Review

Future Rare Diseases

Functional genetics in inborn errors of immunity

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Inborn errors of immunity are genetic defects of the immune system, causing increased susceptibility to infection, autoinflammation, autoimmunity and immune dysregulation. Next-generation sequencing has enabled exponential identification of novel inborn errors of immunity due to mutations in genes encoding for proteins that participate in the immune response. However, genomic sequencing often yields multiple variants in potential candidate genes, hence functional validation of these genetic defects becomes paramount to achieve diagnosis and discovery. Genome-editing technologies such as CRISPR-Cas9 have allowed exponential advances on discovery of new primary immunodeficiencies, enabling appropriate diagnosis and treatment. This review summarizes the heterogeneous clinical presentation of primary immunodeficiencies and contextualizes the rationale for functional validation studies to achieve diagnosis and discovery, subsequently leading to the application of directed therapies.

Lay abstract: Inborn errors of immunity (IEI) are congenital (genetic) defects of the immune response. The immune response can be broken by errors in the genetic code that lead to a defective immune system that is unable to fight infection, leading to an increased susceptibility to infection or to responding excessively and inadequately against an individual's own cells, leading to severe autoimmunity or autoinflammation. Novel gene sequencing technologies have increased the capacity to discover new errors in the genetic code leading to IEI. Genetic errors can be modeled using gene-editing technologies to understand their effects in the human immune system allowing the description of new IEI, this is referred to as functional validation. This manuscript reviews the clinical heterogeneity of IEI and the process by which new discoveries can be achieved going from genetic sequencing to functional validation in order to improve diagnosis and therapeutics.

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The immune system is a sophisticated biological system dedicated to fight against foreign antigens, distinguish self from non-self-antigens and eliminate cells that are not growing properly. Consequently, organisms are protected from potentially damaging microorganisms and from infected or abnormally growing cells. The immune system is also designed to tolerate self-antigens and non-pathogenic microorganisms such as commensal microbiota. This concept is referred to as immune tolerance, which involves apoptosis of self-antigen-specific lymphocytes. This process can also be altered by specific genetic defects of the immune system. In consequence, inborn errors of immunity (IEI) or primary immunodeficiency disorders (PIDD) may drive increased susceptibility to infection, autoinflammation, autoimmunity, malignancy or allergy. They are caused by mutations that result in loss (LOF) or gain of function (GOF) of key molecules participating in the immune response. To date, defects in more than 450 genes have been described to cause different IEI phenotypes. These phenotypes are very heterogeneous including: antibody deficiency, T and B lymphocyte deficiency, complement deficiency, autoimmunity, lymphoproliferative syndromes or immune dysregulation [1,2]. In some cases, immunodeficiency may concur with autoimmunity and/or

Future Medicine

immune dysregulation, especially when genetic defects compromise molecules that regulate the immune response or are involved in tolerance. Although IEI are considered rare diseases and defects in individual genes may be infrequent; collectively, they can affect a considerable number of individuals. Moreover, as a result of improved diagnoses enabled by next-generation sequencing (NGS), prevalence has increased in the last years to approximately 40 per 100,000 mostly due to increased diagnosis [2,3]. Individuals with IEI provide the unique opportunity to study and comprehensively understand the function of the affected molecules in the human immune system providing crucial clues on how it can be modulated to ameliorate disease. This knowledge has allowed the development of precise molecular therapies not only for these specific IEI but can also be applied to other, more common diseases in which similar pathways are disturbed [4].

In this article, we comprehensibly review the use of next-generation DNA-sequencing technologies for diagnoses and discovery of novel genes associated to IEI and describe the rationale for functional validation of novel candidate variants or genes associated to IEI. Altogether, this process allows for precision medicine in different IEI phenotypes.

IEI are phenotypically & genetically heterogeneous

PIDD or IEI are classified by the last International Union of Immunological Societies (IUIS) classification according to clinical manifestations and immune compartments that are affected. The IUIS classifies the different phenotypes associated to IEI as follows: combined immunodeficiencies, combined immunodeficiencies with syndromic features, predominantly antibody deficiencies, diseases of immune dysregulation, congenital defects of phagocytes, defects in intrinsic and innate immunity, autoinflammatory diseases, complement deficiencies, phenocopies of inborn error of immunity and genes that cause bone marrow failure [2]. To date, more than 450 genes have been associated to different forms of IEI (Table 1).

Combined immunodeficiency

Severe combined immunodeficiency (SCID) is a life-threatening disease in which lymphocyte development is affected by defects in molecules that are crucial for different stages of T, B and natural killer (NK) cell development and maturation (Figure 1). Depending on the molecules that are affected, T-cell lymphopenia can concur with or without B and NK cell lymphopenia. Classically, SCID patients present with severe, recurrent viral and bacterial infections as well as opportunistic infections very early in life (before 6 months of age) [5]. Genes causing $T^-B^+NK^-$ SCID include *IL2RG, JAK3, PTPRC, LAT, IL7R, CD3D, CD3E, CD3Z, CORO1A* and *FOXN1*. Mutations in *ADA, AK2* and *RAC2* cause $T^-B^-NK^-$ SCID while defects in *LIG4, NHEJ1, PRKDC, RAG1/RAG2* and *DCLRE1C* cause $T^-B^-NK^+$ SCID [6–8]. Newborn screening for SCID by quantifying T-cell receptor excision circles has significantly increased survival of infants with this lethal form of IEI allowing diagnosis before the appearance of infectious complications [9]. Combined immunodeficiencies (CID) are less severe than SCID. Patients may show low $CD4^+$ or $CD8^+$ T-cell numbers, low immunoglobulins or poor antibody responses and phenotypic manifestations include severe and recurrent respiratory and gastrointestinal infections; some are associated with specific syndromic features that help clinically identify the affected gene/pathway [2].

