocystis, Cryptosporidium spp., Dientamoeba spp, Entamoeba histolytica/dispar, Giardia spp* and presence of bacteria *Salmonella spp, Shigella spp, enteropathogenic Campylobacter spp, Yersinia enterocolitica* and *Pleisomonas shigelloides*.

Calprotectin measurements

Calprotectin levels were using a calprotectin ELISA kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's instructions. After an extraction procedure, an antibody to calprotectin heterodimeric and polymeric complexes was coated onto the microplate and extracts were incubated at room temperature for 30 minutes. After washing, a secondary horseradish peroxidase (HRP) conjugated antibody was added and incubated. After another washing step, tetramethylbenzidine was added followed by a stopping reaction. Absorption was measured at 450nm. Calprotectin levels were divided into 'normal' and 'increased' according to age-related reference values; below 50 µg/g was considered normal for children aged 9 years or older, and less than 166 µg/g normal for children aged 2-9 years[20].

Immunological phenotype and IgA secretion in stool

Immunological phenotyping of patients included in this study was performed as part of the routine screening of our institute. Serum immunoglobulin (IgG, IgM, IgA) levels, B-cell and T-cell phenotype were evaluated. For determination of IgA in faecal samples preparation with the fecal sample preparation kit (Roche) was performed. Samples were thawed, weighted and diluted in 5 ml PBS. The obtained fecal suspension was centrifuged for 5 minutes at 16100 x g. The supernatant was collected and diluted 1:625, 1: 1250, 1:6250, and 1:12500. Dilutions were calculated based on the exact fecal weight. To determine the concentration of sIgA in the fecal samples 96-well microtiter plates were coated with 10 µg/ml (1 µg/well) goat anti-human IgA in PBS and incubated overnight at 4°C. All further incubations were at room temperature. Plates were then washed and blocked with PBS-T with 1% BSA for 30 minutes. After blocking the diluted fecal samples were added and the plates were incubated for 1 hour. After washing, biotin-conjugated goat anti-human pIgR was added in a 50 ng/ml concentration. Plates were incubated for 1 hour, washed and incubated with horseradish peroxidase (HRP)-conjugated avidin (1:500) for 1 hour. After washing, plates were developed using tetramethylbenzidine peroxide solution (TMB), which was incubated for 12 min in the dark. The reaction was stopped using 2M H_2SO_4 and OD values were measured at 450 nm by an ELISA reader (Thermo Multiskan Ex).

Statistics

All statistical analyses were performed with SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL, U.S.A)To compare continuous data between two groups, unpaired t-tests were used for parametric data and Mann-Whitney *U* tests for non-parametric data, respectively. Ordinal categorical data were compared using linear-by-linear association; other categorical data were tested with Pearson's chi-square tests or Fischer exact tests if sample sizes were small. Tests were performed two-tailed, and p-values ≤ 0.05 were considered significant.

RESULTS

Gastrointestinal viruses are more prevalent in antibody deficient patients than in healthy controls

Fifty-four pediatric patients and 66 pediatric controls were included in the study. Patients were diagnosed with CVID (48%), CVID-like disease (41%) or XLA (11%) (Table 1) and all patients presented with recurrent respiratory tract infections. Almost half of the patients had experienced gastrointestinal infections with in some cases severe dehydration, however gastrointestinal infections were not recurrent. So far, one patient developed severe auto-immune manifestations.

Fourteen out of 54 patients versus 6 out of 66 controls tested positive for enteric viruses (24 vs 9%, respectively, p=0.028; Fig 1a). In the CVID+CVID-like group, 12 out of 48 (25%) patients tested positive for enteric viruses. Viral loads in feces were similar between patients and control group. Adenovirus was the most prevalent virus found in both patient- and control group. Virus positive patients suffered more often from gastrointestinal complaints (ache, thin stool, and increased frequency of stool) than virus positive healthy controls (Table 1, Fig 1a). Of note, antibody deficient patients with no viruses detected also reported more gastrointestinal complaints than healthy children without detectable viruses. Symptom scores in patients, with separate analysis for CVID+CVID-like patients, and symptom scores of healthy controls are presented in Figure 1a and Table 1.

We found no pathogenic bacteria in patients'samples, and only one healthy control was infected with Salmonella. Parasitic infections were relatively common in healthy controls and patients, both groups most frequently carried *Disentamoeba spp.* (60% of healthy controls and 35% of patients).*Blastocystis* was mostly found in healthy controls but less in the patient group and *Giardia spp* was present in 5% of patients and in 8% of the controls. *Cryptosporidium spp* and *Entamoeba histolytica/dispar* were not detected in the stool of patients nor healthy controls.

Altogether antibody deficient patients showed a higher prevalence of intestinal viruses than healthy controls, and these viruses did not necessarily result in more gastrointestinal complaints.

Virus positive patients display significantly more signs of inflammation than patients and controls without detectable viruses

To allow for distinction between organic bowel disease and non-organic disease such as irritable bowel syndrome (IBS), levels of calprotectin were measured in stool[20-22]. There was a significant association between calprotectin levels and watery diarrhea in patients (p=0.046) and a trend towards an association with loose stools (p=0.07). Calprotectin levels of controls with and without detectable viruses were comparable with a median of 30 ug/g (Fig 1b). However, virus positive antibody deficient patients displayed significantly higher calprotectin levels with a median of 142 ug/g (30-385)) in comparison to patients without detectable viruses (median 30 ug/g) (p=0.002)(Fig 1a) a similar level as in healthy controls. Of the virus positive individuals, 7 / 14 (50%) of all antibody deficiency patients and 6/12 (50%) CVID+CVID-like patients versus 1 / 6 (16%) of healthy controls had calprotectin levels above the upper limit of the normal value range

Table 1
Baseline characteristics

	Patients (all)	CVID + CVID-like	Healthy donors	P-value
Number	54	48	66	
Male, n (%)	45 (83 %)	39 (81%)	29 (44 %)	<0.001
Age, years ± SD	11.1 ± 3.8	11.2 ± 4.9	9.4 ± 3.8	ns
Diagnosis, n (%)				
CVID	26 (48 %)	26 (54%)		
CVID-like disease	22 (41 %)	22 (46%)		
XLA	6 (11 %)			
Defecation frequency times/wk, **median [range]**	8 [0-74]		8 [0-18]	0.022
Decreased (0-4)	10 (19 %)	10 (21%)	7 (11 %)	
Normal (5-15)	33 (61 %)	30 (62%)	53 (83 %)	
Increased (>15)	11 (20 %)	8 (17%)	4 (6 %)	
Unknown	na	na	2 (3%)	
Abdominal ache or cramps, n(%)				
Never	26 (49 %)	23 (48%)	40 (60.6 %)	0.003
Once a week	6 (11 %)	5 (11%)	12 (18.2%)	
Twice a week	5 (9 %)	5 (10%)	4 (6.1%)	
Three or more	16 (30 %)	15 (31%)	3 (4.5%)	
Unknown	na	na	7 (10.6%)	
Thin stools, n (%)				
Never	23 (43%)	23 (48%)	46 (69.7%)	0.001
Once a week	7 (13%)	7 (15%)	3 (4.5%)	
Twice a week	4 (8%)	4 (8%)	5 (7.6%)	
Three or more	19 (36%)	14 (29%)	6 (9.1%)	
Unknown	na	na	6 (9.1%)	
Watery diarrhea, n (%)				
Never	46 (87 %)	42 (88%)	61 (92.4 %)	0.035
Once a week	3 (6 %)	2 (4%)	0 (0 %)	
Twice a week	1 (2 %)	1 (2%)	0 (0 %)	
Three or more	3 (6 %)	3 (6%)	0 (0 %)	
Unknown	na	na	5 (7.6%)	

SD=standard deviation, CVID=common variable immunodeficiency, XLA=X-linked agammaglobulinemia, na=not applicable. P-values are calculated between patient (all) group and healthy donors, p-values remained significant for comparison between CVID+CVID-like patients and healthy donors.

