Deconstructing B cell responses

Lessons learned from development, drugs and deficiencies

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Chapter 9

General Discussion

B cells are very important players in the adaptive immune system to fight all the infectious threats the human host faces during life. The complex mechanics behind the whole process from a common progenitor cell to a plasma cell producing highly specific antibodies that help to efficiently eradicate pathogens, is another masterpiece of nature. Like with every complex process it can be easily derailed if the crucial components are missing, are modified or get exhausted.

In this thesis we touched upon multiple B cell defects caused by dysregulation of the immune system. Our approach has been to learn more from B cell responses by studying patients with inborn defects in the humoral immune response. Additionally we investigated the most naive cells 'lacking' the antigen experience that follows during life, and modified mature B cell functional outcomes by adding small molecule inhibitors. The findings in this thesis also contribute to the clinical field of immunology, as the categorization of patients with common variable immunodeficiency remains challenging, with consequences for decision making by clinicians regarding diagnostics, manifestations, treatment, monitoring and genetic counseling.

Cord blood cells as a starting point for memory B cell development

In **Chapter 2** we studied the B cell compartment at the very early stage of life and compared it with its adult counterpart, being antigen experienced. Besides exploring the unique capacity of cord blood B cells to produce more IgM than you would expect from naive B cells as discussed in more detail in the concerning chapter, this showed us what antigen unexperienced B cells can and cannot.

The development of antigen-experienced B cell subsets is shaped by the micro-environment and the development of secondary lymphoid organs[1,2], by which simultaneously the functional responses *ex vivo* mature[3]. $CD27^{+}IgD^{+}$ marginal zone B cells start populating the spleen from 2 years of age and onwards[4], but can be found in the circulation before already[3,5]. Those marginal zone B cells have an important role in the defense against encapsulated bacteria by (mainly) producing IgM. The $CD27^{+}$ memory B cells start appearing in the circulation as T-cell dependent responses start taking place in germinal centers of secondary lymphoid tissue and generate plasmablasts and plasma cells producing class-switched IgG, IgA, IgE and to a lesser extent non-class-switched IgM, with concomitant generation of immunological memory.

In a normally developing immune system certain B cell subsets and B cell responses should be represented and if not, the results point at a defect. However, the B cell compartment is a dynamic compartment and could change at any moment during life. As opposed to long lived plasma cells that reside in the bone marrow for years and produce the majority of serum immunoglobulins[6,7], the peripheral blood compartment is more dynamic, influenced by age and environmental factors (e.g. infections, vaccines, nutrition, microbiome)[8,9]. Therefore it gives a more actual indication of the "health" of the B cell compartment at a certain time point.

By investigating the peripheral B cell compartment and its functional responses we were able to identify and characterize immunodeficient patients and address whether genetic lesions correspond to actual differences in B cell phenotype and functionality.

Secondary B cell defects

In **Chapter 3** and **Chapter 4** we investigated two novel diseases in which B cell development is affected, although the majority of the clinical disease burden is not primarily caused by defects in B cells. Although these disorders showed indirect B cell defects, it learned us more about the unexpected relevance of the affected proteins for normal immune development, as this was not known to date, and to which extent the B cell responses *ex vivo* are dependent on normal T cell development *in vivo*.

The immune defect in EXTL3 deficiency lies in T cells

In **Chapter 3** we described a novel immunodeficiency caused by homozygous missense mutations in *EXTL3* (exostosin-like glycosyltransferase 3), important in regulating proteoglycan formation by biosynthesis of heparan sulfate chains. Absence of EXTL3 causes a neuro-immuno-skeletal dysplasia, similar to well-known immunodeficiencies presenting with skeletal abnormalities such as cartilage-hair hypoplasia and Schimke immune-osseous dysplasia[10], all with very different etiologies. The EXTL3 patients that showed clear features of immunodeficiency presented early in life due to severe immune abnormalities.

Patients with immune defects suffered from T-B+NK+ severe combined immunodeficiency (SCID), suggesting EXTL3 plays an important role in T cell development. Indeed, we localized the defect at the thymic stages of T cell development which is compatible with the high EXTL3 protein expression in the thymus. Although EXTL3 is expressed in common lymphoid progenitors, we could not detect EXTL3 in precursor or (activated) peripheral B cells. Using B cell phenotyping and functional assays we confirmed indirect effects of T cell deficiency only, that account for the hypogammaglobinemia reported by us and others[11,12].

The immunological importance of this protein in humans was not yet described. B cells rely on heparin sulfate proteoglycans for survival of long-lived plasma cells and promote cytokine signaling[13,14], but the absence of a clear intrinsic B cell defect and the fact that EXTL3 is not expressed in circulating B cells suggests that these processes are compensated for or guided by other glycosyltransferases instead.