CID with associated features

Among CID with syndromic features are: immune-osseous dysplasia, DNA repair defects, congenital thrombocytopenia, hyper-IgE syndrome (HIES) and defects of vitamin B12. Actin cytoskeleton dynamics are a key biological process for immune system including cell division, phagocytosis and hematopoiesis [10,11], defects in molecules that mediate filament branching, including arp2/3, WIP, NCKAPL1 and Wiskott–Aldrich protein can cause CID with or without thrombocytopenia [12].

HIES is characterized by high levels of IgE with clinical manifestations including recurrent sinopulmonary and skin infections together with atopic dermatitis. Heterozygous mutations in *STAT3,* inherited as an autosomal dominant (AD) or autosomal recessive (AR) trait cause STAT3 deficiency driving AD HIES, also known as Job's syndrome that is characterized by extremely high levels of IgE, eczema and eosinophilia. Recently, biallelic mutations in *ZNF341* have also been described as genetic defect causing HIES [13]. This gene encodes for a transcription factor that binds to STAT3, and mutations in *ZNF431* lead to low levels of STAT3 mRNA [14]. Biallelic LOF variants in *DOCK8* can also cause HIES [15] associated to eosinophilia, and Th-17 differentiation impairment with loss of circulating group 3 innate lymphoid cells (ILC3s) [16,17]. Mutations in *TYK2* have been found in HIES patients exhibiting susceptibility to intracellular pathogen and impairment in IL-12 and IFN-α signaling pathway [18]. Defects on *PGM3*, which encodes for phosphoglucomutase-3, an essential precursor of protein glycosylation, can

Table 1. Disease-causing genes of primary immunodeficiencies according to the 2019 update of the International Union of immunological Societies.

Inborn errors of immunity	Genes
Combined immunodeficiencies SCID	*IL14RG, JAK3, IL7R, CD3D, CD3E, CD3Z, PTPRC, LAT, CORO1A, FOXN1, ADA, AK2, RAC2, LIG4, NHEJ1, PRKDC, RAG1/RAG2, DCLRE1C*
Combined immunodeficiency less severe	*RFXANK, CCIT A, RFXS, RFXAP, LCK, POLD1, POLD2, UNC119, CD8A, ZAP70, TAP2, TAP1, TAPBP, B2M, DOCK8, STK4, IL21, MAP3K14, MSN, CD3G, RHOH, TRAC, OX40, FCHO1, RELA, ITK, DOCK2, CARD11, BCL10, IKBKB, ICOS, TFRC, CD40LG, CD40, MALT1, RELB, IL21R,*
Combined immunodeficiencies with syndromic features	*WAS, WIPF1, ARPC1B, ATM, NBS1, BLM, PMS2, DNMT3B, ZBTB24, CDCA7, HELLS, MCM4, RNF68, POLE1, POLE2, NSMCE3, RMRP, SMARCAL1, RNU4ATAC, EXTL3, MYSM1, TBX1, CHD7, SEMA3E, FOXN1, STAT3, ZNF341, SPINK5, PGM3, CARD11, ERBB21P, IL6R, IL6ST, TGFBR1, TGFBR2, TCN2, SLC46A1, MTHFD1, IKBKG, NFKBIA, IKBB, PNP, ORAI1, STIM1, TTC7A, SP110, STAT5B, BCL11B, CCBE1, FAT4, RNF31, EPG5, KMT2D, KDM6A, KMT2A, NFE2L2, TTC37, SKIV2L*
Predominantly antibody deficiencies	*BTK, IGHM, CD79A, CD79B, BLNK, IGLL1, TCF3, SLC39A7, TCF3, TOP2B, CD20, TNFRSF13B, TNFRSF13C, TWEAK, PIK3R1, PIK3CD, ARHGEF, SH3KBP1, SEC61A1, RAC2, CD19, CD81, TRNT1, NFKB1, NFKB1, NFKB2, IKZF1, ATP6AP1, MOGS, AICDA, UNG, INO80, MSH6, IGKC, CARD11*
Diseases of immune dysregulation	*LYST, RAB27A, AP3B1, AP3D1, PRF1, UNC13D, STX11, STXBP2, FAAP24, SLC7A7, RASGRP1, CD70, CTPS1, TNFRSF9, RLTPR, MAGT1, PRKCD, SH2DIA, XIAP, CD27, TNFRSF6, TNFSF6, CASP10, CASP8, FADD, AIRE, ITCH, TPP2, JAK1, EPD, FOXP3, IL2RA, CTLA4, LRBA, STAT3, BACH2, IL2RB, DEF6, FERMT1, IL10, IL10RA, IL10RB, NFAT5, TGFB1, RIPK1,*
Congenital defects of phagocytes	*DNAJC21, EFL1, SBDS, G6PC3, G6PT1, COH1, CLPB, TAZ, C16ORF57, VPS45, JAGN1, WDR1, SMARCD2, CEBPE, HYOU1, LAMTOR2, ELANE, HAX1, GFI1, WAS, CSF3R, MKL1, CFTR, CTSC, FPR1, ACTB, ITGB2, SLC35C1, FERMT3, GATA2, CSF2RA, CSF2RB, CYBB, NCF1, CYBA, NCF4, NCF2, CYBC1, RAC2, G6PD*
Defects in intrinsic and innate immunity	*IRAK4, MYD88, IRAK1, TIRAP, RPSA, HMOX, STAT1, IL.17F, IL17RA, IL-17RC, ACT1, CARD9, APOL1, TNFRSF11A, PLEKHM1, TCIRG1, CLCN7, OSTM1, SNX10, TNFSF11, PSENEN, NCSTN,PSEN, NBAS, RANBP2, IRF4, IFNGR1, IFNGR2, IL12RB1, IL12B, IL12RB2, IL23R, STAT1, SPPL2A, TYK2, CYBB, IRF8, ISG15, RORC, JAK1, TMC6, TMC8, CIB1, CXCR4, STAT1, STAT2, IRF7, IRF9, IFNAR1, IFNAR2, FCGR3A, IFIH1, POLR3A, POLR3C, POLR3F, IL18BP, UNC93B1, TRAF3, TICAM1, TBK1, IRF3, TL3, DBR1*
Autoinflammatory diseases	*MEFV, MVK, TNFRSF1A, NLRP3, NLRP12, NLRP3, TNFAIP3, PLC3G, NLRP1, PSMB8, PSMG2, PSMB4, PSMB9, PSMA3, POMP, NLRC4, ALP1, TRIM22, HAVCR2, PSTPIP1, LPIN2, IL1RN, SH3BP2, NOD2, CARD14, IL-36N, ADAM17, SLC29A3, OTULIN, AP1S3, TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIH1, DNASE2, ACP5, TMEM173, CECR1, POLA1, USP18, DNASE1L3, OAS1*
Complement deficiencies	*C5, C6, C7, C8A, C8B, C8G, C9, PFC, CFD, C3, MASP2, FCN3, CFB, C1QA, C1QB, C1QC, C1R, C1S, C2, C4A, C4B, CFH, CFHR1-5, THBD, CD46, SERPING1, CD59, CD55, C1R, C1S,*
Phenocopies of inborn errors of immunity	*TNFRSF6, N-RAS, K-RAS, NRLP3, STAT5b,*
Bone marrow failure	*FANCA, FANCC, BRCA2, FANCD2, FANCE, FANCF, XRCC9, FANCI, BRIP1, FANCL, FANCM, PALB2, RAD51C, SLX4, ERCC4, RAD51, BRCA1, UBE2T, XRCC2, MAD2L2, RFWD3, FANCB, DKC1, NOLA2, NOLA3, RTEL1, TERC, TINF2, ACD, TERT, TPP1, DCLRE1B, SNM1, APOLLO, WRAP53, DCAB1, PARN, RTEL1, ACD, SRP72, TP53, SAMD9, STN1, CTC1*