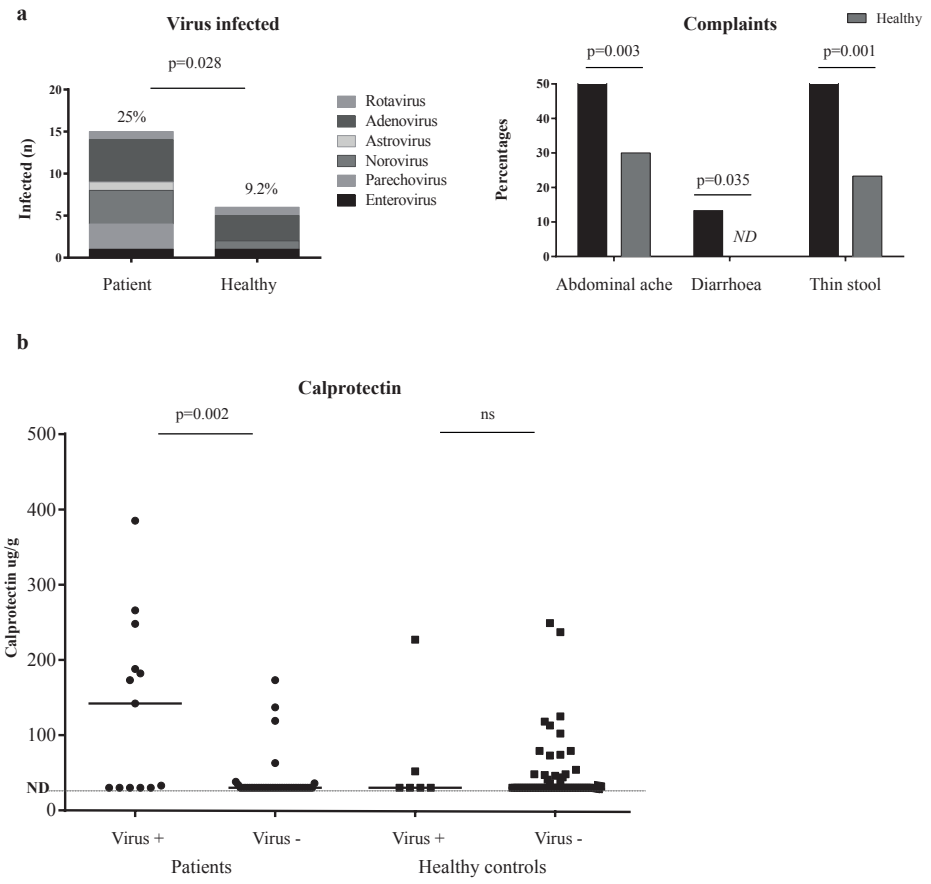

Figure 1

a Virus presence and gastrointestinal complaints in antibody deficient patients and healthy controls. **b** Mucosal inflammation in antibody deficient patients and healthy controls. The lower limit of detection for calprotectin was 30 ug/g and is depicted with the grey dashed line. ND: Not detectable. Lines indicate medians.

The relative severity of antibody deficiency relates to the prevalence of gastrointestinal viruses in immunodeficiency patients

To test whether residual immunoglobulin production in antibody deficient patients relates to the prevalence of gastrointestinal viruses, we evaluated peripheral blood B-cell subsets, serum- and secretory immunoglobulin levels. Diminished serum IgA levels in antibody deficient patients were associated with an increased prevalence of the tested gastrointestinal viruses (p=0.04). In the combined analysis of all antibody deficient patients (i.e. XLA, CVID and CVID like patients), 13/ 14 gastrointestinal virus

positive patients (94%) had serum IgA levels of -2 SD (Fig 2a). When excluding XLA patients, 11/12 patients had low serum IgA levels (91%) Correspondingly, low memory IgA B-cell percentages were also associated with virus positivity in patients (both with and without inclusion of XLA patients) (Fig 2a). Patients with low serum IgA levels did not have significantly increased calprotectin levels or more gastrointestinal complaints when compared to all patients and CVID+CVID-like patients with normal IgA serum levels.. The association between serum IgG levels and virus presence was not tested since almost all patients were on immunoglobulin substitution therapy.

Since IgA is abundantly present at mucosal sites [23,24], we next measured secretory IgA (sIgA) as excreted in faeces of patients and healthy controls. Only virus positive patients had significantly lower sIgA (median of 13 ug/ml) in comparison to patients without viruses detected and healthy controls (median 155 ug/ml) ($p=0.046$) (Fig 2b). This was also observed in the combined CVID+CVID-like group where virus positive subjects had sIgA levels of 30 ug/ml (median) versus 165 ug/ml (median) in the CVID+CVID-like patients without viruses detected ($p=0.034$). The sIgA levels correlated to serum IgA levels ($r=.337$ $p=0.019$) (Fig 2b). In the low serum IgA patients, we observed a trend ($p=0.06$) of lower sIgA levels when compared to the normal serum IgA patients, however this trend was not observed in the CVID+CVID-like group. Thus, virus positive antibody deficient patients have significantly diminished serum and secretory IgA levels.

Prolonged viral shedding occurs in patients and healthy controls

We hypothesized that prolonged viral shedding, due to an inability of effective viral clearance, would eventually lead to enteric abnormalities, and therefore prolonged viral replication in antibody deficient patients was determined and compared to healthy controls.

Three out of 14 virus positive patients at T=0 had prolonged viral replication as defined by continued PCR positivity at T=3 months (Table 2). One patient positive for norovirus at T=0 withdrew consent. Two virus positive XLA patients and one healthy control at T=0 had encountered a different virus at T=3 months, which was cleared at T=6 (Table 2). In addition, one XLA patient that was re-sampled due to the presence of an adenovirus at T=6, excreted enterovirus for at least 3 months (Table 2). Prolonged viral shedding caused gastrointestinal symptoms in the majority of cases, but was not necessarily accompanied by mucosal inflammation. All patients with prolonged viral shedding had low serum IgA levels and secreted almost no IgA in faeces.

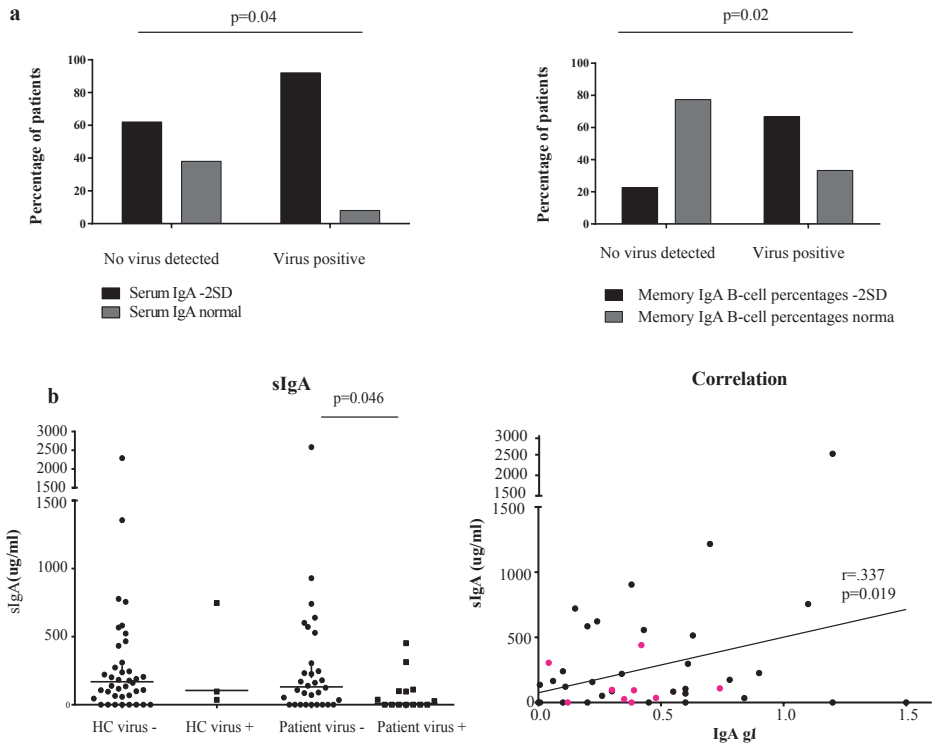

Figure 2

a Memory IgA B-cell percentages and serum IgA levels in antibody deficient patients with and without viruses detected. Memory IgA B-cell percentages were calculated as the fraction of the total B-cells of patients.

b MSecretory IgA levels in antibody deficient patients and healthy controls. Coloured dots depict virus positive patients. SD: Standard deviation. sIgA: secretory IgA. Lines indicate medians.

