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MULTI-AUTHOR REVIEW

New frontiers of primary antibody deficiencies

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Abstract Primary antibody deficiencies (PAD) form the largest group of inherited disorders of the immune system. They are characterized by a marked reduction or absence of serum immunoglobulins (Ig) due to disturbed B cell differentiation and by a poor response to vaccination. PAD can be divided into agammaglobulinemia, Ig class switch recombination deficiencies, and idiopathic hypogammaglobulinemia. Over the past 20 years, defects have been identified in 18 different genes, but in many PAD patients the underlying gene defects have not been found. Diagnosis of known PAD and discovery of new PAD is important for good patient care. In this review, we present the effects of genetic defects in the context of normal B cell differentiation, and we discuss how new technical developments can support understanding and discovering new genetic defects in PAD.

Keywords Primary antibody deficiency · Serum immunoglobulin · Agammaglobulinemia · Immunoglobulin class switch recombination deficiency · Idiopathic hypogammaglobulinemia

Introduction

Primary antibody deficiencies (PAD) form the largest group of inherited disorders of the immune system, i.e., primary immunodeficiencies [1]. They are characterized by a marked reduction or absence of serum immunoglobulins (Ig) and poor response to vaccination. The clinical presentations and the underlying immunopathological causes of PAD are diverse. A division into three categories can be made based on the presence of B-lymphocytes in peripheral blood, on serum Ig levels, and on the type of B-cell defect. Here, we divided PAD into three categories: (1) agammaglobulinemia with defects in precursor B cell differentiation, (2) Ig class switch recombination deficiencies (IgCSR), which were previously called hyper IgM syndromes, and (3) idiopathic hypogammaglobulinemia. In fact, in these categories different stages of B cell differentiation and maturation are affected.

Several genetic defects have been identified in B cell intrinsic genes, but also in genes encoding receptors or ligands expressed on T cells [1]. The genetic defects provided insight into the underlying immunopathological disease mechanisms in various PAD. However, in many cases, especially in the third category of PAD, the underlying mechanism is not (yet) known. Recently, it became clear that modifying genetic factors, the age of the patient, environmental exposures, and other factors also play a role and contribute to the clinical variability of PAD [2, 3].

Patients with a PAD can present either in early childhood or in adulthood with increased susceptibility mainly to bacterial infections that typically involve the upper and lower respiratory tract (otitis, sinusitis, and pneumonia) [4]. Infections might also cause abscesses in the skin or other organs, urinary tract infections, and arthritis. Common infectious agents are *Streptococcus pneumoniae* and *Haemophilus influenzae*, but infections with *Giardia lamblia* are also found [5]. Additionally patients with agammaglobulinemia are susceptible to enteroviral infections. Ig replacement therapy [intravenous Ig (IVIG) or subcutaneous Ig (SCIG)] is

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essential for all PAD patients. Frequently, antibiotic treatment or prophylaxis is necessary to control the number and severity of infections. On top of chronic and recurrent infections, PAD patients can have serious complications such as granulomatous inflammation, autoimmunity, lymphoproliferations, and malignancies. Prognostic factors predicting these clinical complications are scarce.

Diagnostic delays in affected patients with PAD still remain a significant problem due to the variability in clinical features and laboratory findings, but also due to limited awareness [6]. The European Society for Immunodeficiencies (ESID) has designed multi-stage diagnostic protocols for primary immunodeficiency screening, which is an important tool for increasing awareness [7]. However, the diagnostic protocol for PAD does not (yet) take into account abnormalities in peripheral B cell subsets and can be further optimized. In case of a diagnostic delay, PAD can lead to serious morbidity and early mortality. In addition, lack of knowledge of the immunopathological causes and molecular defects hampers accurate diagnosis and appropriate clinical management of patients. This necessitates further investigations and discovery of new genetic defects.

Diagnosing known PAD and discovering new PAD requires understanding of the normal B cell system. In this review, we sketch a historical overview of the identification of genetic defects in PAD. Furthermore, we place the effects of genetic defects in the context of B cell differentiation and discuss technical developments that can lead to novel insights and potentially to the discovery of new genetic defects in PAD.

Identification of genetic defects in PAD over the past 20 years

Over the past 20 years, 18 genetic defects have been identified as underlying PAD (Fig. 1a). The genetic basis of most cases of agammaglobulinemia and Ig CSR deficiency has been unravelled. In contrast, for idiopathic hypogammaglobulinemia, gene defects have only been identified over the past 6 years, and in the majority of patients, a genetic defect has not (yet) been identified. Here, we give a historical overview of the identification of genetic defects and a brief description of the function of the various genes.

X-linked and autosomal recessive agammaglobulinemia

The vast majority of patients with agammaglobulinemia (~90%) are boys with a mutation in the X-linked Bruton's