SCID: Severe combined immunodeficiency.

also cause AR HIES [19]. Patients with such defects additionally present cytopenias, bronchiectasis and neurologic impairment [20]. Heterozygous *CARD11* variants can also cause elevated IgE levels with severe atopic dermatitis and recurrent infections [21].

Diseases of immune dysregulation

Defects in cytolytic pathway including lack of perforin or defects in degranulation are associated with familial hemophagocytic lymphohistiocytosis (HLH). These defects cause uncontrolled T-cell activation, driving IFN-γ production and potent macrophage activation (Figure 1). Persistent macrophage activation triggers a dysregulated release of inflammatory cytokines including IL-6, IL-18, IL-1 and TNF-α causing a life-threatening cytokine storm. Mutations in *PFR1, UNC13D, STX11, STXBP2, RAB27 and LYST* genes were initially described as the genetic cause underlying familial or inherited HLH. However, more recently, several other genetic defects have been described, causing HLH through heterogeneous underlying molecular mechanisms including inflammasome dysregulation. Among inflammasome-related HLH genes are heterozygous GOF mutations in *NLRC4*, encoding for NLRC4 inflammasome, which is relevant in the immune response against enteric pathogens. Pathogenic *NLRC4* variants render constitutive NLRC4 inflammasome activation driving high levels of IL-1β and most importantly IL-18 that results in macrophage activation/HLH phenotype [22,23]. Patients with X-linked lymphoproliferative syndromes (*SH2DIA, BIRC4* and *XIAP*) characteristically associated with increased susceptibility to infection with Epstein Barr virus, dysgammaglobulinemia and lymphoma can also develop life-threatening HLH [24,25].

Among immune dysregulation group, syndromes with autoimmunity or immune dysregulation with colitis are included. Genetic defects have been reported in inflammatory bowel disease, such as *IL10, IL10R, TGFB1* or