DISCUSSION

This is the first observational study on the presence of gastrointestinal viruses in children with antibody deficiencies and healthy controls. Results show that antibody deficient patients have an increased prevalence of gastro intestinal viruses, and that these patients more frequently have gastrointestinal symptoms than healthy controls. Strikingly, these patients did not display a significantly increased infection rate with common bacterial or parasitic gastrointestinal pathogens. Only in antibody deficient patients, the presence of these gastrointestinal viral pathogens displayed a significant association with signs of inflammation. We thus found a significant association between

Table 2
Antibody patients with prolonged viral shedding

Subject	1	2	3	4
Diagnosis	CVID; gradually evolved in late onset CID	CVID-like (SAD+low IgA)	XLA	Healthy control
Gender	Female	Male	Male	Male
Age of patient (years)	13	7.5	4.5	11.5
Virus present 3 months	Adenovirus (and intermittent norovirus)	Norovirus	Enterovirus	Rotavirus
Abdominal ache or cramps?	Yes, often cramps	Currently not, and usually never	Currently not, and usually never	Currently not, and usually never
Thin or watery stools?	Yes, most days of the week thin stools and a few days also watery diarrhea	Currently not, and usually never	Almost always has thin stools, but never diarrhea	Currently not, and usually never
Defecation frequency	Around 2 times a day	Always once a day	Variable, 1 to 4 times a day	Usually 3 times a day
Calprotectin levels (µg/mL)	142 at first and 30 at second measurement, at other timepoints often increased	33; 36	63; 30; 318 with adenovirus infection	30; 30
sIgA (ug/ml)	0	26	0	
Serum IgA (g/l)	0.1	0.35	0.01	

CVID=common variable immunodeficiency, CID=combined immunodeficiency, SAD=specific antibody deficiency, XLA=X-linked agammaglobulinemia, sIgA= secretory IgA.

mucosal inflammation and the presence of enteric viruses in the patients, but not in healthy controls. These findings suggest that gastrointestinal viruses usually do not cause (sustained) intestinal damage in healthy children, but may specifically affect the intestinal epithelium in antibody deficiency syndromes.

Further evaluation of the antibody deficient patients suggested that particularly patients with low IgA were prone to acquire gastrointestinal virus infections, since we found

that both serum IgA and excreted sIgA were significantly diminished in virus positive antibody deficient patients. Furthermore, patients with signs of mucosal inflammation showed a trend towards lower sIgA values than the group without signs of mucosal inflammation.

The local IgA production is of importance for bacterial and viral clearance in the gut [26,27], and since IgG and typically also IgA production is impaired in antibody deficient patients, this might contribute to ineffective viral clearance and facilitate continuous viral replication.

For this matter, serum and secretory IgA levels might both serve as valuable indicators in antibody deficient patients prone to develop viral infections and subsequent inflammation and may thus become of help in stratifying the heterogenous CVID group in those most at risk for CVID related enteropathy.

The strengths of this study include its prospective design, the high return rate of questionnaires (100% of patients and 94% of healthy controls) and stool samples, and the use of realtime (RT)-PCR which allows for ultra sensitive for detection of viral, parasitic and bacterial pathogens in stool. Furthermore, this study includes the use of a reliably method for measuring IgA in stool samples.

As for limitations of the study, extended follow-up of this cohort is required to firmly establish an association between diminished antibody excretion, prolonged viral replication, mucosal inflammation and, eventually, the occurrence of CVID-related enteropathy. To this end, the specific virus strains should be determined to allow for distinction between chronic viral infections and re-infection. Alternatively, evaluation for viral PCR positivity could be performed as case control study between CVID patients with and without enteropathy. Secondly, although calprotectin is a highly sensitive marker for intestinal bowel inflammation and can be used as a surrogate marker for inflammation, it is not very specific and cannot distinguish between infectious or noninfectious causes of mucosal inflammation. Other (additional) parameters to evaluate inflammatory disease, such as alpha 1 antitrypsin could also be considered. Thirdly, we did not measure serum IgA levels of healthy controls and thus asymptomatic IgA deficiency (prevalent in 1 of 600 people) might have been present in healthy controls as well. Although we consider this possibility unlikely to significantly affect our findings, it would be interesting to compare our findings in hypogammaglobulinemia with IgA deficiency in otherwise immunocompetent individuals in future studies. Both serum and excreted sIgA were significantly diminished in virus positive antibody deficient patients, however a causal relation remains to be determined. It is possible that IgA deficient patients are more prone to viral infections; an alternative explanation could be that IgA is low as a consequence of these infections. Finally, microbiome assessment of the

antibody deficient patients and healthy controls would enhance the value of this study. As has been shown before, the development of the microbial flora is closely related to intestinal IgA excretion [26,28-30]. Thus, the microbiome of antibody deficient patients with low intestinal IgA levels might significantly differ from patients with normal intestinal IgA secretion, therewith predicting their susceptibility to viral and bacterial infections and subsequent derailment of the immune response into enteropathy.

In conclusion, we here report an increased prevalence of gastrointestinal viruses and gastrointestinal complaints in antibody deficient patients. The gastrointestinal virus positive patients showed diminished serum- and secretory IgA levels, and virus positivity in patients was associated with signs of mucosal inflammation. This study thus supports the hypothesis that hypogammaglobulinemia, particularly decreased IgA production, is associated with prolonged intestinal virus replication and that this may result in an increased risk for chronic mucosal inflammation such as CVID related enteropathy.

Acknowledgements
The authors would like to thank Jessica van der Aa and Géanne Krabben for assistance with participant inclusions and Karin van Veghel and Lysette Ebskamp of U-DAIR with determination of sIgA levels.

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CHAPTER 9

IgG trough levels and progression of pulmonary disease in pediatric and adult CVID patients

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Published in: Journal of Allergy and Clinical Immunology 2017

To the Editor,

Common variable immunodeficiency disorder (CVID) is the most prevalent symptomatic primary immunodeficiency in adults and children. Patients encounter recurrent bacterial and viral infections of the respiratory tract, with delayed pathogen clearance due to diminished immunoglobulin levels and insufficient specific antibody responses to encapsulated bacteria. As a result, the most prevalent comorbidities in adult CVID are recurrent pneumonia (32%) and chronic lung disease (28%), with lung failure being the most prevalent cause of death[1]. Pulmonary comorbidities include structural airway disease (AD) due to recurrent pulmonary infections and interstitial lung disease (ILD), in particular in patients with autoimmune phenomena[2]. Both disease entities are well detected by high-resolution computed tomography (CT) [2,3]. In this study, we investigated progression of pulmonary disease in pediatric and in adult CVID patients in a 3-5 year follow-up period using a uniform CT-scanning- and scoring protocol [5,6].
Pediatric and adult CVID patients were allocated to low- or high-risk profiles for AD and/or ILD disease progression. This was based on baseline AD- and ILD-scores, defined comorbidities and/or immunological parameters (Table 1 and Methods).

The AD- and ILD-score cut off values for the high-risk for pulmonary disease progression group were respectively 7 and 5, and were determined based on previously published cross sectional evaluations of pulmonary CT scans (see Methods)[3,5,6]. Patients underwent follow-up scanning after 2-3 years (adult and pediatric high-risk patients) or 5 years (low-risk patients). Pulmonary function tests (PFT) were performed at baseline for all patients and at follow up when clinically necessary (24/55; 43% of cases) as determined in our institutional protocol for follow up of CVID patients. PFT test results were mostly within normal ranges at baseline and at follow up (Table E1).

All patients were treated with Ig replacement therapy. Antibiotic prophylaxis was prescribed in patients with a history of recurrent pulmonary infections despite Ig therapy (Table E2). Two adult patients received immunosuppressive medication (azathioprine and prednisolone), for interstitial lung disease. One low-risk pediatric patient (5%) and 1 high-risk adult (4%) suffered from radiologically proven pneumonia during follow-up. Two out of thirteen high-risk pediatric patients (15%) developed autoimmune diseases (Table 1).

In order to quantify the progression of airway- and interstitial lung disease, AD- and ILD-scores were calculated in baseline and in follow-up CT scans. The ILD-scores did not increase significantly in high-risk nor in low risk patients (Fig 1A). In contrast, the AD-scores did increase in all groups after 2-5 years of follow-up (Fig 1B), especially in the pediatric and adult patients with low (<7) AD-scores at baseline (n=35) (p=0.001 Fig 1C). Importantly, in this group only one patient (1/35) suffered from a radiological proven

Table 1

Patient Characteristics at initial CT scan

	Low risk children	High risk children	High risk adults	p-value	Total
CVID patients (n)	19	13	23		55
Gender (male)	13/19 (68%)	9/13 (69%)	8/23 (35%)		30/55 (54%)
Median Age at initial CT in years (range)	13 (6-18)	11 (8-17)	38 (19-76)		
Disease duration in years at initial CT (median, range)	5 (0.5-11)	6.5 (0-10)	9.5 (5-16)?		
Baseline AD score >7	none	6/13 (46%)	10/23 (43%)		
Baseline ILD score >5	none	6/13 (46%)	11/23 (48%)		
Both ILD and AD high	none	2/13 (15%)	5/23 (21%)		
Respiratory tract infections					
URTI (during FU time)					
Sinusitis, rhinitis, otitis	8/19 (42%)	7/13 (54%)	16/23 (70%)	ns	31/55 (56%)
none	5/19 (26%)	2/13 (15%)	6/23 (26%)		13/55 (24%)
<=2/year	6/19 (31%)	4/13 (31%)	1/23 (4%)		11/55 (20%)
>4 times/year					
LRTI (before initial CT scan)	9/19 (47%)	10/13 (77%)	10/23 (44%)	ns	29/55 (53%)
Pneumonia					
Bronchiectasis	none	3/13 (23%)	11/23 (48%)	0.008	15/55 (27%)
Autoimmunity					
Hepatitis, ITP	none	3/13 (23%)	4/23 (17%)	ns	9/55 (16%)
Lymph proliferation	none	2/13 (15%)	4/23 (17%)	0.05	6/55 (11%)
Enteropathy	none	1/13 (8%)	2/23 (9%)	ns	3/55 (5%)
Progression into CID	none	3/13 (23%)	None	ns	3/55 (5%)
Cancers	none	none	3/23 (13%)	ns	3/55 (5%)