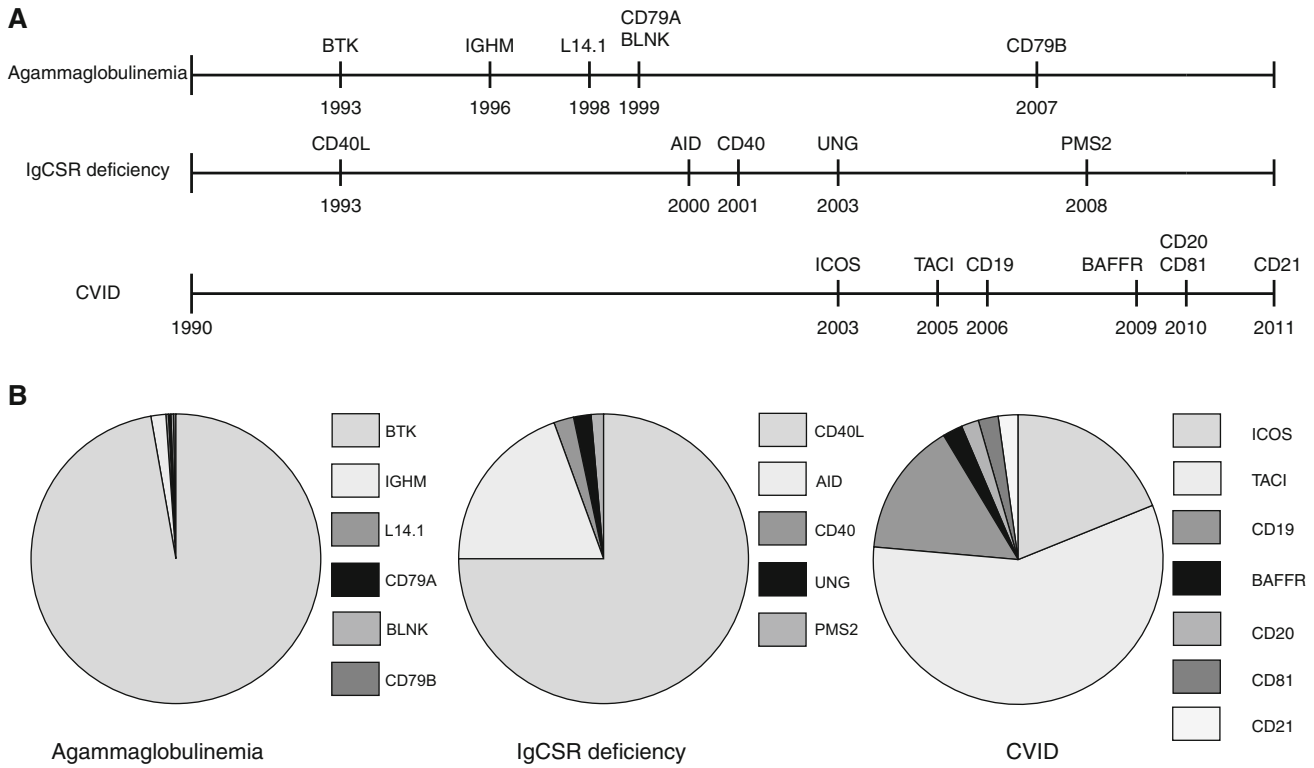

Fig. 1 Historical overview and frequencies of genetic defects in PAD. **a** Identification of genetic defects in agammaglobulinemia, IgCSR deficiencies, and CVID from 1990 to 2010. **b** Frequencies of PAD gene defects in agammaglobulinemia, IgCSR deficiencies, and CVID

tyrosine kinase (*BTK*) gene named after Dr. O.C. Bruton, who described the first agammaglobulinemia patient in 1952 [8]. This 8-year-old boy had recurrent bacterial sepsis and absence of serum immunoglobulins. He was successfully treated with subcutaneous gammaglobulins. When more children with agammaglobulinemia were discovered, it was noticed that boys were predominantly affected and that the disease followed an X-linked pattern of inheritance [9]. The adult onset form of agammaglobulinemia did not show an X-linked inheritance pattern and later became known as common variable immunodeficiency (CVID) [10]. In 1978, it was noted that XLA patients have a defect in bone marrow precursor B cells and consequently lack peripheral B cells [11]. The gene that was linked to this disease, *BTK,* was described in 1993 by two independent groups (Fig. 1a) [12, 13]. In the same period, Btk was also shown to be defective in the murine immunodeficiency model Xid [14, 15]. BTK is a signal transduction molecule downstream of both the pre-B cell receptor (preBCR) and the BCR and is essential for precursor B cell differentiation in bone marrow.

Following the identification of *BTK* mutations, other components of the preBCR signaling complex became candidate genes for autosomal recessive (AR) agammaglobulinemia. The preBCR signaling complex is composed of two identical Igμ chains and the surrogate light chain proteins VpreB and λ14.1 together with the anchoring molecules CD79a and CD79b. In 1996, the first genetic defects were indeed identified in AR agammaglobulinemia in the Igμ heavy chain (*IGHM*) [16]. To date, 26 families with mutations in the μ heavy chain have been reported (summarized by Conley et al.) [2]. Half of these mutations concerned gross deletions ranging from 70 to over 700 kb, including the V, D, and J regions [17]. The frequency of gross deletions is generally only 6% of all genetic disruptions underlying human disease [18]. Detailed sequence analysis of the deletion breakpoints revealed that most *IGH* deletion breakpoints were located in or near sequences derived from transposable elements (TEs) [17]. These TEs appeared to be specifically overrepresented in the *IGH* gene as compared to the average in the human genome. The increased frequency of gross deletions in a specific gene was shown to result from a high total TE content [17].

In the following years, two other genetic defects affecting preBCR expression were described, i.e., in λ14.1 and CD79a [19, 20]. In addition, a mutation was described in BLNK, which is a signaling molecule downstream of the preBCR [21]. It was not until 2007 that the first mutation in CD79b was described [22]. Genetic defects identified in agammaglobulinemia patients all affect preBCR expression or downstream signaling, which results in a block in precursor B cell differentiation.

IgCSR deficiency

IgCSR deficiencies were previously called hyper IgM syndromes because the patients are generally characterized by increased levels of serum IgM in combination with reduced levels of IgG and IgA. However, patients with a IgCSR defect can also have a normal serum IgM level. Therefore, the term IgCSR deficiency has been introduced in the WHO classification [1].

In 1993, the first genetic defect in patients with an IgCSR deficiency was identified in the X-linked CD40L gene (Fig. 1a) [23]. Eight years later, in 2001, a mutation in the receptor of CD40L, CD40, was identified [24]. CD40-CD40L interaction plays an important role in T-cell-dependent B cell proliferation and differentiation and in the induction of CSR and somatic hyper mutations (SHM) (see below). However, CD40 triggering also plays a central role in T-cell-mediated activation of monocytes and dendritic cells. In fact, these two genetic defects represent CD40-CD40L interaction defects and therefore do not exclusively belong to the category of PAD. In contrast, IgCSR deficiencies due to intrinsic B-cell defects have officially been classified as PAD. In 2000, homozygosity mapping in eight consanguineous families with patients with a hyper IgM syndrome pointed to a genomic region harboring the *AID* gene [25]. From mouse studies it was known that AID expression is strictly restricted to B cells and induces CSR and SHM, therefore this gene was sequenced in these families and was found to be mutated. This made *AID* the first candidate gene for autosomal recessive B-cell-intrinsic IgCSR deficiencies. In 2003, the group of Durandy described a second candidate gene [26]. They defined a new phenotype of the "hyper IgM syndrome" patients that was characterized by impairment of CSR and a partial disturbance of the SHM pattern. As this phenotype resembled the phenotype of *Ung*-deficient mice [27], the possibility of UNG deficiency in these patients was explored, and mutations were indeed identified. In 2008, they described—in patients with an IgCSR defect without a disturbed SHM process—mutations in the *PMS2* gene [28], which is a component of the mismatch repair system known to play a role in CSR [29]. There is still a group of patients with a defined IgCSR defect in combination with a normal SHM frequency in whom the genetic defect has not yet been unravelled (reviewed by Kracker et al.) [30].