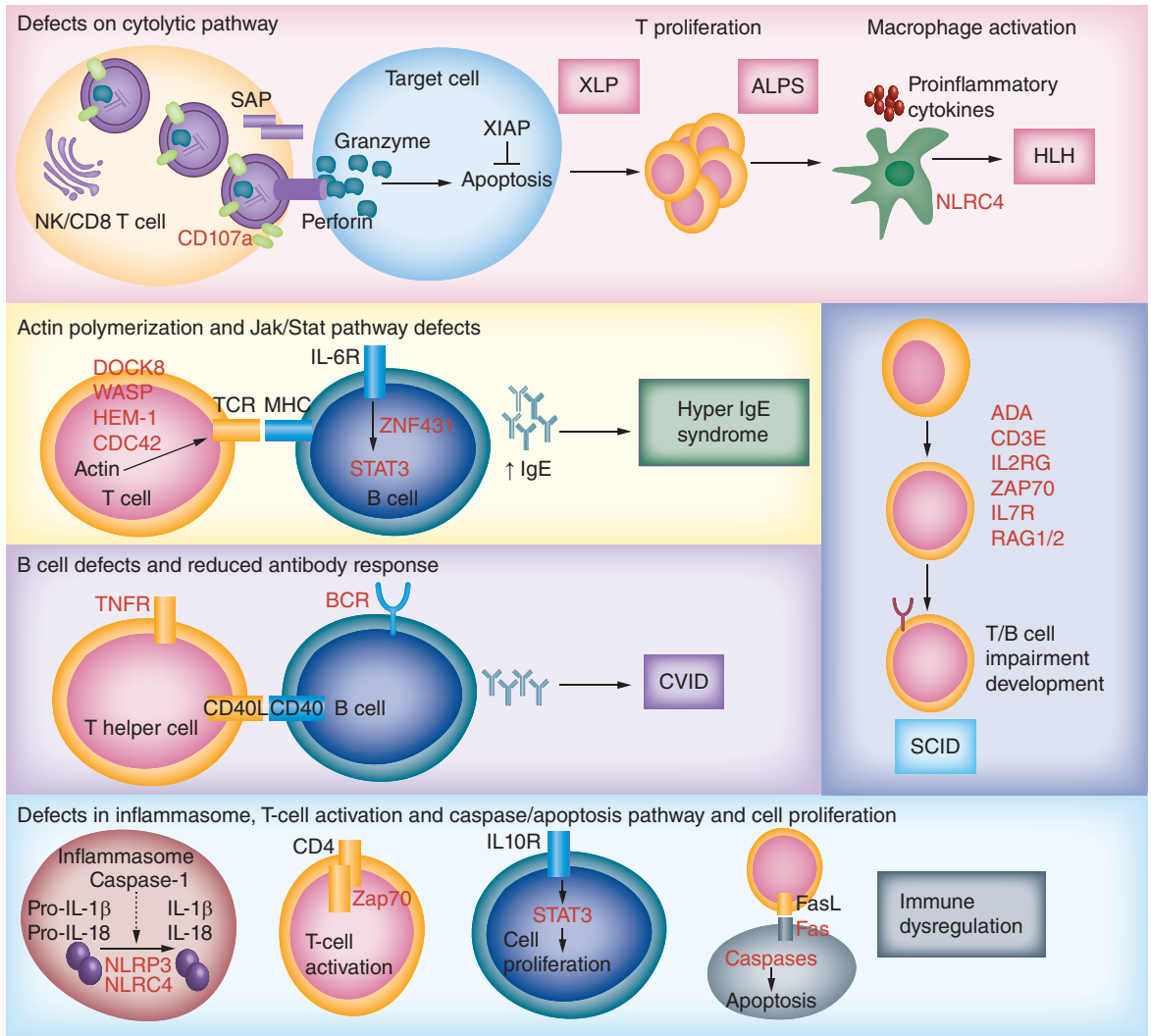

Figure 1. Immunopathogenesis in primary immunodeficiency and immune dysregulation. Simplified figure of most representative molecules or pathways related with immunopathogenesis of HLH, HIES, CVID, SCID and immune dysregulation. Defects of cytolitic pathway including perforin expression, CD107a degranulation causes constant macrophage activation with elevated levels of pro-inflammatory cytokines causing HLH. Genetic defects in XIAP (apoptosis inhibitor) or SAP derives in XLP syndrome. ALPS is characterized by T-cell proliferation. Defects on actin polymerization and JAK/STAT pathway derive in elevated IgE levels causing HIES. Genetic defects on B cells derive in B-cell response impairment and reduced antibody responses causing CVID. When genetic defects involve alteration of T- and B-cell development including mutations in ADA, ZAP70, IL7R, CD3E, IL2R or RAG1/2 individuals present with SCID. Selected gene defects are shown in red.
ALPS: Autoimmune lymphoproliferative syndrome; CVID: Common variable immune disease; HIES: Hyper-IgE syndrome; HLH: Hemophagocytic lymphohistiocytosis; SCID: Severe combined immunodeficiency; XLP: X-linked lymphoproliferative disease.

NFAT5 deficiencies. Congenital errors in *CASP10, CASP8, FADD* or *TNFRSF6* have been reported in autoimmune lymphoproliferative syndrome (ALPS). Other IEI characterized by autoimmunity can be caused by defects Treg development due to mutations in *FOXP3* leading to polyendocrinopathy, enteropathy and X-linked syndrome (IPEX).

Autoinflammatory diseases

Autoinflammatory diseases comprise a heterogeneous group of disorders characterized by dysregulation of innate immune system with recurrent inflammation [26]. Autosomal recessive or AD mutations in *MEFV* cause familial Mediterranean fever, the most common hereditary autoinflammatory disease. *MEFV* encodes for a pyrin

inflammasome, and these genetic defects destabilize its structure rendering it more prone to activation with subsequent dysregulated IL-1 production. Clinical manifestations of familial Mediterranean fever include polyserositis and recurrent fevers [27]. Missense mutations in *NLPR3* encoding for NLRP3 inflammasome cause cryopyrin-associated periodic syndromes also characterized by periodic fevers [28]. Another autoinflammatory disease is the TNF receptor-associated periodic syndrome, caused by mutations in *TNFRSF1A* [29].