Baseline characteristics. AD: Airway disease; CID: Combined Immunodeficiency Disease; CVID: Common variable Immunodeficiency; CT: Computed Tomography; ILD: Interstitial Lung Disease; ITP: immune thrombocytopenic purpura; LRTI: Lower respiratory tract infections; SE: standard error of the mean; URTI: upper respiratory tract infections.

lower respiratory tract infection during follow-up time, all other patients were clinically stable. In support of the increased AD-scores, we found that the AD-score progression correlated to a decrease in predicted FEV1/FVC% , although still within normal range in the group with low AD-scores at baseline (r= -.6 p=0.048, see Figure E1). Thus, the

Table E1

Pulmonary function tests at baseline and follow up scans

	FEV1/FVC baseline (%predicted)	FEV1/FVC FU (%predicted)	FEV1 baseline (%predicted)	FEV1 FU (%predicted)	FVC baseline (%predicted)	FVC FU (%predicted)	RV/TLC baseline (%predicted)	RV/TLC FU (%predicted)
Low-risk children (n=7/19) PFT: normal	99 (90-117)	99 (90-105)	92 (75-130)	89 (75-96)	94 (77-124)	92 (78-110)		
High-risk children (n=8/13) Obstructive n=2	98 (73-114)	93 (74-104)	93 (69-102)	83 (57-111)	88 (76-100)	86 (77-111)		
High-risk adults (n=3/23)	96 (78-99)	97 (85-103)	102 (86-120)	107 (96-109)	113 (110-126)	116 (108-116)	71 (20-134)	83 (66-121)

Supplementary Table I Pulmonary function tests of CVID patients. The two patients with obstructive pulmonary disease had high AD-scores at baseline and AD-scores increased at follow-up. Depicted are FEV1 and FVC's relative (%) to predicted values and medians with range.
FEV1: Forced Expiratory Volume; FVC: Forced Vital Capacity; FU: follow-up; PFT: Pulmonary Function Test.

AD-score increase could not be predicted by upper- or lower respiratory tract infection episodes or by abnormal pulmonary function tests.

Since no radiological proven pneumonias occurred during follow-up time and immunological phenotypes were normal in most cases in this group, we explored whether treatment parameters influenced AD-score progression. Antibiotic prophylaxis did not contribute to differences in AD-scores. The IgG trough levels at baseline and follow-up in high- and low-risk patients are depicted in Fig 1D. Interestingly, the difference (delta) in AD-scores of the pediatric and adult patient group with low AD-scores at baseline were inversely correlated to follow-up IgG trough levels (r= -.48 p=0.009), (Fig 1E) ie AD-score progression correlated to lower IgG trough levels at follow-up CT scan. No correlation was detected between IgG trough levels and AD-scores at baseline. When dividing

Table E2

Treatment regimens

	Low risk children (number, percentage)	High risk children	High risk adults	Total
AB prophylaxis:				
Continuous use	4/19 (21%)	6/13 (46%)	1/23 (4%)	11/55 (20%)
Stopped during FU	9/19 (47%)	5/13 (39%)	7/23 (30%)	21/55 (38%)
None	6/19 (32%)	2/13 (15%)	15/23 (65%)	23/55 (42%)
Immunosuppressiva - Prednisolon - Azathioprine	none	none	2/23 (9%)	
IgG levels between initial and FU CT (mean SD)	11.4 (2.2)	11 (2.9)	8.7 (1.3)	

Treatment regimens of the patients. AB: antibiotic; CT: computed tomography; FU: follow-up; SD: standard deviation.

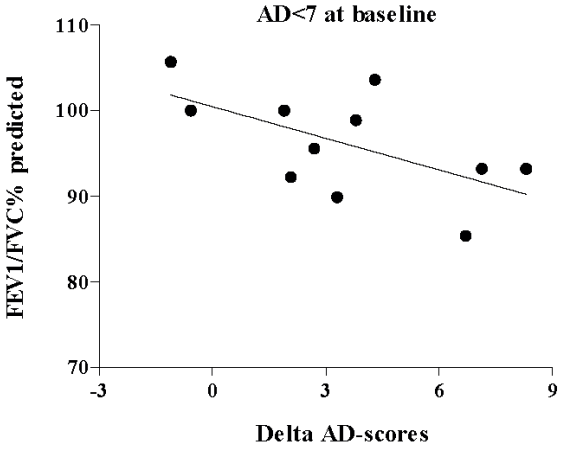

Figure E1

Pulmonary Function Tests and AD-score increase

FEV1/FVC% predicted of 11/35 CVID patients with low-risk for airway disease progression correlated to AD-score increase (delta AD-scores) r= -0.6 p=0.048

AD: Airway disease; FEV1= Forced Expiratory Volume; FVC=Forced Vital Capacity

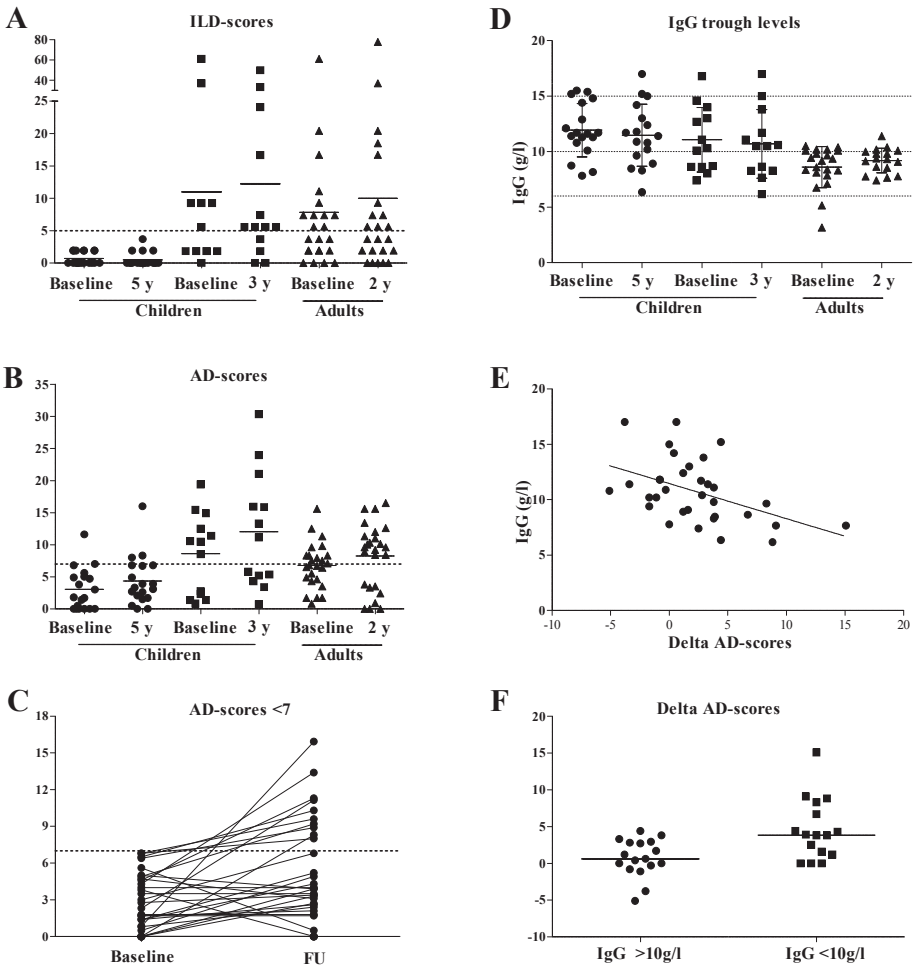

Figure 1

ILD- and AD-scores of pediatric and adult CVID patients at baseline and follow-up CT scan