Idiopathic hypogammaglobulinemia

Idiopathic hypogammaglobulinemia is the largest category of symptomatic PADs and includes patients with possible or probable CVID according to the ESID-PAGID criteria (http://www.esid.org) [31]. CVID is characterized by enormous clinical and immunophenotypical heterogeneity

[32]. In contrast to the other PAD categories, of which in the majority a genetic defect can be identified, in >95% of CVID patients the genetic defect is not (yet) identified. In fact, CVID is a diagnosis *per exclusionem*. The immunophenotype of B cell subsets was used in several classifications, which aimed to make correlations between the immunophenotype and the clinical presentation of subgroups of CVID patients [33–36]. However, these studies have not yet resulted in the identification of new genetic defects in the CVID subgroups.

The first genetic defect in patients with CVID was identified by Grimbacher et al. and concerned a homozygous deletion of exons 2 and 3 of the "inducible costimulator" or *ICOS* gene (Fig. 1a) [37]. Initially, the same mutation had been reported in nine patients from four families, indicating a founder effect in these families [37–39]. In 2009, a second *ICOS* mutation was identified in two Japanese siblings [40]. ICOS is expressed on activated T cells and interacts with ICOSL on B cells and dendritic cells [41]. ICOS-ICOSL is important for T-B-co-activation, CD40-mediated CSR, secretion of cytokines, and development of a Th2 immune response [40, 42, 43].

In 2005, mutations in *TACI* (transmembrane activator and CAML interactor) were identified in patients with CVID and IgA deficiency by two independent groups [44, 45]. TACI belongs together with BAFF-R and BCMA to the TNF receptor superfamily, and interaction with the ligands BAFF and APRIL is crucial for development and maintenance of humoral immune response [45]. Heterozygous *TACI* mutations result in increased disease susceptibility, but are not likely to be disease causing, because these heterozygous mutations are also found in healthy individuals [46]. In 2009, a homozygous *BAFF-R* mutation was reported in two siblings with reduced serum IgM and IgG levels but with normal IgA concentrations [47]. The deficiency was identified by screening the CVID cohort for individuals with potential defects in genes regulating B cell survival and homeostasis. Only one of these patients had recurrent infections, which indicates that a BAFF-R deficiency does not always result in a clinically manifest immunodeficiency [47].

Another category of CVID concerns deficiencies of the CD19 complex. This complex, consisting of CD19, CD21, CD81, and CD225, reduces the threshold for antigen-dependent stimulation via the B cell receptor. In 2006, we described the first genetic defects in the *CD19* gene, which illustrated that a defect in the CD19 complex gives rise to antibody deficiencies [48]. In the following years, a total of seven different mutations were described in nine patients [48–51]. In 2010, we identified a mutation in the *CD81* gene [52]. CD81 is essential for CD19 expression, but for the other two complex members it is not known whether they are as essential for CD19 expression as CD81. This year, the first human CD21 deficiency has been reported [134].

CD20 was one of the first B-cell-specific B cell differentiation antigens, and in 2010 the first mutation in this gene was described [53, 54]. A CD20 deficiency results in an impaired T-cell-independent (TI) antibody response [54].

In summary, the identified genetic defects in CVID affect different steps or processes of B cell differentiation (Fig. 2). This illustrates that on top of clinical and immunophenotypical heterogeneity, the underlying immunopathological mechanisms and genetic defects of CVID are heterogeneous as well. We propose to classify patients with an identified gene defect no longer as CVID, but rather classify them according to the affected B cell differentiation pathway or process as a defined entity.

Frequencies of PAD gene defects

Information about the identified mutations in PAD genes is available on various websites. In Fig. 1b, we summarized the relative frequencies of the identified gene defects based on the human Gene Mutation Database (HGMD Professional 2010.2) [55]. Making an estimation of PAD patients with an unknown genetic cause is more complicated. The frequency of agammaglobulinemia of unknown cause is estimated to be 10–15% [2], for IgCSR it is 15% [30], and for CVID the frequency is estimated to be >95%.

Positioning PAD gene defects in the context of B cell differentiation

To understand the consequences of PAD gene defects, it is important to find out which crucial processes are disturbed. All PAD gene defects somehow impair B cell differentiation (Fig. 2), and especially the genes affected in the CVID category are involved in many different processes. Therefore, we will discuss important pathways, the effects of identified gene defects on these pathways, and likely new candidate genes. Finally, we will present how new (technical) developments can support the identification of new defects.

B cell commitment

The first critical step in B cell differentiation is the commitment of a hematopoietic stem cell (HSC) in bone marrow to the B cell lineage through several consecutive steps (Fig. 3) [56]. HSCs are long-lived and self-renewing cells that generate multilineage progenitor cells (MLPs), which have the potential to develop into myeloid or erythroid cells via a common myeloid progenitor (CMP) or into lymphoid cells via the common lymphoid progenitor