A combined immunodeficiency caused by homozygous *ARPC1B* mutations

In **Chapter 4** a novel syndrome with deficient actin polymerization was further characterized by reporting on the defects in adaptive immune responsiveness in a series of patients with biallelic pathogenic *ARPC1B* variants. The clinical phenotype is broad with recurrent bacterial and viral infections, thrombocytopenia, eczema, allergies and skin vasculitis. After we and other groups first published the defect of actin polymerization in neutrophils and thrombocytes[15,16], we hypothesized that the recurrent bacterial infections, mainly of the upper respiratory tract, could also be partly explained by defects in cytoskeletal rearrangements in B cells.

Upon examining general B cell parameters we did not find a large impact of ARPC1B deficiency on B cell functioning. However, absolute number of B cells were high with elevated IgG, IgA and IgE in patients. This could not only be explained by the defects in T cells[17,18], suggesting that detailed investigations might reveal subtle B cell effects. ARPC1B is part of the Arp2/3-complex, important for nucleation and branching of actin filaments[19]. It is well known that actin polymerization is important for BCR ligation and immune synapse stability[20,21]. Strengthened by the fact that we found atypical CD19 expression, it could very well be that cell-cell interactions and/or migration will show abnormalities by more detailed investigations.

The T and B cells in ARPC1B deficiency showed many characteristics of WASp deficiency (Wiskott-Aldrich Syndrome), the upstream regulator of the Arp2/3-complex, such as decreased T cell function, elevated serum IgA and IgE and an expansion of the $CD21^{low}$ population (unpublished data). However, the B cell polysaccharide response and *ex vivo* B cell responses were normal in most ARPC1B-defective patients. The abnormal IgA and IgE could therefore be due to abnormal function of T_{Reg} cells, shared by both diseases.

The slightly broader phenotype in ARPC1B deficiency compared to defects in WASp may be spurious and there is considerable overlap. In WASp deficiency the regulator of the complete Arp2/3 complex is impaired, while in ARPC1B deficiency, part of the complex may still be present and allow (partial) function compensated by ARPC1A expression, but this induced expression in ARPC1B-deficient patients is not sufficient to rescue these patients from disease.

Primary B cell defects

In **Chapter 5** and **Chapter 6** we touched upon two diseases of which the identity was hidden under the umbrella “common variable immunodeficiency” (CVID) until a couple of years ago. The findings presented in these two chapters translate directly into our current understanding of B cell responses, since these mutations are central in two major signaling pathways in B cells: the canonical and non-canonical NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway.

NFKB1 loss-of-function is the most common monogenic cause of CVID to date

Disease-causing heterozygous *NFKB1* variants were first described by Fliegauf *et al.* by whole-exome sequencing in three unrelated families with CVID and/or hypogammaglobinemia[22]. By careful analysis, the splice-variant in *NFKB1* in intron 8 segregated with disease status. In **Chapter 5** we showed it is the commonest monogenic cause of CVID-like disease accounting for 4% of cases classified as CVID and half of all monogenic causes identified in an unbiased primary immunodeficiency disorder (PID) series sent in for whole-genome sequencing (WGS) diagnostics. Furthermore, we characterized the B cell phenotype and function in the largest cohort of NFKB1-defective patients to date. Additional papers have now been published and it became evident that NFKB1 loss-of-function comes with highly variable disease manifestations and a high frequency of non-infectious complications, including inflammation, auto-immunity and malignancies.