A) ILD-scores B) AD-scores , low-risk children (5y follow-up) p=0.06 C) AD-score increase of pediatric and adult CVID patients with low AD-scores of <7 at initial (baseline) CT scan (p=0.001) D) IgG trough levels at baseline and follow-up E) Correlation between delta AD-scores (follow-up minus baseline AD-scores) and IgG trough levels of patients with low AD-scores at baseline r=-.48 p=0.009. F) Delta AD-scores categorized according to IgG trough levels (< or > 10 g/l) p=0.004. Depicted are medians (A,B,F) paired data from baseline and follow-up CT scans (C) and means ±SD (D)

AD: Airway Disease; CVID: common variable immunodeficiency; FU: Follow-up; ILD: Interstitial Lung disease; y: years; SD: Standard Deviation.

these same patients by IgG trough levels (<10 vs >10 g/L), we found that individuals with IgG trough levels >10g/l showed significantly less AD-score progression than pediatric patients with IgG trough levels <10g/l (p=0.004) (Fig 1F). This observation is in line with a meta-analysis by Orange *et al*. that showed that patients with higher IgG trough levels up to at least 10 g/l had decreased rates of clinical pneumonia [8] Furthermore, lower IgG trough levels were associated with progression of ILD in CVID patients in a recent study[7]. Our findings question the current opinion that optimal IgG trough levels should be adjusted to the individual need of the patient to be symptom free (with a minimum advised IgG trough level of 7 g/l and depending on the presence of bronchiectasis [9]), as our findings indicate that there may be an additional protective effect of higher IgG trough levels.

Taken together, we found that silent progression of pulmonary airway disease occurs on CT scans of pediatric and adult CVID patients, despite seemingly optimal treatment regimens and no apparent clinical pulmonary infections during follow-up. Especially in patients that were considered to be at low-risk for pulmonary airway disease progression, the AD-scores increased significantly.

Although the majority of patients did not develop clinically symptomatic lung disease in this 3-5 year follow up period (with mean AD-scores remaining <7 and stable pulmonary function tests), this finding is likely to become clinically relevant when taken into consideration that with a longer follow-up time, continuously increasing AD-scores will eventually result in clinical complications and significant decrease of PFT's. Furthermore, our study suggests a correlation between increasing AD-scores and lower IgG trough levels at follow up. As such, these findings stress the urgency for a randomized controlled study in pediatric and adult CVID patients in order to define optimal IgG trough levels for prevention of (silent) AD progression, and possibly ILD progression, as quantified by CT-scans and PFT's. Such a study would also provide further insight on the optimal follow-up period of CVID patients for monitoring of pulmonary disease.

METHODS

Study population

All patients were treated at a tertiary care center (University Medical Center of Utrecht, the Netherlands). As the study involved analysis of clinical data derived from routing clinical care, informed consent was waived by the Medical Ethical Research Committee of the UMC Utrecht.

A longitudinal observational cohort study was conducted in pediatric and adult CVID patients who underwent structured follow-up at the outpatient clinic. Common variable immunodeficiency was diagnosed according to ESID criteria. In short, CVID was diagnosed in case of 1) a clinical history of recurrent pulmonary and/or gastrointestinal infections, 2) serum IgG and IgA and/or IgM levels below -2SD for age 3) insufficient specific responses upon vaccinations and 4) exclusion of other defined causes of hypogammaglobulinemia. Structured follow-up included monitoring of CVID-related symptoms (see Table 1), IgG trough levels and measurement of B- and T-lymphocyte subsets. Furthermore, to monitor pulmonary disease, CT scannnig was performed at baseline (in 2008-2009) as described previously [3,5,6]. Follow-up CT's were scheduled after two years (in adults) and three years (in pediatric patients) with high-risk for pulmonary disease progression (2011-2013). In all other patients without increased risk for pulmonary disease progression, follow up CT scans were planned after five years (2014).

High-risk pulmonary disease patients

Patients were defined as high-risk for pulmonary disease at initial CT scan, in case of:

- Recurrent (>2) lower airway infections during any time of immunoglobulin replacement therapy
- Interstitial Lung Disease scores > 5 **and/or**
- Airway Disease scores >7 **and/or**
- Presence of CVID-related autoimmune or autoimmune complications, hepatosplenomegaly or malignancy **and/or**
- Class-switched memory B-cells <2% of total B cells

Low-risk pulmonary disease patients

Patients were defined as low-risk for pulmonary complications at the initial CT scan in case of

- No recurrent lower airway infections during immunoglobulin replacement therapy
- Interstitial Lung Disease scores <5
- Airway Disease scores <7
- No other CVID-related complications
- Class-switched memory B-cells >2% of total B cells

Follow-up CT scans were evaluated of 32 pediatric CVID patients: 13 high-risk and 19 low-risk for pulmonary disease progression and 23 high-risk adult CVID patients. These numbers were reached as follows. At baseline, 54 pediatric patients (34 low-risk and 20 high-risk) and 24 high-risk adult patients were evaluated for CT scanning. Of the pediatric patients, 22/54 patients did not undergo follow-up CT scanning for one of the following reasons: 1) relocation to another hospital (n=9), 2) death due to CVID complications (n=3) or 3) because the treating clinician or parents decided to postpone scanning to later because of excellent clinical performance (n=10). One high-risk adult patient was not evaluated for follow-up scan because of relocation abroad.

CT scanning

Chest CT scanning was performed by multi detector-row computed tomography (CT) scanners (16 256-detector row scanners; Philips, Cleveland, OH, USA) conforming to a dedicated volumetric protocol. Scans were obtained during both inspiration and expiration using a breath-hold instruction. In children inspiratory scans were acquired in a caudocranial direction with a collimation of 16*0.75 mm, pitch 0.9, rotation time 0.5 s, 90 kVp and milli-amperage per second depending on body weight (range 16–60 $mA*s^{-1}$). Expiratory scans were acquired in a caudocranial direction with a collimation of 1660.75 mm, pitch 1.2, rotation time 0.4 s, 90 kVp and 11 $mA*s^{-1}$. The expiratory scan was obtained at end expiration[18]. In adults thin slice volumetric data were acquired in inspiration at 100-120 kVp and 130 mAs and in expiration at 80-120 kVp and 20-40 mAs depending on body weight.

CT scoring

All CT scans were anonimised and scored by the same reader (radiology resident with a PhD in pulmonary imaging) after randomisation of baseline and follow-up scans. CT scoring was executed as described previously[20]. In short, each pulmonary lobe was scored 0 to 3 for the following items: size of largest bronciectasis, size of average bronciectasis, extent of bronciectasis, most severe airway wall thickening, average severity airway wall thickening, extent of airway wall thickening, extent of mucus plugging, extent of tree-in-bud, extent opacities, extent of ground glass, average size of lung nodules, extent of septa thickening, number bulla/cyst. Extent of airtrapping was given a score between 0 and 5. Subsequently, airway disease scores were calculated from a total bronciectasis score, total airway wall thickening score, total mucus score and airtrapping. Interstitial lung disease scores were calculated from the total extent opacities score, total extent of ground glass score, total lung nodules score, total extent of septa thickening score.

Pulmonary Function Tests

Pulmonary function tests (PFTs) were measured with a Jeager Masterscreen CS and Masterlab Systems (Wurzburg, Germany) and carried out according to the American Thoracic Society criteria in order to determine pulmonary health condition. Measurement

of PFT contained the following parameters: % predicted forced vital capacity (FVC%), % predicted forced expiratory volume in 1 second (FEV1%), % predicte total lung capacity (TLC%), % FEV1 as part of vital capacity (FEV1 / VC %), % predicted peak expiratory flow rate (PEFR%) and % residual volume (RV%) as part of total lung capacity (RV / TLC %) .

Statistical Analysis

All statistical analyses were performed with SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL, U.S.A). To compare continuous data between two groups, unpaired t-tests were used for parametric data and Mann-Whitney *U* tests for non-parametric data, respectively. Paired data were tested with the Wilcoxon signed rank test. Categorical data were tested with Pearson's chi-square test. For correlations the Spearman rank correlation was used for non-parametric data and Pearson for parametric data. Tests were performed two-tailed, and p-values ≤ 0.05 were considered significant.

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CHAPTER 10

General Discussion

Primary immunodeficiencies encompass a large group of diseases with diverse clinical presentations; patients can suffer from severe (lethal) disease to relatively mild disease with primarily recurrent infections and subsequent risk for complications at later age. Because of the wide range of disease expression a solid classification system is imperative, not in the least to optimize treatment for each group of diseases. The International Union of Immunological Societies (IUIS) Expert committee on PID subdivides known PIDs into nine major categories and especially in the categories harboring more severe immunodeficiency diseases, the identification of distinct genetic mutations offers improved insight into pathogenesis and future cure is a possibility[1,2]. CVID disorder patients however usually cannot be classified according to monogenetic defects, for reason that single genetic causes are identified at an only infrequent rate. Diagnosis and treatment are therefore based on the clinical symptoms and immunological phenotype of the patients, with most usually immunoglobulin substitution therapy for prevention of recurrent infections and several immunosuppressive agents for autoimmune complications. With this thesis we hope to contribute to the understanding of the underlying B- and T-cell defects of a subgroup of CVID patients and to critically evaluate the diagnostic and therapeutic options in CVID with suggestions for future studies.