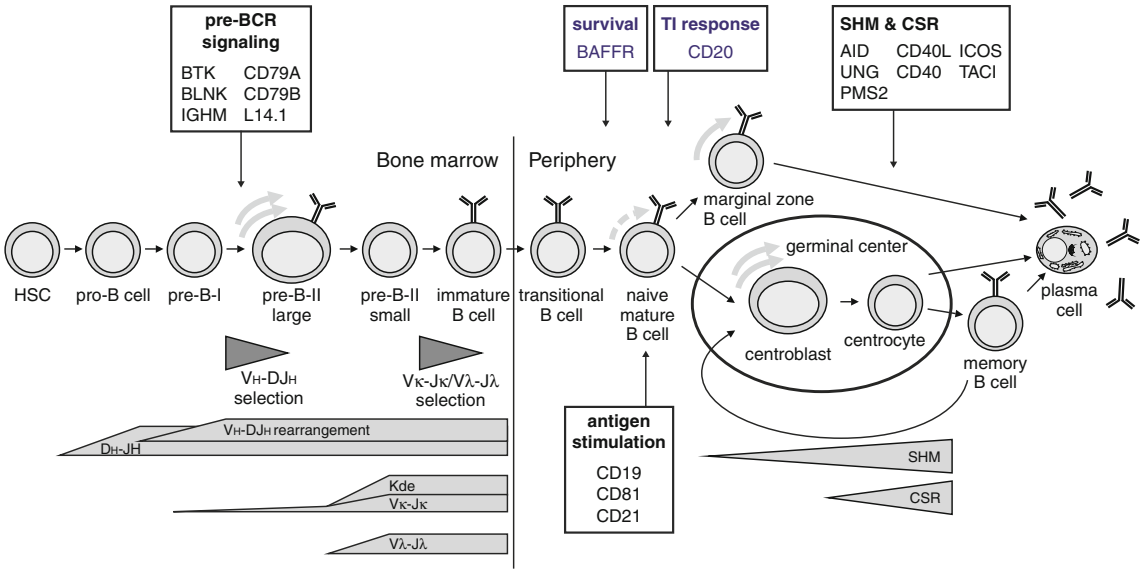

Fig. 2 B cell differentiation. Molecular processes during the stepwise differentiation of B cells from hematopoietic stem cells (HSC) to memory B cells and plasma cells. The Ig gene rearrangements and the selection of their functionality in the bone marrow compartment are followed by antigen-induced proliferation and selection processes in the periphery. The identified PAD gene defects and the impaired differentiation steps are indicated in *boxes*

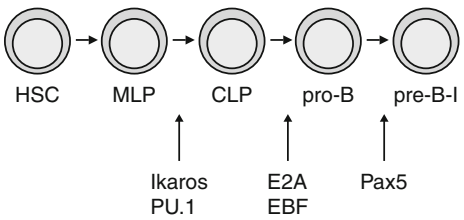

Fig. 3 B cell commitment. Schematic overview of differentiation and commitment from hematopoietic stem cells (HSC) to the B cell lineage and the role of the transcription factors Ikaros, PU.1, E2A, EBF, and PAX5. For details, see text

(CLP) [57]. The lymphoid restriction that occurs at the MLP-CLP transition is the first step towards B cell commitment and is directed by the transcription factors Ikaros and Purine box factor 1 (PU.1) (Fig. 3) [58–60]. Subsequently, the transcription factors E-box binding protein 2A (E2A) and early B-cell factor-1 (EBF1) direct specification of CLPs into pro-B cells [61–63]. Although these first steps towards B cell commitment are already induced at an early stage, the progenitor cells retain lineage plasticity: the myeloid potential is not lost until the pro-B-cell stage. In pro-B cells, E47, one of the splice variants of E2A, and EBF regulate expression of multiple B-cell-specific genes, including components of the pre-B cell receptor (pre-BCR) and the transcription factor Pax5 [64]. Pax5 is the commitment factor that activates B-cell-specific genes and represses genes associated with other lineages [65]. In doing so, Pax5 expression commits the cells to the B cell lineage and enables differentiation of pro-B into pre-B-I cells.

Despite their crucial role in B-lineage specification and commitment, no genetic defects have been reported in one of the above-mentioned transcription factors in patients with antibody deficiencies. Defects in these genes are typically expected in patients with agammaglobulinemia, who completely lack bone marrow precursor B cells and consequently peripheral B cells and serum Ig [66, 67]. However, through the use of mouse models, it has been shown that genetic knock-outs for transcription factors involved in B cell commitment generally exhibit a more severe phenotype in which it is not only B cell commitment that is affected (Table 1). Although mouse data should be interpreted with care and cannot always be directly extended to humans, they give at least some direction. Hypomorphic mutations with residual activity can never be excluded, and therefore testing for defects in these genes remains valid.

The frequency of agammaglobulinemia patients with a complete lack of precursor B cells is low (<2% of all agammaglobulinemia patients), and so far no genetic defect has been identified in these patients. This could imply that in these patients other B cell commitment factors are mutated, which might not even be known to be involved in this process. Alternatively, another pathway can be affected in these patients. Flow cytometric analysis of the precursor B cell compartment in bone marrow is an important tool to discriminate the rare agammaglobulinemia patients without precursor B cells from those with precursor B cells, who have a block later in differentiation (see below). A pitfall in this analysis is that the lymphoid

Table 1 Phenotype of knock-out mice deficient in genes involved in early B cell differentiation

Gene	Knock-out mouse phenotype	Reference
IKAROS	Homozygous mutants lack T and B lymphocytes, NK cells, and their progenitors. Erythroid and myeloid lineages are intact. Heterozygotes for one allele exhibit dominant negative effects and mice develop lymphoproliferative disorders.	[58, 124]
PU.1	Homozygous mutant embryos die at late gestational stage. Multilineage defect in the generation of progenitors for B and T lymphocytes, monocytes, and granulocytes.	[125]
EBF	EBF-deficient mice lack B cells that have rearranged their immunoglobulin DH and JH gene segments. Various non-lymphoid tissues that express EBF are apparently normal. Mutants are smaller than normal, and many die prior to 4 weeks of age.	[126]
E2A	Homozygotes for targeted null mutations are devoid of B cells due to an early differentiation block. They have a partial early block in T-lymphocyte development and show high postnatal mortality. Granulocyte, macrophage, and erythroid lineages are intact.	[127]
PAX5	Null mutants exhibit impaired development of the midbrain resulting in a reduced inferior colliculus and an altered cerebellar folial pattern, failure of B cell differentiation, and high postnatal mortality with few survivors.	[128]
SYK	Homozygous null mice have high rates of postnatal lethality and exhibit developmental defects of B cells.	[129]
LYN	Homozygotes for targeted null mutations exhibit splenomegaly, reduced numbers of peripheral B cells, impaired immune responses, IgM hyperglobulinemia, and autoimmunity with glomerulonephritis.	[130]
PLCγ2	PLCγ2-deficient mice have a partial block in B cell development at the transitional B cell stage and show reduced Ab response.	[131]
VpreB	Homozygous null mutants have fewer cells with functional pre-B cell receptors. Double knockouts homozygous for null mutations at Vpreb1 and Vpreb2 show impaired B cell development. Fewer B cells are found in bone marrow, spleen, and peritoneum.	[79, 80]
PI3 K	Homozygotes for a targeted null mutation exhibit perinatal lethality associated with hepatic necrosis, chylous ascites, enlarged muscle fibers, calcification of cardiac tissue, and hypoglycemia. Mutants lacking only the major isoform are immunodeficient.	[132, 133]

compartment in bone marrow can be suppressed by the myeloid compartment in case of severe infections. In addition, immunosuppressive therapy can severely influence the size and composition of the precursor B cell compartment. Therefore, it is recommended to repeat flow cytometric analysis to confirm the complete absence of precursor B cells. To identify a genetic defect in patients without bone marrow precursor B cells, genetic approaches such as homozygosity mapping via SNP arrays in patients from consanguineous families, linkage studies, or genome-wide sequencing could be applied.