Genetically determined defects in proteasome assembly or its different components are also associated with autoinflammation and are collectively referred to as proteasome-associated autoinflammatory syndromes (PRAAS). These were initially identified in individuals with biallelic mutations in *PSMB8* encoding for β5i proteasome subunit causing Chronic Autoinflammation Neutrophilic Dermatosis, Lipodystrophy and Elevated temperature (CANDLE) [30]. Later, AR or digenic mutations in *PSMB4, PSMB9, PSMB10, PSMA3* and heterozygous truncating mutations in *POMP and PSMG2/PAC2* [31,32] were found to cause PRAAS. Proteasome deficiency in PRAAS results in ubiquitinated protein accumulation and a characteristic upregulation in the expression of type-1-IFN-inducible genes and have thus been classified as interferonopathies [31]. Mutations in genes encoding for intracellular DNA/RNA sensors, molecules in the ubiquitination pathway and retrograde Golgi to ER transport have also been associated with an increased type-1 interferon response and classified among interferonopathies. These defects include mutations in *TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIH1* (MDA-5), *DDX58* (RIG-1), causing Aicardi Goutières; *TMEM173* (STING) causing STING-associated vasculopathy with onset in infancy; *IGS15 and USP18* causing dysfunction of IFN-α/β pathways; *POLA1* causing pigmentary disorder; *SKIV2L* causing Trichohepatoenteric syndrome; *PSMG2* - associated proteasome assembly dysfunction, *ACP5* (TRAP) causing immune-osseous diseases, complex autoimmune disorder with arthritis and pulmonary hemorrhages due to mutations in *COPA* and *PNPT1* causing neurodevelopment diseases with mitochondrial dysfunction [33].

Predominantly antibody deficiencies

Defects on early B-cell development cause B cell and antibody deficiency, manifesting as recurrent bacterial and viral respiratory and gastrointestinal infections. The major genetic cause of B-cell deficiency is X-linked agammaglobulinemia due to mutations in *BTK* encoding for Bruton's tyrosine kinase. Mutations in other genes involved in B-cell development or function can also cause antibody deficiency including *IGHM, IGLL1, CD79A, CD79B, BLNK, PIK3R1* and *FNIP* [34–36].

Common variable immunodeficiency is the most common symptomatic PIDD, the age of onset of symptoms ranges from the childhood to the adulthood [37]. Patients present with hypogammaglobulinemia, B-cell defects and reduced antibody responses leading to recurrent infections. Around 10–20% patients with common variable immune disease (CVID) phenotype harbor an identifiable monogenic cause, and digenic causes with epistatic interactions have also been reported [38].

The following genes have been described as monogenic causes of CVID: *AICDA*, *IKBKG*, *TNFRSF13B, TRAF3IP2, NFKB1* and *NFKB2* [2,39]. Interestingly, wide characterization of 157 individuals with mutations in *NFKB1* revealed 56 distinct heterozygous *NFKB1* mutations displaying diverse phenotypes: hypogammaglobulinemia, respiratory and gastrointestinal infections, lymphoproliferation, autoinflammation and malignancy [40]. Recently, heterozygous protein-truncating *SOCS1* variants were found in patients with CVID and lung and liver inflammation [41]. SOCS1 inhibits the phosphorylation of STAT1, a transcription factor of IFN-γ signaling, thereby SOCS1 GOF drives skewing to Th1 response and inflammation [41]. In this same pathway, autosomal recessive STAT1 LOF causes primary immunodeficiency with severe infections, HLH and inflammation [42]. In STAT1 deficiency, there is an impairment in the regulation of type-1 IFN genes leading to increased inflammatory cytokine levels including TNF-α, IL-6 and IL-18 [42]. On the other hand, AD STAT1 GOF mutations cause increased susceptibility to infections including mycobacteria, chronic mucocutaneous candidiasis and autoimmunity [43]. Variants in *PTPN2*, which inhibits cytokine signaling by protein dephosphorylation, have also been associated with CVID phenotype [41]. Activated PI3Kδ syndrome can also present with common variable immunodeficiency phenotype, benign lymphoproliferation, recurrent ear and lung infections, herpes family virus, cytopenia and mild development delay. GOF and LOF have been described for *PIK3CD* [44].

Defects in intrinsic & innate immunity

Predisposition to invasive bacterial, parasitic and fungal, viral and mycobacterial infections are present in individuals with congenital errors in innate immunity. Mutations in genes related with IFN-γ such as *IFNGR2, IRF7* or *IRF9*

lead to severe phenotypes of viral and mycobacterial infections. To date, mutations in *IL12RB1, IL12B, IL12RB2, SPPL2A, IFNGR1, IFNGR2, STAT1, NEMO, CYBB* and *TYK2* are associated with Mendelian susceptibility to mycobacterial disease. Importantly, some of these mutations are also associated with infections due to intracellular bacteria such as Salmonella.

Defects in the Janus Kinase family (JAK)/signal transducer and activator transcription (STAT) pathway can cause very heterogeneous phenotypes ranging from SCID to immune dysregulation and early-onset autoimmunity. This pathway plays a crucial role in signal transduction of cytokines, binding to cytokine receptors with JAK intracellular tyrosine kinases and activating a serial of STATs, driving downstream signaling [45]. LOF mutation in *TYK2* resulting in defective STAT3 activation and reduction of Type I IFN response has been described in defects in intrinsic and innate immunity [18]. Defects in *STAT1* or *STAT2* cause susceptibility to candidiasis, mycobacterial and viral infection [46].

Complement deficiencies

Individuals with defects in genes that encode for molecules involved in complement system also suffer from increased susceptibility to infection. Such complement deficiencies include mutations in *C5, C6, C7* or *C8*, which are clinically characterized by disseminated Neisseria infections [47]. Deficiency of other proteins of complement pathway such as ficolin or properdin has also been associated with increased infectious susceptibility associated with neurological or autoimmune complications [48]. Some monogenic forms of systemic lupus erythematosus-like syndrome can be caused by mutations in the complement pathway [49]. Hereditary angioedema is commonly associated with deficiency in C1 esterase inhibitor (*SERPING1*) [50].