In this chapter the findings of the previous chapters are shortly summarized and completed with a discussion of these findings.

In search of genetic defects in CVID- is it useful?

The majority of CVID disorder patients do not seem to harbor monogenetic disease (<10%). However, in recent years, helped by the development and application of new genetics-based studies, several new CVID-associated genes were proposed. A large genome wide association study found a strong association with the HLA/MHC region and ADAM genes and a recent whole exome sequencing study exploring the 269 known PID associated genes, identified 17 new probable disease-causing mono-allelic mutations in 50 CVID patients including STAT3 and PIK3CD amongst others[3,4]. We confirm the PIK3CD and STAT3 gene mutations in a cohort of 40 CVID/CID patients where we screened 25 pediatric CVID patients using a next gen sequencing (NGS)-based DNA sequencing technique on 171 known PID genes and 300 candidate PID genes.

We consider genetic screenings most useful when potentially disease causing gene variants can be translated into proven disease causing gene mutations through functional experiments. Genetic defects that were proven to be CVID disease causing include CD19, CD20, CD81, ICOS, LRBA, PRKCD and PLCG2[5]. Recently, CLEC16A was added as a potential new disease causing gene, at least in mice[6]. In our quest to translate potential disease genes into proven ones, we sought to provide evidence for 3 three CVID-associated gene mutations, one in the B-lymphocyte kinase BLK (Chapter 2) and two in PSTPIP1 (chapter 3). The functional assays performed supports a pivotal role

of BLK in human B-cell differentiation, contributing to CVID disease through hampered BCR activation and further processing. Data from various complementing cellular assays comparing the common PSTPIP1 and the mutated PSTPIP1 protein variants, show a correlation to the T-cell phenotype of the patients. We found that PSTPIP1 has a pivotal role controlling the immune synapse stability between T-cell and APC. We can only hypothesize on the connection to B-cell functioning, where we propose that the hampered T-cell activation and further altered T-cell compartment as described in chapter 3 will result in abolished CD4 T-cell help, as essential for proper B-cell differentiation into class switched memory IgG and IgA B-cells.

The identification of 3 novel gene mutations out of 30 patients suggests to us that CVID patients may be a rich source of new gene mutations, although this cannot be firmly stated in such a small number of patients tested. However, also other studies find relatively high numbers of potential disease causing variants, albeit in a preselected group of CVID patients with pronounced B-cell maturation defects and/or autoimmunity. Furthermore the mutations are found in divers pathways[34].

The latter is in line with the very heterogeneous presentation of CVID patients, and therefore it may not be surprising that also the potential genetic defects are located in different pathways and immune cells. Thus the clustering of genes according to a more general downstream effect (e.g., f-actin remodeling or cytosolic calcium mobilization), as we approached in a first attempt in chapter 5, might provide a categorization option. As we describe in this review in chapter 5, PID related gene defects appear in T-cells presenting with f-actin polymerization defects, with abrogation of the immune synapse and subsequently resulting in immunodeficiency. Furthermore, we hypothesize that early signaling gene defects might cluster in accordance to abrogated cytosolic calcium mobilization, offering another potential diagnostic stratification method of the heterogeneous CVID patient group.

The CVID related gene mutations we found and that have been described before do seem to mostly locate in the early signaling pathways or in cell surface receptors, which seems to fit the predominantly naive B-cell phenotype and diminished T-cell proliferation numbers as can be found in many CVID patients. After all, when the first antigen presenting contact fails to translate into proper downstream signaling, the B- or T-cell will not differentiate and maturate.

Secondly, the (potential) genetic defects identified mostly occur in the CVID patients with a pronounced B- and/or T-cell phenotype and/or autoimmunity, in some cases on the borderline of a combined immunodeficiency (CID). The relatively mild pediatric CVID patients with recurrent infections and normal B-and T-cell subsets will probably not benefit from this genetic approach, unless a genetic defect would for example predict a

detrimental course of disease at later adult age. Thus, for the CVID patients with no genetic basis for disease a tailored treatment strategy as based on the clinical presentation and residual immunoglobulin functioning in the local mucosa might be more beneficial, with the main purpose to prevent future infectious (and non-infectious) complications, as will be discussed in the clinical section of the discussion.

Diagnostic tools in CVID - Pneumococcal Polysaccharide Serotype vaccination responses revisited

The diagnostic use of measuring Pneumococcal Polysaccharide Serotype (PnPS) vaccination responses for CVID is submissive to a continuous debate. The European Society for Immunodeficiency (ESID) criteria for CVID do include insufficient specific antibody responses to vaccinations however those are not required to meet the diagnoses of CVID[78]. When one does decide to measure PnPS vaccination responses, there are several techniques available for use [910][11][12]. Also on this matter a uniform statement is not yet formulated: some institutes prefer to measure all combined PnPS responses as a whole where others prefer separate PnPS responses per serotype [13–15]. In chapter 6 of this thesis, we added our educated advice in this matter, by direct comparison of two methods for serotype measurements. We argue that individual serotype measurements are preferred over the combined serotype measurement, for reason that high responses to some (<50%) serotypes could mask the overall very poor responses on the other (>50%) of serotypes measured and thus unjustifiably classify a patient as 'non-immunodeficient'. In support, we show that individual PnPS vaccination response measurements are more informative to evaluate in the context of confirming CVID diagnosis, compared to combined anti-PnPS IgG responses. As we show in chapter 7 also the serum anti-PnPS IgA responses are of importance, mainly to discriminate CVID from transient antibody deficiency (TAD). While the individual serum PnPS-IgG responses of the TAD patients showed quite some overlap with the control group and as such would not be identified as antibody deficient especially in case of a combined PnPS serotype measurement, the serum anti-PnPS IgA responses did not show any overlap with controls nor with the persistent CVID group and thus discriminated the TAD patients superiorly from the two.

Thus we plead for the measurement of individual IgG and IgA PnPS-vaccination responses as part of the diagnostic trajectory of a suspected immunodeficient patient, not only to confirm disease but also to stratify the relatively milder CVID patients into potentially transient or persistent disease. One obvious question is, why the IgA anti-PnPS responses are more discriminative? One of the answers might lie in the kinetics of serum anti-IgA responses. Healthy adults display a serum anti-PnPS IgA peak after 7 days, elderly have been shown to have a delayed anti-PnPS IgA response up to 28 days, while the anti-PnPS IgG response dynamics remained unaltered[16,17]. The measurement of the anti-PnPS IgA responses in our retrospective cohort was from the same sample as from which the anti-PnPS IgG levels were measured, approximately 4 weeks after

PnPS vaccination when the peak level of anti-PnPS IgG response first occurs. The time of measurement-although optimal for IgG is not optimal for IgA. Therefore a study on the of kinetics of anti-IgM, -IgA and -IgG PnPS responses in antibody deficiency patients would be useful to further investigate if the anti-PnPS IgA responses are delayed or indeed absent or respectively less in persistent CVID or TAD patients. Furthermore an assessment of the local respiratory tract anti-IgM, -IgA and -IgG PnPS titers through oropharyngeal swabs and bronchial alveolar lavage, would shed further light on how the serum responses would relate to the actual response in the local pulmonary mucosa (see future perspectives) as has been investigated in saliva of pediatric CVID patients[18].

Local immunoglobulin homeostasis in CVID patients

CVID patients encounter recurrent bacterial and viral infections of the upper- and lower respiratory and gastrointestinal tract, which are ascribed to their diminished immunoglobulin levels. Those immunoglobulin levels are measured in serum and are easy to obtain since it involves a 'simple' venous puncture. However these do not always reflect what happens at the mucosal site of the patient, where the actual interaction with hazardous pathogens such as encapsulated bacteria and viruses takes place.

The gut mucosa

In chapter 8 we explored the IgA levels of CVID patients that carried enteric viruses, while the patients did not report active complaints as would occur with an acute gastroenteritis. The presence of viruses was significantly higher in asymptomatic CVID patients than in healthy controls, and was accompanied by signs of inflammation as measured by feces calprotectin levels that were significantly higher, yet not as high as in eg Crohn's disease. Next to that the patients carrying viruses exhibited significantly lower faecal IgA levels than healthy controls or CVID patients without viruses. These findings are most intriguing since the mucosal IgA production balances with the mucosal microbial flora to keep a healthy mucosal immune homeostasis[19,2021]. Perhaps the diminished or even absent IgA production results in 1) a significantly different commensal gut flora skewing the mucosal immune environment towards a pro-inflammatory state which could eventually result in CVID-related enteropathy and/or 2) on the other hand offering less protection against invading microorganisms and viruses thus leaving the gut more prone for active recurrent infections and inflammation.