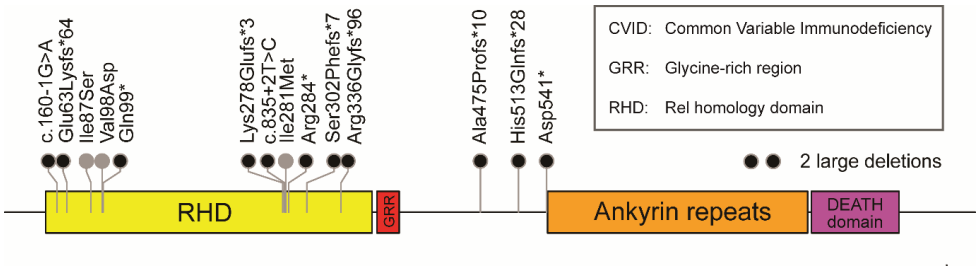


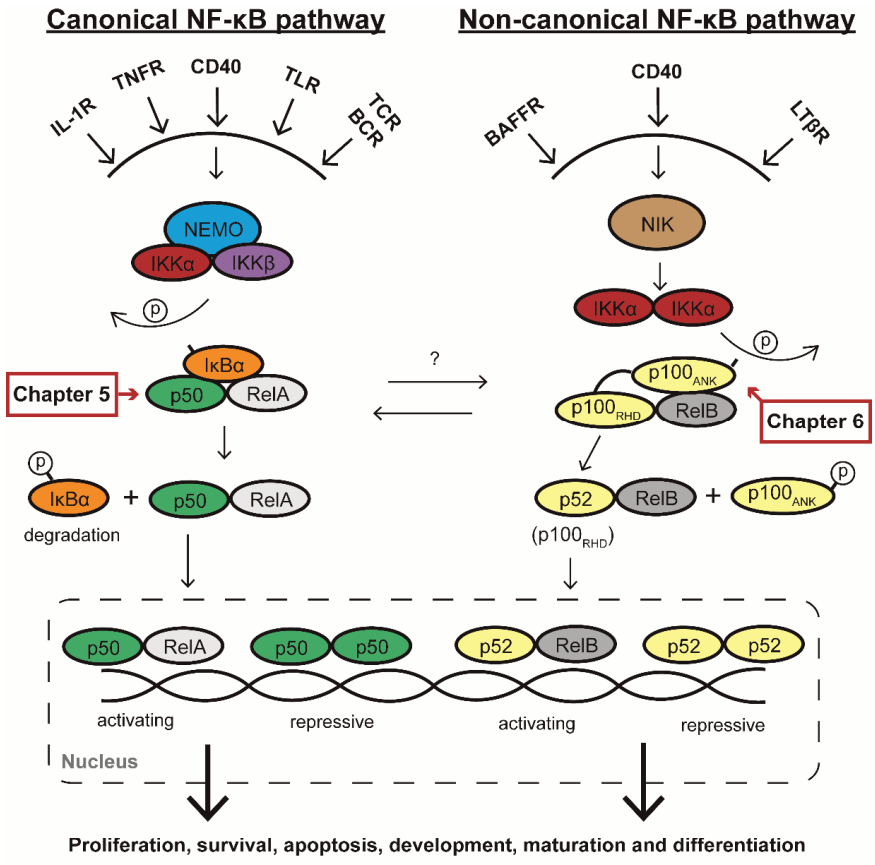
Figure 1. Location of pathogenic *NFKB1* variants described in this thesis. Black dots indicate truncating variants or deletions, grey dots indicate missense variants. Part of the Graphical Abstract of Chapter 5.

All pathogenic *NFKB1* variants in our cohort caused a reduction of both the p50 and p105 protein at baseline, leading to haploinsufficiency. This is now established as the disease mechanism, although it is questioned whether pathogenic variants around the cleavage site also lead to haploinsufficiency[23]. Insight in the function of NF-κB pathway and regulation of NF-κB activation helps understanding how defects in this pathway can lead to immune deficiency with impaired B cell responses. There seems to be predominance of variants that at least affect the active p50 part of the protein, mainly around the Rel homology domain (**Figure 1**). The Rel homology domain is important for the specific DNA binding as well as dimerization with p50 itself or Rel-proteins (RelA/p65, c-Rel, RelB)(**Figure 2**). The latter is highly important since p50 (similar to p52, see below) lacks a transactivation domain (TAD), as opposed to Rel-proteins. Therefore, NFKB-signaling is balanced by either the formation of heterodimers with Rel proteins (mainly RelA and RelB) for active transcription or – instead – formation of p50 homodimers to suppress transcription[24-26]. It is yet unknown how exactly NFKB1-haploinsufficiency influences the proportion of p50 hetero- and homodimers present in the nucleus and/or cytoplasm.

The B cell phenotype is characterized by a progressive defect to generate class-switched memory B cells coinciding with a decrease in serum immunoglobulins preceded by absent production *ex vivo*. As opposed to individuals that we identified having pathogenic *NFKB1* variants by pedigree analysis without any indication of disease, patients with clinical symptoms all showed an increase in $CD21^{low}$ B cells, associated with splenomegaly and granulomatous disease in the context of CVID[27]. Although the phenotype of these $CD21^{low}$ cells is more clear now[28-30], it is not known whether the expansion of this population can be used as a prognostic marker in this disease. The fact that we identified three young adolescent individuals carrying a pathogenic *NFKB1* variant without any clinical manifestations (and without a $CD21^{low}$ B cell expansion) as of now, holds great promise to follow cellular alterations and disease progression over time.

Pathogenic *NFKB2* variants causes a variable antibody deficiency

Heterozygous pathogenic variants in *NFKB2* are less common, but one of the more prevalent monogenic causes of CVID when excluding *NFKB1* gene defects. Many pathogenic (frameshift and missense) variants have been found in the C-terminal part of the protein just before or



Gene	Protein	Inher.	Defect
IKBKG	NEMO	XL	EDA-ID
IKBKB	IKKβ	AR/AD	(S)CID
NFKBIA	IκBα	AD	EDA-ID
NFKB1	p50 (and p105)	AD	CVID
RELA	RelA	AD?	Inflammation?