Ig gene rearrangements

As soon as a precursor B cell has been generated from an HSC, it undergoes further antigen-independent differentiation in the bone marrow (Fig. 2). The main objective of precursor B cell differentiation is to create a unique B cell antigen receptor (BCR), which is composed of two immunoglobulin (Ig) heavy chains (IgH) and two Ig light chains (Igκ or Igλ). A highly broad and diverse BCR repertoire is necessary to recognize all different pathogens. Generation of such a repertoire is achieved by V(D)J recombination in the Ig genes [68].

Ig gene rearrangements are initiated in pro-B cells at the *IGH* locus with DH to JH rearrangements, followed by VH to DHJH rearrangements at the pre-B-I cell stage (Fig. 2) [68]. During the rearrangement process, nucleotides are randomly deleted and inserted in the coupling sites of VH, DH, and JH gene segments resulting in unique junctions. Ig light chain rearrangements (*IGK* and *IGL*) are initiated following completion of *IGH* gene rearrangements in the pre-B-II small cell stage (Fig. 2) [68].

The effect of defects in the V(D)J recombination process is generally not restricted to the B-lineage because T cell receptors are rearranged in a similar fashion in differentiating T cells in the thymus. V(D)J recombination defects are therefore typically found to underlie T-B-severe combined immunodeficiency (T-B-SCID). Patients with T-B-SCID present with opportunistic infections, growth delay, and failure to thrive already during the first months of life [1, 69–71]. However, recently it became clear that V(D)J recombination defects can result in a milder clinical presentation due to hypomorphic mutations, and in these cases agammaglobulinemia is one of the presenting features [72, 135, 136]. Therefore, in patients with agammaglobulinemia and unexplained progression of the disease, mild V(D)J recombination defects need to be considered.

PreBCR signaling

Upon formation of a functional *IGH* gene rearrangement, an Igμ chain is expressed as a preBCR together with surrogate light chain proteins VpreB and λ14, and the

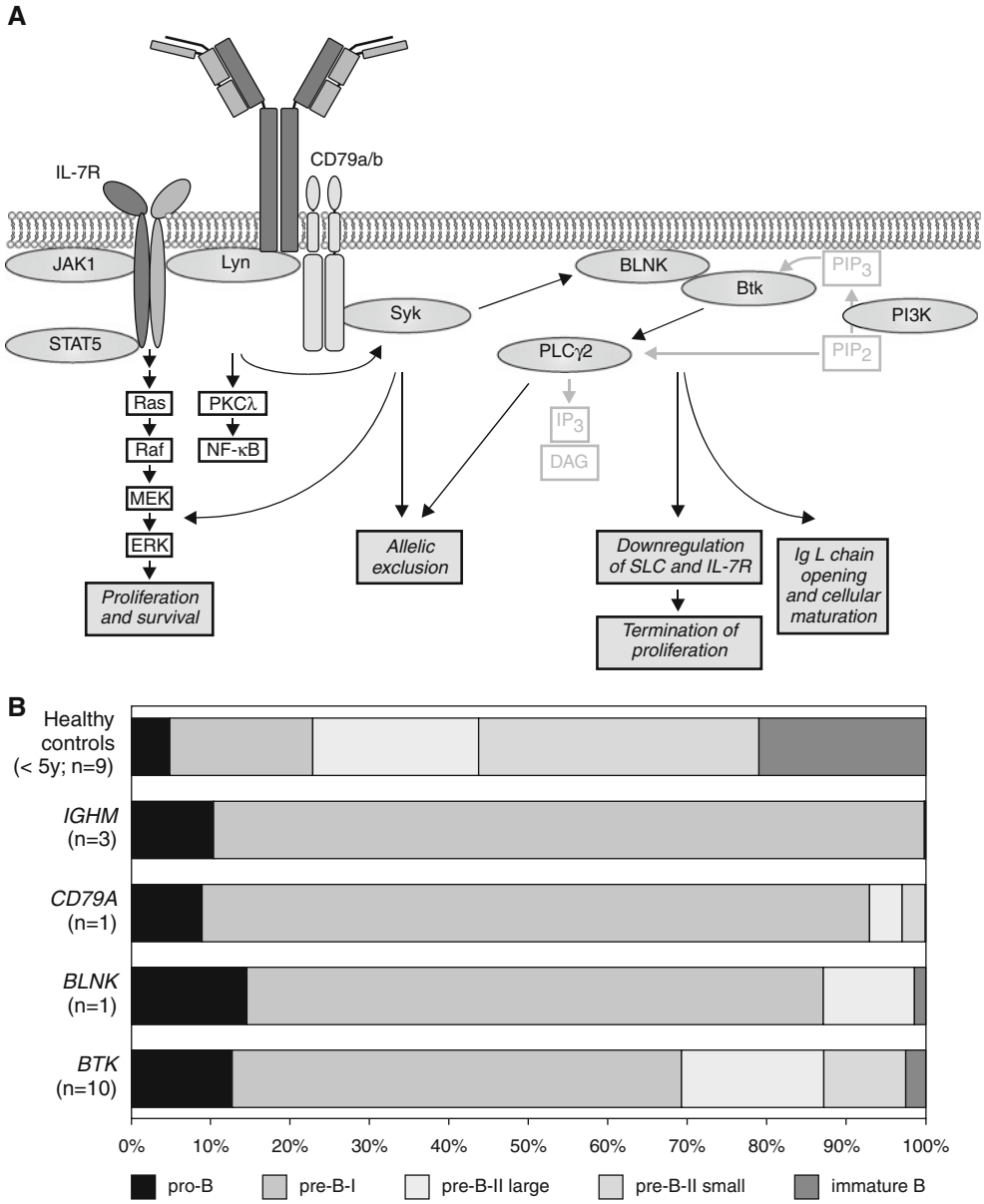

Fig. 4a, b preBCR signaling and precursor B cell differentiation blocks in agammaglobulinemia. **a** Schematic overview of factors and downstream processes regulated by preBCR signaling. The preBCR and the IL7R signal via Lyn, Stat5, and the Ras-Raf-MEK-ERK pathway for proliferation. Signaling of the preBCR via Syk is required for allelic exclusion. Furthermore, the preBCR signals via BLNK and Btk to limit proliferation and induce Ig light chain rearrangements. Figure adapted from Hendriks and Middendorp [73]. **b** Composition of the bone marrow precursor-B cell compartment in healthy children ($n = 9$; <5 years) and in agammaglobulinemia patients with genetic defects in *IGHM* ($n = 3$), *CD79A* ($n = 1$), *BLNK* ($n = 1$), and *BTK* ($n = 10$)