From the gut to another mucosal site: the lung

Chapter 9 of this thesis includes a study on the pulmonary complications of CVID. Pulmonary damage due to recurrent respiratory tract infections and/or CVID related immune dysregulation is the most prevalent complication of CVID. In order to reduce the recurrent respiratory tract infections and prevent future derailment of pulmonary disease, treatment consists of IgG replacement therapy[22]. In chapter 9 we address an important issue in this matter: the optimal IgG trough levels for effective prevention of recurrent

respiratory tract infections and pulmonary damage. The opinions vary on this topic and no concluding studies to answer this question have been performed up to date [22–24]. We show in a prospective study, although not of a randomized controlled trial design, that high (=>10g/l) IgG trough levels seem to reduce the progression of subtle pulmonary damage as measured on CT-scans, likely due to recurrent, potentially also subclinical infections. Although this finding is very intriguing, there are some major limitations in this study because of the study design, which was not set up to evaluate treatment regimes, but to assess progression of pulmonary damage in CVID patients. Thus a future study with a randomized controlled study design is warranted and will be discussed at the end of this chapter.

Our study leaves some other questions unanswered. First the patients in our study progress in the airway disease score as quantified on CT-scans, even without clinically symptomatic recurrent pulmonary infections. The airway disease score is composed from scoring items that relate to infectious damage (airway wall thickening, bronchiectasis etc). Thus, although the infections are not apparent to the patient or the doctor, a chronic viral/bacterial dysbiosis might be present in the lung due to delayed pathogen clearance as we found in the gut (chapter 8), triggering silent pulmonary disease progression through chronic inflammation that eventually might result in (severe) apparent pulmonary airway disease. Alternatively, a continuous state of inflammation might also result from a lower tolerance for the commensal respiratory tract flora thus triggering the adaptive/innate immune system into an inflammatory state. Or a chronic dysbiosis that results in a chronic (pro)- inflammatory status of the lung, similar to that described in de gastro intestinal tract.

Second the finding that higher serum IgG levels through immunoglobulin replacement therapy might reduce pulmonary airway disease progression makes one wonder how this would translate to the local pulmonary mucosa of CVID patients. Most probably the higher serum IgG levels will result in increased IgG diffusion into the bronchia, similar to that described in oral cavity[25] while a small part of the IgG's present in the lung is produced in the local pulmonary mucosa IgG supplementation as offered through the serum will partially improve the local pulmonary defense. A proposed mechanisms is offered in a recent paper of Mitsi et al, where oral clearance of pneumococcal colonization is mediated by pneumococcal agglutination by anti-capsular IgG antibodies in the nasopharyngeal tract after parenteral pneumococcal vaccination. Higher IgG serum levels after vaccination correlate with secondary higher oral levels of secreted serum IgG and improves clearance. Thus, it would be interesting to quantify the local pulmonary IgG presence of antibody deficient patients upon IgG supplementation and assess how this relates to the efficiency of pathogen clearance.

Thirdly, although IgG's are mostly represented in the lower respiratory tract, also IgA, IgM and IgD contribute in the host pathogen defense. It is unknown how these

immunoglobulins behave in the different CVID patients. IgD could be of interest to study in antibody deficiencies since it has been reported as an important immunoglobulin in the upper respiratory tract, where it contributes to pathogen clearance through interaction with basophils which then produce B-cell stimulating cytokines (IL4, IL13) and pro-inflammatory cytokines such as TNFalpha and IL1beta, contributing to pathogen clearance and inflammatory responses. In inherited auto-inflammatory syndromes with hyper IgD, basophils are in a hyper activated state secreting enhanced amounts of IL1beta and TNFalpha[26,27]. Local IgD levels in CVID are unknown. Decreased IgD levels might contribute to an opposite mechanism as in the auto-inflammatory setting, with less B-cell maturation cytokine induction and a lesser inflammatory response to pathogens with subsequent less clearance. In contrast, high IgD levels could have a similar effect as in the inherited autoinflammatory patients, facilitating a chronic inflammatory state in the respiratory tract and subsequent mucosal damage.

FUTURE STUDIES

This thesis describes several aspects, clinical and molecular, of (pediatric) CVID patients. Next to the continuous search for a molecular basis for disease, future studies should also be directed towards improvement of treatment strategies and better understanding of the mechanisms defining the lack of local defense at the mucosal sites of the lung and gut.

A randomized controlled intervention study for treatment optimization of CVID patients

Q1 *Do higher IgG trough levels provide better protection against (silent) pulmonary airway disease as evaluated by CT and Pulmonary Function Tests?*

This study will focus on IgG replacement therapy in pediatric and adult CVID patients. Patients with and without pulmonary complications will be assigned equally to two study arms, one with current standard IgG substitution and one with levels higher than 10 g/l. Once the appropriate IgG levels will be reached patients will be scanned with CT and perform pulmonary function tests (PFT) at baseline and this will be repeated after two years. The CT scans will be scored for airway and interstitial lung disease components as we have described before (chapter 9) and intermittently serum immunoglobulin levels will be assessed. Major possible confounders are the pulmonary condition at start of the study, use of antibiotics, age, respiratory tract infections and immunophenotype,

Q2 *Does IgG supplementation improve the local pulmonary mucosal homeostasis of the CVID respiratory tract? And is this different between pediatric and adult CVID?*

Another part of this study would be to assess the mucosal microbiota of pediatric and adult CVID patients and controls. Ideally bronchial alveolar lavage (BAL) or otherwise nasal plus oro-pharyngeal swabs will be obtained at both CT scan timepoints and assessed for:

-Local levels of IgG, IgA, IgM, IgD → is there indeed significantly more IgG secreted with higher Ig replacement therapy? What is the correlation to serum values?
-Viral and bacterial presence in bronchial fluid: which common and pathogenic species are present? Does higher IgG supplementation alter the present commensal/pathogenic flora?
- Inflammatory state (local cytokines, activated adaptive/innate immune cells, CRP in serum) does IgG supplementation reduce local inflammation?
-Specific antigen responses: assess the local anti-PnPS IgM, IgA and IgG titers present in the lung → what is the correlation to serum?

Last but not least, CVID disorder is a highly heterogeneous disease group and will remain so unless the definition is redefined. The efforts to stratify for different subgroups according to genetic screening, B- and T-cell studies and clinical presentations, are resulting in new ways to diagnose and treat CVID. Some patients will benefit from genetic screening, others from optimized therapeutic approaches. Most likely, future treatment options will bring a combination of both, maybe through directed therapy with modulation of molecular signaling pathways and on the other hand through optimized (local) immunoglobulin substitution therapy.

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CHAPTER 11

Nederlandse Samenvatting

DEEL I

Hoofdstuk 1 geeft een introductie tot het onderwerp van dit proefschrift. Common Variable Immunodeficiency disorder (CVID) is de meest voorkomende symptomatische primaire immuundeficientie. Patienten presenteren zich doorgaans met recurrente infecties van met name de bovenste- en onderste luchtwegen maar ook van de gastrointestinale tractus. Daarnaast komen autoimmuun symptomen voor bij circa 30 % van de patienten. Door de recurrente infecties van de luchtwegen krijgt een deel van de patienten op de langere termijn te maken met longschade. Dit kan resulteren in een dusdanig ernstige vorm dat zij terminaal longfalen ontwikkelen en vervroegd overlijden. CVID patienten voldoen aan een aantal vastgestelde criteria, namelijk recurrente infecties van de luchtwegen/ gastrointestinale tractus, sterk verlaagde immuunglobuline G (IgG) en immuunglobuline A (IgA) en/of immuunglobuline M (IgM) waarden en gestoorde vaccinatie responsen. De oorzaak van CVID is slechts deels bekend maar wordt doorgaans gezien als een dysfunctioneren van B-cellen, voor circa 10% van de patienten is een genetische basis voor de ziekte gevonden. Daarom richt dit proefschrift zich op 1) het verder uitwerken van een moleculaire basis voor CVID en 2) het klinisch verder duiden van klinische symptomen van CVID.

In **Hoofdstuk 2** wordt de mogelijke rol van het BLK eiwit in de ziekte CVID beschreven. Via een genetische screening van CVID patienten hebben wij een nieuwe mutatie gevonden van het BLK eiwit. Het BLK eiwit is met name van belang in de vroege signaal transductie van de B-cel. Als deze niet goed plaats vindt, kan de B-cel niet efficient op een antigen (zoals een bacterie) reageren en hier ook onvoldoende specifieke immuunglobulinen tegen aanmaken. Door een aantal functionele proeven tonen wij aan dat het gemuteerde BLK eiwit inderdaad bijdraagt aan een verminderde signaal tranductie in de B-cel en daardoor minder immuunglobuline productie zoals bij CVID patienten voorkomt.