Gene	Protein	Inher.	Defect
MAP3K14	NIK	AR	CID
IKBKA	IKKα	AR	Embryonically lethal
NFKB2	p52 (and p100)	AD	CVID
RELB	RelB	AR	CID

Figure 2. Canonical and non-canonical NF-κB pathway and defects leading to human disease. Schematic and simplistic overview of both pathways[31,32]. On top several receptors that lead to activation of the specific pathway, p: phosphorylation, ANK: ankyrin repeat domain, RHD: Rel homology domain. Modes of inheritance currently known, AD: autosomal-dominant, AR: autosomal-recessive, XL: X-linked. Defects in different components leading to several different PIDs in humans[24], CID: combined immunodeficiency, CVID: combined variable immunodeficiency, EDA-ID: anhidrotic ectodermal dysplasia with immune deficiency, SCID: severe combined immunodeficiency.

inside the NIK-responsive domain (amino acids 866 to 870)[33,34], essential for NIK mediated phosphorylation of p100 which results in cleavage into the active, DNA-binding, protein p52[24] (**Figure 2**). These patients suffer from antibody deficiency with respiratory infections and a variable degree of (mainly T cell driven) autoimmunity, also showing non-immunological features such as mild ectodermal dysplasia and central adrenal insufficiency[34]. In **Chapter 6** we described two families of patients with a pathogenic variant outside this protein region, a mutation which was reported once before with the same pathogenic variant in the ankyrin repeat domain[35], and we were able to determine the phenotype and function of B cells in detail.

Excluding pathogenic variants that confine the C-terminal part of the protein affecting processing by NIK, studies on patients affected by variants in other regions are limited. Although we describe two families with an infection-only CVID and might conclude this is distinctive for these pathogenic variants, others do report on non-infectious complications as well[34,35]. Kuehn *et al* describe a patient with the same Arg635X variant who suffered from severe viral (EBV, CMV) infections, granulomatous disease, interstitial lung disease and T-cell leukemia, which clearly differed from the clinical presentations that we observed in our families. Recently, two additional cases of proximal truncating variants were described with patients presenting with hemophagocytic lymphohistiocytosis and vitiligo initially or during follow-up, respectively[34].

In our families the functional B cell defect *ex vivo* was located at the stage of immunoglobulin production, as plasmablast formation was seemingly normal based on proliferation and increase of CD38. However, compatible with the reduced presence of class-switched memory B cells, IgG was not produced *ex vivo*. Absence of an expanded $CD21^{low}$ population corresponded with the infection-only phenotype in the two families we investigated, which contrasts with the NFKB1 defects mentioned above. Contribution of T cell defects could not be excluded, since suboptimal TCR stimulation led to low proliferation rates in the index cases. Other groups did not report extensively on the B and T cell phenotype, hence future experiments need to establish whether patients with both pathogenic variants in and outside the NIK-responsive domain show a similar B and T cell phenotype and function.

Differences and similarities between NFKB1/2 deficiency and their place as NF-κB-opathies

Both NFKB1 and NFKB2 deficiency can be classified as NF-κB-opathies. Despite the heterogeneity within this group of diseases, the differences in clinical and cellular phenotypes can be largely explained by the distinct functions of the different defective proteins (**Figure 2**; bottom panel). Although we were able to characterize a large group of NFKB1-deficient patients supplemented by other publications, it is too early to draw firm conclusions regarding the less common NFKB2-deficiency being a (partial) genocopy or a completely different disease entity. The cellular differences and similarities in NFKB1 and NFKB2 deficiency we describe in this thesis, are summarized in **Figure 3**.

The fact that there are overlapping features is not surprising. Cross-talk on a molecular level between the canonical and non-canonical NFKB pathway is well-established. For example, p100 processing enhances nuclear localization of RelA, the main binding partner of p105/p50 in the canonical NF-κB pathway[36,37]. Many other interactions in B cells may go undiscovered.