anchoring molecules CD79a and CD79b (Fig. 4a). The expression of a functional preBCR is crucial for further precursor B cell differentiation, through initiation of several events.

PreBCR cross-linking initiates phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmatic domains of CD79a and CD79b [73]. Syk is subsequently activated by the phosphorylated ITAM motifs (Fig. 4a). Activated Syk phosphorylates multiple tyrosine residues in BLNK, which then recruits BTK, which becomes phosphorylated by Lyn, Syk, or both [74]. PLCγ2 is phosphorylated by BTK and hydrolyzes PIP_2 to IP_3 and diacylglycerol (DAG), which causes calcium mobilization and activation of calcium-dependent enzymes.

The first event regulated by preBCR signaling is induction of proliferation and survival via activation of the Ras-Raf-MEK-ERK pathway, which is mediated by Lyn recruitment (Fig. 4a) [75, 76]. In addition, preBCR signaling ensures downregulation of RAG1 and RAG2, thereby inhibiting further *IGH* rearrangements.

Additionally, the *IGH* locus becomes inaccessible for the recombination machinery, a process referred to as allelic exclusion [77]. Allelic exclusion is established through Syk and PLCγ2 signaling, which demonstrates the existence of two separate signaling pathways, one mediating proliferation and the other allelic exclusion. This proliferation or clonal expansion phase is followed by cell cycle arrest in the G1 phase, during which the surrogate light chain is downregulated and the rearrangement process is continued at the Ig light chain loci (*IGK* followed by *IGL* in small pre-B-II cells). This process is regulated by BLNK and BTK signaling [73].

In most patients with X-linked and autosomal recessive agammaglobulinemia, a component of the preBCR is mutated, thereby resulting in a block in precursor B cell differentiation at the pre-B-I cell stage. The stringency of the differentiation block seems to be dependent on the genetic defect (Fig. 4b) [78]. *IGHM* gene defects result in a complete block with absence of pre-B-II and immature B cells. A defect in CD79a expression also results in a severe block, but since it is not complete, it is referred to as "leaky" based on the presence of low numbers of pre-B-II cells. A similar block in precursor B cell differentiation can be expected for a CD79b deficiency. A mutation in the *BLNK* gene also results in a leaky block with the presence of low numbers of pre-B-II and even immature B cells. The differentiation block in XLA patients appears to be the least strict with on average 30% of precursor-B cells being pre-B-II or immature B cells. These data show that the differentiation block is more complete when the defect is more upstream in the preBCR signaling cascade (Fig. 4a, b). This can probably be explained by redundancy in downstream components of the preBCR signaling pathway. Despite the variable leakiness of the differentiation block at the pre-B cell stage, agammaglobulinemia patients have (virtually) no transitional B cells and naïve mature B cells in their blood.

Flow cytometric analysis of the bone marrow precursor B cell compartment has added value for the diagnostic process of autosomal recessive agammaglobulinemia. On one hand, protein expression of the preBCR components and downstream molecules can directly be measured by flow cytometry. On the other hand, the composition of the precursor B cell compartment also guides selection of the right candidate gene. However, not in all patients with a block in precursor B cell differentiation a genetic defect has been identified. VpreB is one of the likely candidate genes, and a VpreB deficiency is expected to have a similar effect as the λ14.1 deficiency [79, 80]. Knock-out mouse models with defects in other signaling molecules generally exhibit more extensive problems beyond the B cell lineage (Table 1). Hypomorphic mutations in these genes that result in expression of proteins with residual activity might give rise to a milder phenotype and can still be considered as candidate genes in agammaglobulinemia patients. In summary, the genetic defects identified in agammaglobulinemia all interfere with preBCR signaling, but other processes might be impaired in currently unsolved agammaglobulinemia patients.

BCR selection and tolerance induction

The BCR repertoire is formed during precursor B cell differentiation by V(D)J recombination. This random process also leads to the generation of large numbers of autoreactive B cells. In particular, long stretches of complimentarity determining region (CDR) 3 with positively charged amino acids are associated with autoreactivity or poly-reactivity. Selection against autoreactive BCRs is essential for preventing autoimmunity [81, 82].

Selection against autoreactive BCRs is achieved by tolerance induction through one of three mechanisms: deletion, receptor editing, and anergy [83]. Clonal deletion of autoreactive B cells is based on removal of autoreactive B cells via apoptosis, whereas receptor editing describes the process through which a B cell modifies its BCR by secondary rearrangements of Ig light chain genes. In contrast to the first two mechanisms, anergy induction does not remove autoreactive B cell clones but renders them unresponsive to antigenic stimulation. Receptor editing is the dominant B cell tolerance mechanism towards membrane-bound antigens, and when it fails, clonal deletion by apoptosis is initiated [84]. Anergy is induced in B cells that have moderate autoreactivity towards soluble antigens [84]. Tolerance induction through each one of these three mechanisms is dependent on intact BCR signaling.

Analysis of the low number of peripheral B cells in BTK-deficient patients, who are defective in BCR signaling, indeed showed that their BCR repertoire is characterized by extensive secondary rearrangements and enrichment of autoreactive clones with long CDR3 regions with positively charged amino acids [85]. These B cells might even be selected to express such autoreactive antibodies because in the absence of BCR signaling they would be unable to survive [85]. This indicates that in the absence of appropriate BCR signaling, the counter selection for autoreactive B cells does not function properly. Consequently, aberrant BCR signaling might predispose patients to autoimmunity. About 20% of CVID patients are affected by autoimmunity [32, 86], and in these patients, BCR signaling might be disturbed as well.