Hoofdstuk 3 beschrijft de rol van het PSTPIP1 eiwit in T-cellen van 2 CVID patienten. PSTPIP1 heeft een belangrijke rol in het opbouwen van actine filamenten. Actine filamenten zijn belangrijk voor cel motiliteit en het vormen van een immuun synaps met omliggende cellen, zodat deze cellen elkaar kunnen activeren/helpen. Wij beschrijven twee nieuwe PSTPIP1 mutaties in twee CVID patienten en het gevolg hiervan voor de T-cel, namelijk dat de cel niet meer goed in staat is om een immuun synaps te vormen met bijvoorbeeld een B-cel. Dit heeft dan vermoedelijk weer tot gevolg dat de T-cel geen adequate hulp kan bieden aan de B-cel om specifieke immuunglobulinen te produceren en draagt hiermee bij aan de ziekte CVID.

In **Hoofdstuk 4** richten wij ons op een ander eiwit, namelijk STAT3. Dit eiwit zorgt er in de cel kern voor dat DNA wordt afgeschreven en de cel geactiveerd. Wij beschrijven een nieuwe STAT3 mutatie welke bijdraagt een aan de hoge excretie van cytokinen (IL17,

IL21) door een bepaald type T-cel (Th17). Vermoedelijk zorgt dit er ook voor dat de patient autoimmuniteit symptomen krijgt en een overproductie aan immuunglobulinen.

Hoofdstuk 5 is geeft een overzicht (review) van reeds beschreven T-cel eiwit mutaties en hun rol in primaire immuundeficienties.

DEEL II

In **Hoofdstuk 6** worden twee verschillende meet methoden voor vaccinatie responsen met elkaar vergeleken. Aangezien een verstoorde vaccinatie respons onderdeel is van de diagnose CVID, is het van belang hier een zo uniform mogelijk meetmethode voor af te spreken. Op dit moment is daar nog geen consensus over. In dit hoofdstuk beschrijven wij dat het meten van de vaccinatie responsen op individuele pneumococcen serotypen te verkiezen is boven het meten van de totale respons van de individuele pneumococcen serotypen bij elkaar opgeteld. Dit omdat CVID patienten op sommige serotypen een nog hoge respons aanmaken, welke dan een slechte respons op de overige serotypen maskeert, en dus een onterecht goede uitslag geeft.

Hoofdstuk 7 gaat verder in op het meten van vaccinatie responsen bij CVID patienten. De huidige vaccinatie responsen worden gemeten als een anti-pneumococcen IgG respons. Echter er worden ook anti-pneumococcen IgA en IgM responsen gemaakt. Wij beschrijven in dit hoofdstuk dat de anti-pneumococcen IgA responsen tijdelijk imuun deficiente patienten kan onderscheiden van blijvend immuundeficient. Dit kan interessant zijn omdat er een kleine groep immuun deficiente patienten is die de ziekte ontgroeit en deze dan beter herkend zou kunnen worden.

In H**oofdstuk 8** hebben we gekeken naar gastrointestinale infecties bij CVID patienten. Zoals bij de introductie genoemd zijn CVID patienten gevoelig voor recurrente gastrointestinale infecties. Wij beschrijven dat CVID patienten inderdaad meer gastrointestinale virussen bij zich dragen dan gezonde controles en dat deze patienten tekenen van ontsteking vertonen, met daarnaast een significant verlaagde IgA excretie in de ontlasting. IgA is een belangrijk immuunglobuline in de darm en enerzijds nodig om de microbiele balans in stand te houden, anderzijds om schadelijke pathogenen te neutraliseren en met de ontlasting uit te scheiden.

Hoofdstuk 9 beschrijft een prospectieve studie naar de toename van longschade op CT scans van CVID patienten in een follow up periode van 3-5 jaar. In deze studie vinden wij ten eerste dat in ons cohort van patienten longschade toeneemt op CT, ondanks dat er geen klinische verschijnselen van luchtweginfecties/longontstekingen zijn geweest. Ten tweede lijkt er een verband te bestaan tussen de hoogte van de IgG substitutie therapie;

hoe hoger de IgG substitutie spiegels, hoe minder progressie van de longschade. Een verdere grote gerandomiseerde studie om dit te bevestigen zal nog moeten worden verricht.

Hoofdstuk 10 geeft een overkoepelende discussie van de voorgaande hoofdstukken en sluit af met een voorstel voor toekomstig verder onderzoek naar CVID

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CHAPTER 12

Curriculum Vitae

Publication List

Dankwoord

CURRICULUM VITAE

Willemijn Janssen werd op 21 mei 1983 geboren te Nijmegen. Opgroeiende in Wageningen werden er van jongs af aan vele fietstochten gemaakt. Eenmaal op de middelbare school hield zij zich naast het gymnasium ook bezig met viool spelen aan de conservatorium vooropleiding. Omdat de keuze tussen de studie geneeskunde en conservatorium niet gemakkelijk te maken viel, combineerde zij in in eerste instantie beide studies. Inmiddels is zij in opleiding tot reumatoloog bij het UMC Utrecht.

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DANKWOORD

Dank voor het vertrouwen Joris van Montfrans, Marianne Boes en Lieke Sanders.
Annick: Dank voor al je werk, de altijd weer uren durende uit de hand gelopen koffie dates, ik ga je vinden in Groningen!
Arjan: mijn medestander voor een beter milieu
Caro: tja Caro, we moeten de pizza boer nog steeds bellen om ons excuus aan te bieden...
Chiarra: a western blot is never really a western blot without you
De dames en heer in het eiwitlab: altijd weer een goede morgen als jullie er zitten
De oud Boes mannen: voor het zijn van Boes mannen
Elena: spicy greek and in love with the Dutch weather
Emmerik: voor je rust in de Boes groep
Erinke: je eindeloze tolerantie voor mijn zingen en geouwehoer
Eveline: altijd weer een goed gesprek
Ewoud: B-cel mattie!
Francesca: gezelligheid om je erbij te hebben bij de Boesers
Gerdien: lieve Gerdien, zorgzaam en altijd een luisterend oor
Hadewijch: mijn allerliefste allerfijnste 'we go way back' muziek maatje
Josefine: zakken chips, overpijnzingen , altijd voor iemand klaar staan, en de almanak
Judith: STAT3 stress, late uit de hand lopende gesprekken en dan nog later naar huis.
Karin: Flux mattie! Dank! Vakanties zijn ook belangrijk..
Kerstin: dank voor het verdragen van mijn vreselijke duits, je sjaal die ik heb, en voor nog vele etentjes!
Kuldeep: thank you for all your knowledge, also when requested at 07:30h in the morning
Larike: avonturen en vormende (ont) puberjaren
Lieneke: de kunst van het liefdevol uitlachen. PSTPIP1 (tegen wil en dank). Dank voor je hulp!
Lotte: mijn allerbeste lab mattie, koffie maatje en mycoplasma lover
Mateuz: nice to have another Slovak in the lab..still looking to get you tvaroh
Maud: nog steeds niet in de bossen van Driebergen gelopen, waardevolle FACS lessen
Marlot: grote lach
Marthe: lessen over exosomen...
Na: my little sister
Nadia: Iranian dishes are binding us
Noortje: zonder jou had ik sowieso het verkeerde vak gekozen!
Nienke: wat een gastvrijheid, ik sta nog steeds bij je in het krijt
Pawel: thank you for the many coffees we never had…
Rianne: ahhhh poekie!
Sabrine: wat een huisgenootje wel niet kan betekenen in je leven

Sandra: Thank you for all the fun and little glasses of horrible wine on Sunday
Sjors: volgens mij draait Captain America nu echt niet meer in de bios...
Stefan Nierkens: zonder UDAIR had ik geen boekje
Sytze: promoveren is ook gewoon werk, salaris zeik woensdag, eenzaam zwaaien op de P&R
Tessa: je weet zoveel! Gelukkig geen confocal meer...
Vanessa: liefde van mijn leven

And everyone else (equally important):

Thank you for making this 4+ years into an unforgettable experience

Liron, Eitan, Maayan, mijn moeder

Geluk is geen kathedraal,
misschien een klein kapelletje.
Geen kermis luid en kolossaal,
misschien een carrouselletje.

Geluk is geen zomer van smetteloos
blauw, maar nu en dan een zonnetje.
Geluk dat is geen zeppelin,
't is hooguit 'n ballonnetje.

(Toon Hermans)