Congenital defects of phagocyte, bone marrow failure & phenocopies of PIDD

Among congenital defects of phagocytes, congenital neutropenias, defects of motility, defects of respiratory burst and other non-lymphoid defects are included. Mutations in different genes such as *CYBB* and *NCF1* lead to phagocyte defects of NADPH oxidase activity causing chronic granulomatous disease [51]. Pathogenic mutations are found in 50% of cases with bone marrow failure, herein the importance of conducting genetic studies in such patients [52]. Different gene mutations have been reported for Fanconi anemia, Diamond Blackfan anemia and diskeratosis congenital [52].

Despite all advances in genetic sequencing, some patients may present with a PIDD phenotype and lack germline mutations in reported genes, it is possible that they harbor pathogenic variants in yet undescribed *IEI* genes or they could otherwise be affected by reported causes of PIDD phenocopies, including somatic mutations in *PIDD* genes or the presence of auto-antibodies against different cytokines including IL-17, IL-22, IL-6, IFN-γ or GM-CSF [53]. Immunodeficiency caused by auto-antibodies can lead to heterogeneous phenotypes depending on the affected cytokine including susceptibility to mycobacteria, chronic mucocutaneous candidiasis and severe forms of the novel SARS-Cov-2 coronavirus [54].

Genetic diagnosis & functional validation of novel IEI

IEI comprise a heterogeneous group of phenotypes and genotypes, hence the management of each individual disease is challenging as it is often variable according to the biology that is perturbed [1]. In this regard, a thorough immunologic workup is necessary to define the immune compartments that are compromised and this should be correlated with genetic studies to establish a molecular diagnosis (Figure 2). For example, the absence or increase/decrease of certain lymphocyte populations detected by flow cytometry help hypothesize pathways that are perturbed and subsequently predict possible genetic defects [55,56].

Genetic panels for known PIDD-associated genes are available and they usually include between 200 and 407 genes. It is important to consider that some patients may not have a specific change in the DNA sequence but have a copy number variants in which they lack an important portion of DNA sequence that comprises immune-related genes. Sequencing analysis in some of the PIDD panels available is able to detect copy number variants with certain limitations [57,58]. With NGS price drop, it is plausible to consider an unbiased initial approach using exome or genome sequencing (Figure 2). In most scenarios, NGS (exome and genome sequencing) is considered if panel testing does not result in a diagnosis and a monogenic cause is clinically suspected. Diagnostic rates for exome sequencing (ES) in IEI are varies between 25 and 40% patients depending on phenotype severity [59–62]. In a cohort of 278 families, screened ES diagnosis was achieved in 40% of probands and aided clinical management in 25% of patients [63]. In an HLH cohort, ES provided molecular explanation for 58% patients, which would have