These studies nicely illustrate that a better understanding of fundamental processes during B cell differentiation can be obtained through detailed analysis of patients with a defined genetic defect. For the near future, we wish to stress the importance of new insights into the diversity of the Ig repertoire. Thus far, Ig repertoire studies have been

based on PCR analysis followed by spectratyping [87] or sequencing of individual clones. These approaches appear to be informative but not sufficiently sensitive to provide insight into the diversity and broadness of the Ig repertoire of an individual. Recent developments in next generation or high-throughput pyrosequencing allow analysis of large numbers of sequences (tens of thousands of sequences per patient) in a single experiment [88]. This technology can be further developed and has the potential to demonstrate limited or restricted Ig repertoire diversity in PAD or other immunological disorders [89]. A limited Ig repertoire could be specifically expected in PAD patients with unknown genetic defects, who show a poor response to vaccination. Therefore, we anticipate that analysis of Ig repertoire diversity by next generation sequencing will become a new tool in research on pathological mechanisms of PAD.

B cell activation

Transitional B cells are the earliest bone-marrow emigrants in the periphery (Fig. 2). They are immature in their migration capacity and response to antigen and develop rapidly into naïve mature B cells, which form the majority of B lymphocytes in peripheral blood. Naïve mature B cells are short-lived, and they critically depend on BAFF–BAFF-R interaction for survival [90, 91]. In BAFF-R-deficient patients, the number of peripheral B cells was indeed reduced more than fourfold, and the differentiation block of most B cells was at the transitional B cell stage [47]. Naïve mature B cells undergo an immune response upon recognition of antigen with their specific BCR.

The response of a B cell upon antigen recognition depends greatly on the strength of the interaction and the presence of co-stimulatory signals. Upon antigen binding, the BCR complex translocates to glycolipid and cholesterol-rich membrane microdomains ("lipid rafts"). The B cell co-receptor complex, consisting of CD19, CD21, CD81, and CD225 signals in conjunction with the BCR and prolongs the association of the BCR with lipid rafts [92], thereby reducing the threshold for antigen-dependent stimulation [93, 94]. Downstream signaling is mediated by the tyrosine residues in the cytoplasmic tail of CD19. Co-ligation of the BCR-CD19 complex can occur by complement-tagged antigens that are recognized by the BCR and CD21, which is a receptor for the C3d component of complement (Fig. 5). It remains unclear whether co-ligation via CD21 is always required for CD19-dependent co-signaling. Co-localization of CD19 and the BCR might be achieved via different mechanisms, especially in membrane-bound antigen recognition [95].

In man, genetic defects in the *CD19* or *CD81* genes have been shown to result in an antibody deficiency syndrome [48, 49, 52, 96]. B cells from CD19-deficient and CD81-deficient patients have impaired Ca^{2+} influx upon stimulation of the BCR [48, 52]. Consequently, these patients are hypogammaglobulinemic, have decreased memory-B cell numbers and impaired Ig responses to vaccinations. B cells from CD19-deficient patients have normal expression levels of CD225 and CD81, but decreased CD21 expression [48]. CD81-deficient B cells also have normal CD225 expression, but completely lack CD19 expression and have near-normal levels of CD21 [52]. Thus CD19 expression critically depends on CD81, whereas CD21 only partially depends on CD19.

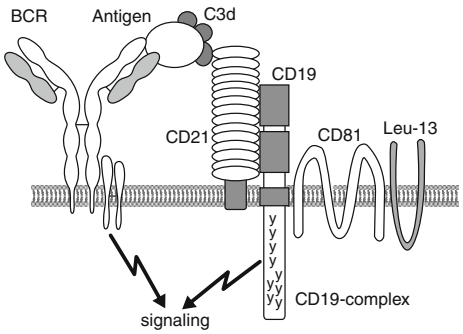

Fig. 5 Signaling via BCR and the CD19 complex. The B cell co-receptor complex consists of CD19, CD21, CD81, and CD225 and signals in conjunction with the BCR, thereby reducing the threshold for antigen-dependent stimulation

A recent study identified a BCR-signaling defect in a subgroup of CVID patients [97]. These so-called type 1a patients have increased frequencies of B cells with low CD21 expression. Specifically these CD21-low B cells demonstrated an impaired Ca^{2+} flux upon stimulation with anti-IgM [97]. The lack of CD21 could be a major factor in making these cells unresponsive to BCR stimulation. However, the CD21-low B cells also have increased amounts of many ITIM receptors including CD22, FcR2B/CD32, and CD72, which could further dampen the activation of these B cells [98, 99].

Thus, the CD19 receptor complex is required for proper BCR-mediated activation of B cells. Defects in CD81 and CD19 severely impair this signaling, whereas it is unknown whether CD225 is critical in this complex. CD21 appears important for optimal BCR signalling in response to low antigen concentrations. As a consequence, the clinical phenotype of CD21 deficiency is less severe than that of the CD19- and CD81-deficient patients and the CD21-deficient patient is able to mount specific antibody responses upon vaccinations in vivo [134].

T-cell-dependent response

The response to antigens depends greatly on the strength of the interaction and the presence of co-stimulatory signals (CD40-CD40L and ICOS-ICOSL, Fig. 6). Most B cell

responses are mediated with cognate CD4+ T cell help [93, 94, 100]. Upon cognate B-T interaction, activated B cells undergo extensive proliferation and form germinal centers (GCs) in the secondary lymphoid organs, i.e., germinal center reaction. In these GCs, dark and light zones can be identified. The dark zone mainly consists of proliferating B cells (centroblasts) in a network of follicular dendritic cells. These cells are of stromal origin and present complete, unprocessed antigen via Fc and complement receptors to stimulate proliferation and survival of antigen-specific B cells. The proliferating centroblasts induce somatic hypermutation (SHM) in their Ig genes, which changes their affinity for antigen (Fig. 2) [101]. The centroblasts become resting centrocytes and can undergo Ig class switch recombination (CSR) and selection based on high affinity for antigen in the light zone of the GC [102]. The CD40-CD40L signaling induces translocation of NF-κB to the nucleus, where NF-κB activates transcription of target genes, including AID and UNG, which are involved in CSR and SHM (Fig. 6) [103].