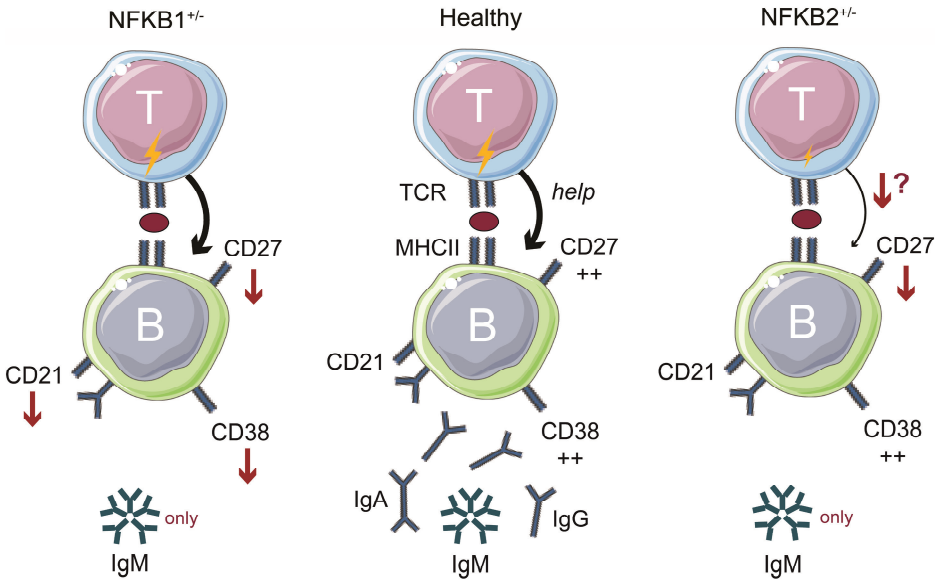


Figure 3. Cellular differences and similarities between NFKB1 and NFKB2 deficiency. Schematic overview of the main effects of both heterozygous pathogenic *NFKB1* (left) and *NFKB2* (right) variants on B and T cell function, as described in this thesis. Immunoglobulins at the bottom indicate secreted IgG, IgA and IgM. TCR: T-cell receptor, MHCII: major histocompatibility complex II. Created by Servier Medical Art.

Alterations in one or the other can disturb the delicate balance between all possible combinations of NF-κB dimers and therefore have an effect on multiple pathways.

Differences can very well be explained by the fact that both have very distinct signaling cascades, activated by their own selective set of ligands, with different roles in B cell activation and differentiation. The canonical NF-κB response is fast, while the non-canonical pathway is slower and is involved in organogenesis besides its role in immunology, which is for instance illustrated by the central adrenal insufficiency in some of the NFKB2-deficient patients or the absence of secondary lymphoid tissue in NIK deficiency[34,38,39].

More detailed molecular experiments are needed to pinpoint how specific pathogenic *NFKB1* variants alter non-canonical NF-κB signaling and vice versa. In general we can conclude that NFKB1 and NFKB2 deficiency are related and can be classified as NF-κB-opathy, yet it remains the question whether they account for distinct disease entities with specific features or do overlap.

Three major challenges in CVID

The field of clinical immunology, and studies on PIDs in particular, has made enormous progress over the last two decades, which was greatly enhanced by the fact that more and more is known about (how to map) the human genome in health and disease. Nevertheless, it leaves us with a couple of challenges in how to interpret the data that becomes available and

how to proceed. The following three major challenges will be discussed, since all have played a substantial role in the chapters in this thesis: (1) how can we explain the (major) genotype-phenotype differences and related, (2) how to handle gene variants in the era of next-generation sequencing, and finally, (3) how to classify CVID best, being the most heterogeneous group of all PIDs, to optimize patient monitoring and treatment.

The complex and variable relation between genotype and phenotype

In the majority of genetic lesions causing an immunodeficiency described in this thesis, we found much variation in the clinical phenotype resulting from exactly the same pathogenic variant between families, but also within families. This clinical phenotype within families showed extremes with both severely affected cases and clinically unaffected cases, while being the same "inborn" PID. As of now, we can only speculate which factors contribute to these clinical differences, ranging from differences in (epi)genetics to the impact of the environment, which may depend on the age of exposure to infectious agents, on food intake, oxidative stress, and several other co-factors changing the impact of a genetic defect on the long-term disease manifestations.

Phenotypic heterogeneity, i.e. pathogenic variants in the same gene causing different phenotypes, can be explained by differences in the effect (e.g. deletions, truncating and missense variants) and the position of the genetic alteration within the gene involved. In NFKB2 deficiency for example, it is clear that pathogenic variants in the C-terminal part of the protein are causing a distinct clinical phenotype, probably explained by the fact that these mutations affect the NIK-responsive domain. Also, the remaining function of the possible alternative protein product transcribed from the affected gene, is important to consider.

Differences between non-related patients (but also related individuals to some extent) with the same genetic variants might as well be explained by a different genetic make-up of the rest of the genome. Genome-wide association studies have shown that variations in certain regions are associated with CVID and/or antibody deficiency, including *MHC*, *TNFRSF13B* (TACI), *EOMES*, *ETS1*, *SOCS1*, *PTPN2* and metalloproteinase (ADAM) genes[40,41]. However, even having a completely identical genetic make-up shows discordance in clinical phenotype, as shown by a study in monozygotic twins[42]. Increased DNA methylation of genes involved in B cell activation such as *PIK3CD*, *BCL2L1*, *RPS6KB2*, *TCF3* and *KCNN4*, was observed in the sibling with CVID compared to the healthy sibling. These findings suggest that epigenetic alterations at least mediate the development of a specific clinical phenotype.