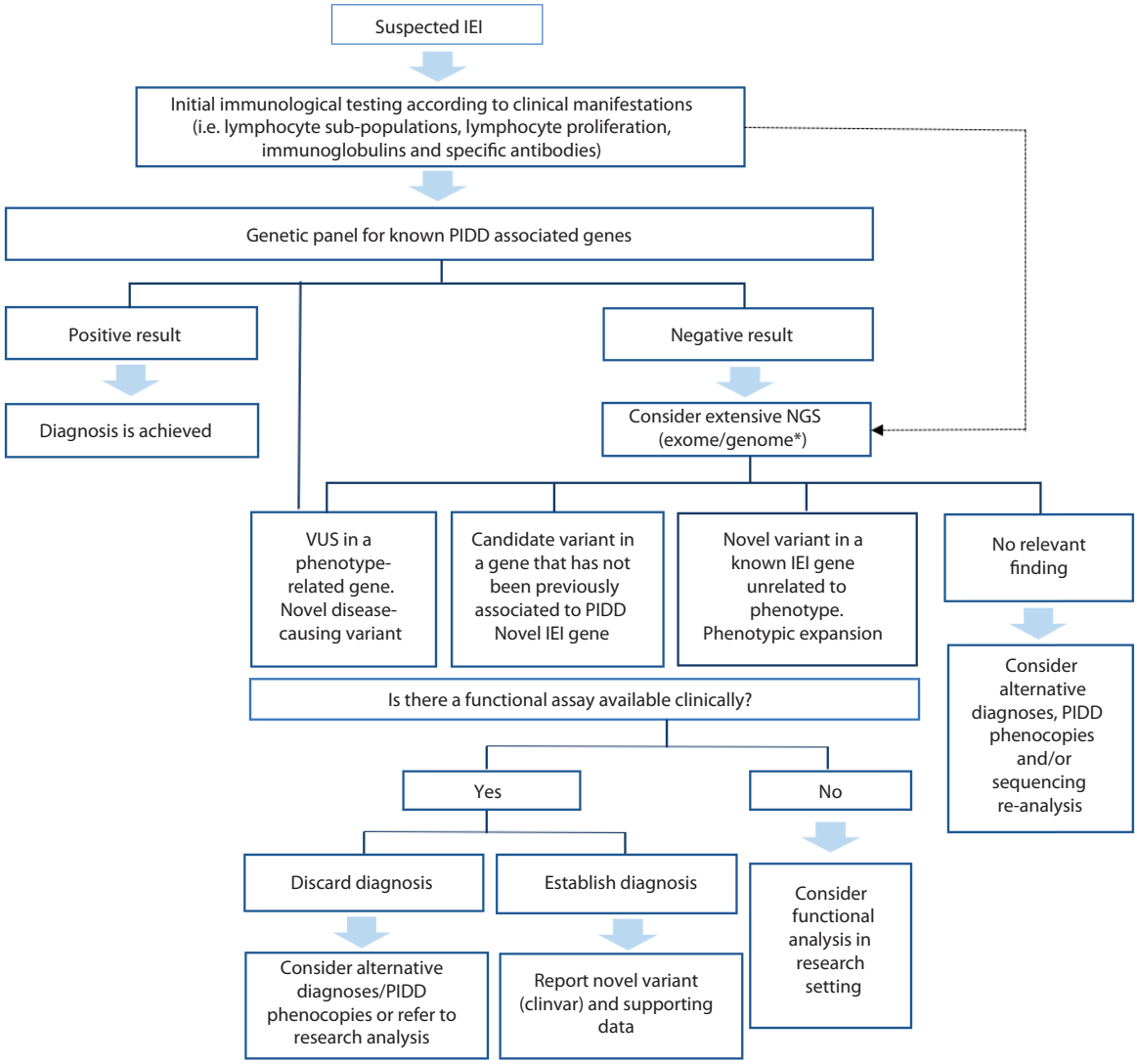

Figure 2. Proposed workflow of inborn error of immunity disease diagnoses. For IEI diagnosis, immunologic testing together with clinical manifestations is required to evaluate which cells are affected. These data together with genetic studies (genetic panels, exome or genome sequencing) are important to establish diagnosis. After genetic testing, whether no relevant gene is found, diagnosis should be reconsidered. Finally, if genetic tests arise variants of uncertain significance in a PIDD phenotype-related gene or novel variant, functional studies should be performed in clinical or research setting. *There are places where cost of exome and genome are close, so genome sequencing is preferred (dotted line).
IEI: Inborn errors of immunity; NGS: Next-generation sequencing; PIDD: Primary immunodeficiency disorder; VUS: Variant of uncertain significance.

been underestimated if only a specific HLH gene-panel had been used [64]. ES conducted in 2392 newborns with abnormal lymphocyte counts or infections yielded a PIDD diagnosis in 51 patients (2.13%) and 68.6% of these patients were cured or improved their outcomes when having an early diagnosis [65].

Mapping of rare and undiagnosed disease-causing genes with sequencing technologies often yields multiple hits. Some can be known pathogenic variants in known PIDD genes and this would yield a diagnosis directly but often variants of unknown significance are identified in PIDD genes or novel candidate genes and functional testing, along with a clinical correlation, are needed to establish diagnosis (Figure 2).

In order to prove causality, studies must demonstrate that a specific mutation impairs a specific immune process to result in the associated phenotype. This is referred to as functional validation. It includes assessment of protein amounts and function including pathway-signaling analysis. Functional analysis can be as simple as determining

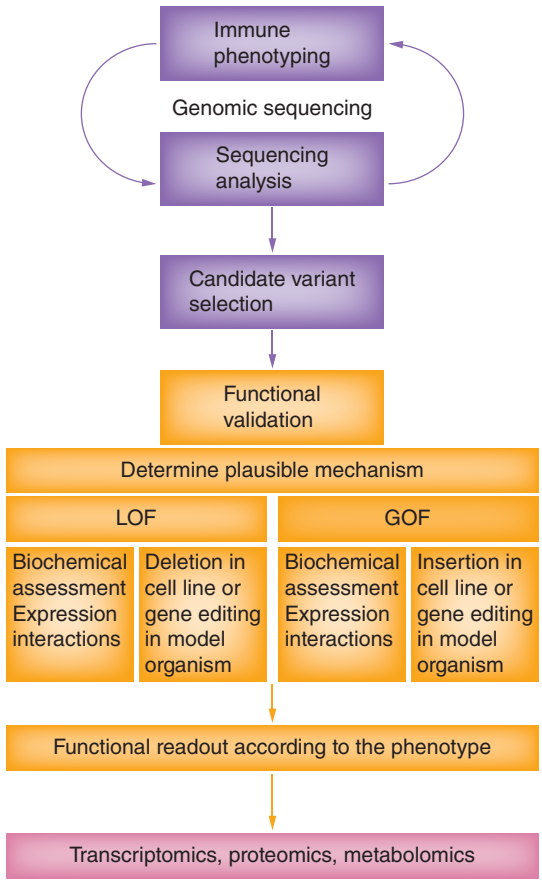

Figure 3. Workflow for discovery of novel candidate genes by exome sequencing. According to the phenotype of patient determined by phenotypic assessment and after sequencing analysis, the candidate variant selection is done. Together with the variant selected and the phenotype, a plausible mechanism is proposed, which can be a LOF or GOF. LOF of the protein-encoded gene selected is determined using biochemical assessment (flow cytometry or western blot techniques).
GOF: Gain of function; LOF: Loss of function.

the presence or absence of a protein and these determinations may be available clinically; such is the case when novel variants are identified in known PIDD genes including *BTK, XIAP, CD40L, SH2D1A* (SAP), *PRF1* (Perforin) or *WASP*, which can be easily detected by flow cytometry [66–68]. If such assays are not available clinically, research analysis may be considered (Figure 2). At this point, extended flow cytometry assays may provide additional immuno-phenotypic characterization [69,70]. For example, for variants in *STAT3*, LOF and GOF of *STAT3* can be detected by a decreased or increased of STAT3 phosphorylation, respectively, using flow cytometry, however, these studies are often non-diagnosic and specific *in vitro* variant testing is needed [71,72].