Three successive steps are required in the molecular process of CSR and SHM, i.e., transcription of the targeted DNA, DNA lesion and cleavage by AID and UNG, and DNA repair [30]. The first steps are shared by CSR and SHM, but DNA repair differs for both events [102]. CSR is dependent on nonhomologous end joining (NHEJ) of DNA double-strand breaks, whereas SHM is dependent on error-prone polymerases and mismatch repair enzymes (MMR) [104, 105]. The MMR protein PMS2 is implicated in CSR [28]. Several gene defects have been reported in IgCSR deficiencies, but not all defects affect CSR and SHM to the same extent (Fig. 2). Mutations in CD40, CD40L, and AID affect both CSR and SHM, whereas UNG mutations cause a CSR defect and result in a skewed pattern of SHM. PMS2 mutations only cause a CSR defect but do not influence the SHM process. Recently, TACI has been shown to trigger CSR through the adaptor molecular MyD88 [106]. As indicated in the previous section, it is not in every IgCSR-deficient patient that the gene defect has been unravelled, but detailed analysis of the various steps in CSR in these patients indicates that in some the defect is located downstream from the DNA lesion and cleavage step, which is suggestive for a DNA repair defect [103, 107].

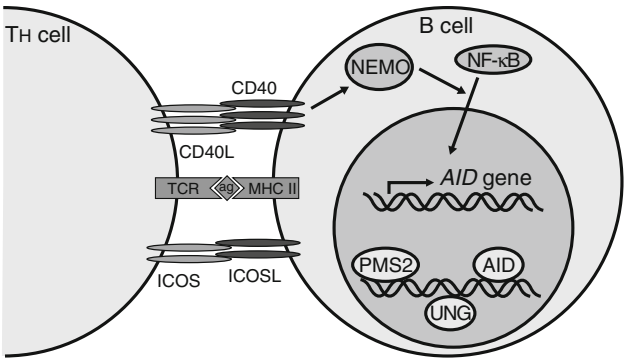

Fig. 6 Induction of CSR and SHM by TH cell–B cell interaction in germinal centers. Upon CD40-CD40L interaction, NEMO supports translocation of NF-κB to the nucleus, where it activates *AID* gene transcription. AID introduces single-strand DNA lesions in Ig genes, which can result in CSR or SHM when repaired by error-prone mechanisms involving UNG and PMS2

Defects in SHM or CSR are not only expected in IgCSR deficiencies but also in (a subgroup of) CVID patients. These processes are crucial for an appropriate antibody response and might therefore be impaired in CVID. New technologies have become available to analyze SHM at the cDNA as well as at the DNA level. These assays range from rapid tests that are based on determining the mutation frequency of a hotspot motif to detailed analysis of SHM patterns at the single nucleotide level [108–111]. For analysis of CSR, several techniques are used to identify the affected step of CSR (see work of Durandy and coworkers) [103, 107]. In addition, analysis of switch junctions gives detailed information of the CSR process [112].

Finally, proliferation is another crucial process for the generation of a B cell pool and an appropriate antibody response. To study this process, we developed a kappa-deleting recombination excision circles (KREC) assay to quantify the replication history of B-lymphocyte subsets in vivo. Analyzing the replication history of B lymphocytes gives insight into potential defects in homeostatic or antigen-induced proliferation, which might be the case in some CVID or other PAD patients. It can be anticipated that systematically analyzing SHM, CSR, and proliferation history, preferably in sorted peripheral B cell subsets, will contribute to defining immunological defects in (subgroups of) CVID patients.

T-cell-independent response

B cell responses can also occur independently of T cell help in the marginal zone of the spleen or in the lamina propria in the gut [113, 114]. These B cells can be sufficiently activated by the repetitive nature of antigens recognized on blood-borne pathogens [115]. Alternatively, these B cells recognize antigens on pathogens, which also stimulate other receptors of the B cell, such as Toll-like receptors [116]. Marginal zone B cells can be found recirculating in peripheral blood (defined as "natural effector B cells"), have a memory phenotype (CD27+IgM+IgD+), and carry SHM [117]. However, within the CD27+IgM+IgD+ subset of healthy individuals, a substantial fraction contain molecular footprints of (early) GC generation [118]. Therefore, one has to be careful with interpreting changes in size of the CD27+IgM+IgD+ subset.

Recently, Kuijpers et al. described an antibody-deficient patient with a genetic defect in CD20. Whereas this patient had hypogammaglobulinemia and decreased numbers of both IgD+ and IgD− memory B cells, she only showed impaired vaccination responses to polysaccharide antigens [54]. Similarly impaired T-cell-independent antibody responses were observed in CD20-deficient mice [54]. Therefore, it is tempting to speculate that CD20 is specifically required for T-cell-independent humoral immune responses. However, the patient's immunological characteristics suggest an overall defect in antigen-dependent B cell maturation. Still, T cell help can drive sufficiently strong B cell responses, but the problem only becomes apparent in the absence of T cell help.

Concluding remarks

The majority of patients with a primary immunodeficiency have a PAD. Although, 18 genes have already been identified to be affected in PAD, for many patients a molecular diagnosis is still not made. A molecular diagnosis is of great importance to both patient and family because it offers a precise cause and it forms the basis of adequate treatment and estimation of prognosis [119]. Furthermore, understanding the molecular defect supports the development of long-term preventive strategies to limit complications and irreversible organ damage, and it contributes to treatment compliance and enables genetic counseling [119]. The general treatment for PAD is Ig replacement therapy in combination with prophylactic antibiotics, which is essential to prevent bacterial and viral infections. However, for some PAD patients corrective treatments, such as hematopoietic stem cell transplantation, may be necessary. An alternative form of corrective treatment is gene therapy, for which a molecular diagnosis is an absolute requirement. Recent studies demonstrate the correction of B cell development in Btk-deficient mice, which indicates that there might be possibilities for gene therapy for XLA patients [120, 121]. Although gene therapy for primary immunodeficiencies has faced some setbacks, it is still the most promising new corrective treatment [122, 123].

In conclusion, diagnosing known PAD and identifying new genetic defects in PAD is essential and necessitates further development of diagnostics and research projects. Current and future research on unravelling immunopathological and genetic causes of PAD will focus on crucial processes of B cell differentiation using new technologies and knowledge of knock-out mouse models. Importantly, better understanding of PAD can only be reached when placed in the context of B cell differentiation research.

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