Environmental factors might contribute as well, as some pathogens might trigger an acceleration of clinical symptoms against a certain genetic make-up. In NFKB1 deficiency it has been shown that EBV can cause a considerable threat to develop EBV-driven proliferative disease[31,43]. Furthermore, CMV infection and a hyper-reactive CMV-specific immune response has been associated with inflammatory complications in CVID[44,45]. In this context, it was remarkable that a relative of an NFKB1-deficient index case carrying exactly the same pathogenic variant, who showed no clinical symptoms of NFKB1-deficiency and only slightly abnormal cellular phenotype, was CMV seronegative at the age of 68 years (unpublished data). In line with this, late-onset of disease could partly be explained by the fact that some pathogens become rare in our 'protected' and clean Western society and an 'accidental' encounter with an uncommon pathogen might cause the immediate stress to the already genetically

comprised adaptive immune system, by which the immunodeficiency suddenly becomes clinically apparent.

Lastly, we established that some cases of CVID or CVID-like diseases are progressive over time. In NFKB deficiency we observed a decline in all immune parameters, corresponding with the clinical symptoms that appeared. This is similarly described for heterozygous IKAROS deficiency, another recently characterized CVID-like disorder[46]. This means, that patients clinically unaffected as of now, can develop clinical features of CVID at any point during life, being influenced by various factors including those mentioned above.

How to handle genetic variants in the era of next-generation sequencing

The introduction of next-generation sequencing (NGS) has been a revolutionary development in biological studies in general, with major impact on how we interpret genetic defects in multiple diseases. With NGS it is possible to sequence millions of DNA molecules in parallel as opposed to conventional techniques, with the advantage of having high depth of coverage and of having an unbiased approach[47,48]. As opposed to whole-exome sequencing (WES), WGS covers the entire genome instead of the protein-coding exons only.

As a partner of the NIHR BioResource Rare Diseases consortium we had the privilege to join an immensely powerful study to identify rare diseases by whole genome sequencing, including PIDs as inborn errors of immunity. This highlighted hundreds to thousands of candidate genes, likely to be pathogenic, stratified by newly developed methods[49]. Despite the great potential to identify new pathogenic variants in PID or CVID in particular, the biochemistry and biology to link the genetic variants to cellular and clinical phenotypes is required to prove a causal relation, otherwise we have to judge these genetic findings with great caution to avoid false disease-causing variant calling.

In **Chapter 7** we presented the case of a boy with IPEX syndrome showing the value of functional tests to validate genetic lesions, as he was carrying a hemizygous pathogenic missense variant in *FOXP3* (located at the X chromosome). We observed normal numbers of T_{Reg} cells and FOXP3 protein levels which together with the absence of diabetes made the diagnosis IPEX-syndrome less likely in the first place. However, by extensive studies into the functional capacity of these T_{Reg} cells after a classical disease-causing *FOXP3* variant was revealed by genetic testing, we observed reduced ability of T_{Reg} to suppress other T cells despite normal nuclear translocation of FOXP3. Indeed, in another publication it was predicted that this hemizygous variant affects the DNA-binding affinity only, while leaving the rest of the protein and shuttling to the nucleus unaffected[50].

Proving disease causality can be challenging in variants of unknown significance (VUS), especially when the gene is not yet described to be involved in human disease. This challenge directly follows from the above mentioned differences between genotype and phenotype. Therefore tools are needed to support the hypothesis of a detected VUS being truly causative and in this thesis we showcased some approaches to underpin this causative relationship as such. Careful analysis of the presence and dynamics of the altered protein helps to verify whether the genetic defect is translated or without any effect. In addition, B and T cell phenotyping and functional tests show whether this affects the expected cell type based on what is known about the protein and links the genotype to the clinical phenotype. In familial CVID (5-25% of cases[51]), pedigree analysis can be helpful to verify whether the genetic lesion

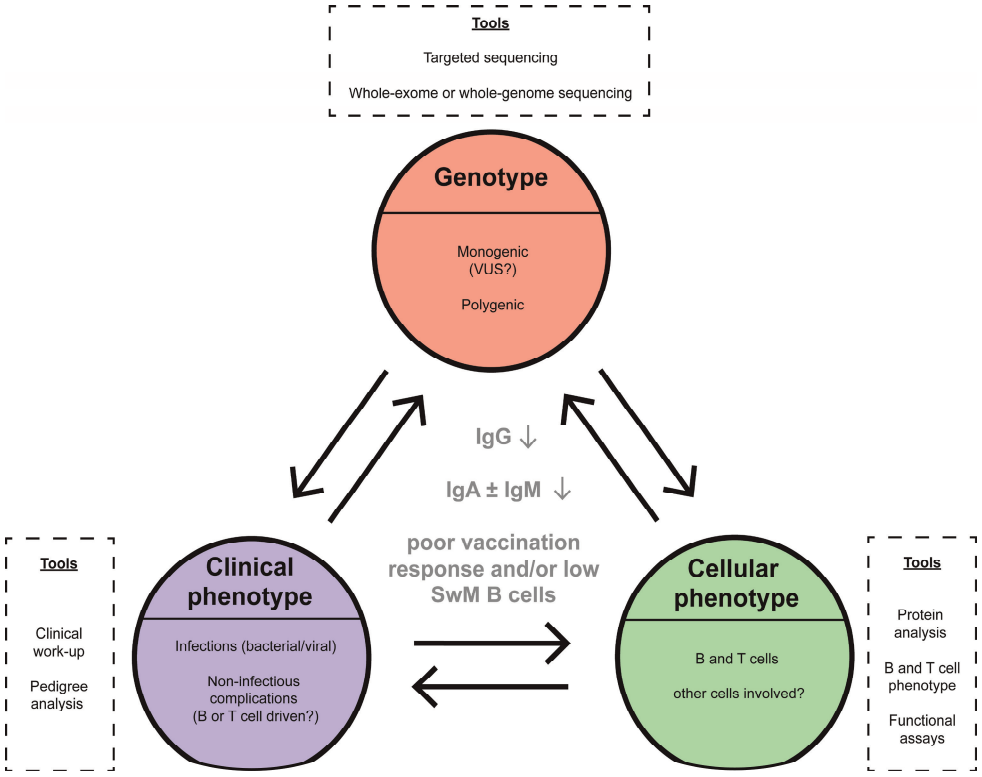


Figure 4. Interpretation of CVID and CVID-like diseases. Methods used in this thesis to characterize patients with potential pathogenic variants that might explain CVID by collecting information on genetics, clinical features and cellular phenotype/function. Arrows symbolize the thorough evaluation of whether findings in one category can be caused or followed by findings in another category. *SwM* switched memory B cells, *VUS* variant of unknown significance.

segregates with the presence of disease, even when not immediately recognized due to incomplete penetrance. In **Figure 4** we summarized all approaches used in this thesis, which might help to assign CVID(-like) patients to a profile that narrows down on the expected course of disease.

Classification of CVID: towards a more molecular and functional approach

In this thesis we described two monogenic CVID-like diseases, previously categorized as being classical CVID. However, current estimates are still that only 10-30% of cases can be explained by single genes[41,52-54]. Although monogenic causes of CVID have been identified in an extreme fast pace in the past decade, the general assumption is that a large part of CVID cases is otherwise polygenic. The clinical heterogeneity as described and the predominantly sporadic nature of this disorder, makes this even more challenging. Nevertheless, it is important to

characterize subgroups of this highly variable group of disorders to (1) reduce diagnostic delay by recognition of specific features by clinicians, (2) stratify patients with the highest probability of finding a monogenic cause by targeted, whole-exome or whole-genome sequencing and (3) initiate the most effective type of treatment as early as possible, in order to reduce morbidity and mortality.

In the past, very solid classifications have been published (Freiburg[55], Paris[56], EUROClass[27], Rotterdam[57]) to categorize CVID patients by cellular phenotype and hereby differentiate in probability of having specific complications. For example, the EUROClass trial from 2008 linked patients with an expansion of $CD21^{low}$ B cells with splenomegaly and an expansion of transitional B cells with lymphadenopathy[27]. These attempts tried to relate B cell phenotype to clinical features, which ultimately segregates between CVID patients with a better or worse prognosis.

Now, almost 10 years later, monogenic causes of CVID showed us that a biological imprint comes with an enrichment of certain clinical features, albeit still very heterogeneous. In **Chapter 5** we observed autoimmune manifestation in 48% of clinically affected cases and a high frequency of (hematologic) malignancies in heterozygous NFKB1 loss-of-function, while defects in BAFF-R, CD20 and CD21 result in CVID with relatively less autoimmune complications, mainly suffering from recurrent infections only[58-60]. Therefore, localizing the molecular, cellular and functional defects can have major implications on prognosis and we would argue in favor of combining previous classifications in a concept where CVID patients are categorized based on the molecular and functional basis of the defects relating to specific aspects of the B cell response. Even with the underlying cause being polygenic, it categorizes patients that might benefit from the same treatment options.

Therapeutic options in development for PIDs

Without discussing the entire spectrum of potential future therapeutic options, we discuss two of the most promising approaches here, as they directly relate to findings in this thesis: specific drug targeting and genetic editing. The most commonly applied therapeutic options currently used, are non-specific and mainly based on clinical presentation, while more insight in the immunopathological mechanism causing immunodeficiency could lead to more specific therapeutics. This could potentially ameliorate the treatment of CVID complicated by non-infectious manifestations not treated by immunoglobulin replacement and antibiotic prophylaxis, the group with the highest morbidity and mortality[61,62].

Specific drug targeting

In **Chapter 8** we screened for unexplored kinases important for plasmablast development by adding a variety of kinase inhibitors to our culture system and thereby identified potential therapeutic targets for auto-antibody mediated autoimmune diseases. This study emphasized the role of mTOR in B cells and hinted towards some interesting leads for further studies. As briefly discussed in the specific chapter on small inhibitor compounds, this assay could very well be of great importance in the context of specific drug targeting in immunodeficiencies, as the effects on B-cell function could be tested *ex vivo* as a kind of 'proof of principle' approach

before being administered to patients. Partial or full restoration of function in this or alternative molecular and functional assays can hold promise for effective therapeutic application *in vivo.*

In activated PI3K-δ syndrome it has already been shown that B cell responses could be modulated by drugs specifically targeting the defective pathway. The p110δ-inhibitor leniolisib restores hyperactive PI3K-AKT-mTOR signaling in B cells *ex vivo* with clear improvements of immune cell phenotype and other clinical parameters[63,64]. One might argue that in immunodeficiencies where loss-of-function is the disease mechanism, it would be more complex to restore function. However, in CTLA-4 haploinsufficiency, treatment with a CTLA4-Ig (abatacept) has been shown to be an effective treatment to dampen autoimmune symptoms [65-67].

Both examples of specific drug targeting are based on understandings of the defective pathway, and therefore the importance of gaining insight in molecular and functional defects could not be emphasized more.

Gene editing

The application of hematopoietic stem cell transplantation (HSCT) in the most severe forms of PID have improved significantly over the years for several reasons, i.e. improvements in HLA-typing, less toxic conditioning regimens, improved supportive care, and better availability of donors and stem cell sources[68]. This facilitated the application of HSCT in other types of PID, i.e. combined immunodeficiency and immune dysregulation syndromes, yet patients should still be carefully selected due to significant transplant-related complications.

During the assembly of this thesis, therapy using genetically modified autologous hematopoietic stem cells has made major steps forward in a select group of PIDs, e.g. X-linked SCID, adenosine deaminase (ADA)-SCID, WASp-deficiency and X-linked chronic granulomatous disease with variable success however[69]. Instead of using retroviral vectors, gene therapy in these diseases with lentiviral vectors have shown very encouraging results regarding the overall clinical outcome[69,70]. In addition, recent developments in genome editing with CRISPR/Cas9 provide novel possibilities to improve the use of gene therapy even further.

The treatment of CVID with HSCT, possibly including gene editing therapy in the select group of monogenic diseases, is more complex and probably needs more time and considerations to be effectively applied. The problem lies in the incomplete penetrance and variable expressivity in monogenic disease, against having end-organ damage, which further complicates treatment in adults with late-onset CVID[71]. Current multicenter experience with HSCT in CVID indicates high overall mortality, yet with beneficial treatment outcomes in the group of surviving patients. Furthermore, it is not clear to which extent the non-hematopoietic contribute to the disease burden and most severe complications, at least in some subtypes or monogenic CVID-like diseases, as for instance in LRBA deficiency[72]. As a treatment for CVID, specific drug targeting might be more realistic to be applied in the near future. Nevertheless, as our insights in the pathophysiology of CVID and other PIDs advances, more and more (specific) therapeutic options will become available.

Future perspectives

Besides the application of genetically editing hematopoietic stem cells before transplantation as discussed above, CRISPR/Cas9 also gives many opportunities in experimental models. Healthy primary B or T cells can be modified to simulate a specific genetic defect and narrow down on whether the variants as seen in patients are really causative using molecular or functional assays as a read-out. Our focus on investigating PIDs for the upcoming years, together with expanding our read-out systems with molecular analysis of signaling pathways, will hopefully help to elucidate some of the aspects of why certain defects in patients result in overt disease and what we should advise clinicians regarding the best therapy of choice to initiate.

The importance of a better understanding of CVID or CVID-like diseases and their prognosis is exemplified by patients asking treating clinicians whether having a specific mutation should affect their family planning. A patient with a certain pathogenic *NFKB1* variant that we now have identified, will ask to which extent the mutation will affect his or her children too, is it heritable? Aside from the fact that we can confirm the latter as we know that the pathogenic variant is inherited in an autosomal dominant manner, we are currently unable to formulate a definite prognosis and by that help with decisive decision-making. Upcoming developments and therapeutic advances will determine whether practitioners might have better answers for these understandable questions in the near future.

Concluding remarks

As the field of research studying PIDs has been growing over the last couple of decades, the next couple of years will not be any different. It remains important for the clinical immunologist to keep in close contact with immunobiologists, molecular biologist and geneticists to keep benefiting from the advances in immunological sciences in general.

The studies in this thesis show the strength of collaboration. Not only our own analyses could use more depth as we proceed. Data on immunodeficiencies presented on conferences and in high impact journals show assays that are relatively basic and could use more advanced techniques for further testing, of course, this is frequently limited by the amount of primary material that can be used. Although the search for novel genes and variants is needed to get the whole (immune) picture more complete, digging deeper into the molecular mechanisms of already described genes and the encoded proteins is of just as much, if not more, value to understand disease and its variability in more detail.

Working on PIDs is for that reason not ‘another study’ on a very rare disease, but rather teaches us how things work in human immunology – both in health and disease - as a highly valuable addition to murine and modified human cell line studies which may only be of limited value for reasons of their species differences and transformed background.

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