When a PIDD phenotype is suspected to be associated to a gene that has not been previously associated to PIDD, thorough functional studies, usually in a research setting, must be performed to confirm this association and define new diseases. Studying mutations of the immune system has a unique advantage for research and this is that immune cells are readily available, usually requiring a simple blood draw to obtain relevant tissue cells, as opposed to mutations affecting other tissues that are more difficult to access [73]. Determining causality of novel mutations is easier when a number of unrelated families with similar genetic variants and phenotype are identified. However, novel diseases can be described in single individuals. Some limitations of single patient studies are the lack of statistical power or the presence of confounding genetic modifiers, impairing the possibility to define a specific variant as a disease-causing mutation. Modeling these mutations in cell lines or animal models and conducting rescue experiments overcomes these limitations. To validate a novel gene causing PIDD in a single individual, the fulfilment of the following criteria is required: the genotype found cannot be in individuals who do not have the clinical phenotype; experimental studies must demonstrate that this variant damages, destroys or alters the function or expression of the gene product; the causal relationship between the genotype and the clinical phenotype must be confirmed in a relevant cellular or animal model [74].

Functional assays required to validate causality will be different depending on the pathway that is affected (Figure 3). Functional testing must be customized according to patient phenotype, tissue expression and predicted genetic mechanism of disease (i.e., LOF or GOF). For LOF variants, biochemical assessment in patient cells is useful to gauge protein levels, inform protein localization and interactions using multiple methodologies including

co-immunoprecipitation and microscopy. Gene silencing strategies can then be used to confirm these findings. For GOF variants, site-directed mutagenesis coupled to transfection experiments may be useful to assess a biologic pathway, for example, phosphorylation of STAT1 [75]. Gene-editing techniques have greatly improved the ability of the field to study the consequences of individual variants, most importantly CRISPR CAS 9 that has enabled to disrupt the genetic code in specific sites, allowing genetic silencing (CRISPR knock-out) or editing (CRISPR knock-in).

CRISPR CAS 9 & induced pluripotent stem cells as novel tools for primary immunodeficiency discoveries

CRISPR/Cas9 gene editing can be used for stable genetic modification of cell lines and model organisms. For LOF variants, the gene of interest can be knocked out to reveal its function in deficient cells/organisms. Subsequently, wild-type and mutant transcripts can be reintroduced to rescue a given phenotype. Alternatively, targeted DNA modifications can be introduced using CRISPR CAS9 coupled with the introduction of homologous strands carrying specific DNA changes to resemble patient mutations and understand the functional impact of specific DNA changes. GOF can be assessed through gene dosage experiments or by generating CRISPR knock-in models appropriate readouts should be assessed to define GOF alterations [76]. One example for this is the assessment of missense variants in *STAT3*, potentially causing *STAT3* GOF [76]. Different model organ embryos can be efficiently edited with CRISPR/Cas9 to recapitulate phenotypic features of human disease and thus reveal the impact of these variations in different cell types and in embryonic development. Generating perturbations at the embryonic cell level provides the unique opportunity to uncover relevant functions of poorly studied genes in different tissues.

Induced pluripotent stem cells (iPSCs) can be generated from patient cells or healthy iPSCs can be modified using CRISPR Cas9, these cells can then be differentiated into the cell type of interest to recapitulate a given phenotype or to study the function of a particular gene, respectively. Reprograming iPSC from patients with immunodeficiency is a valuable tool for understanding pathogenesis and designing more suitable treatments [77]. The effect of mutation in *STAT3* was investigated in iPSCs derived from a patient and authors used of CRISPR/Cas9 for correct the mutation reversing the phenotype [76].

There are multiple animal models for PIDD such as scurfy mice with dysfunctional Treg cells resembling IPEX syndrome or different animal models mimicking SCID (ADA-SCID or RAG1/2 SCID) [78,79]. Recently, Zhou *et al.* achieved the knockdown in zebrafish of ADA2 homolog describing same phenotypes of patients with ADA2 deficiency with mutations in *CECR1*, thereby associating this mutation to vascular and inflammatory phenotype [80]. These examples show the relevance of animal models for PIDD discovery and therapeutic studies.

Cellular or animal models can then be used for pathway assessment. The use of RNA-seq, metabolomics and proteomic studies may uncover perturbed pathways associated to PIDD-associated genes and variants of interest. For example, these technologies provided the association of homozygous mutations in Nck-associated protein 1-like (NCKAP1L) also known as Hem-1, a component of actin cytoskeleton machinery, with immune dysregulation and lympoproliferation. Proteomic studies in this case revealed an unsuspected interaction between Hem-1 and mTOR pathway [81].

Importantly, biological models generated when studying particular genetic perturbations are invaluable resources for future biomarker discovery and high-throughput drug screening allowing the possibility of individualized therapeutic targets.

Precision medicine for IEI

Gene discovery and pathway analysis provide the grounds for therapeutic application and discovery. They provide the opportunity to apply directed pathway modifying drugs or develop new modifiers of pathways that are affected. These therapies can then be applied to more common diseases in which such pathways are also affected.

Cytokine & other molecules

Cytokine pathways characteristically perturbed in some IEI, especially autoinflammatory disorders and can be specifically modulated [82]. Autoinflammatory disorders associated to exacerbated IL-1β secretion can be successfully treated by blocking IL-1 receptor [83]. This same pathway can also be altered in other, more common diseases in which these same treatments can be applied. For example, excessive levels of IL-1β in peripheral blood mononuclear cells (PBMC) from patients with systemic juvenile idiopathic arthritis have been identified and IL-1 blockers have been successfully used to treat a subset of these patients [84–87]. Similarly, high levels of IL-1β, together with IL-18

is increased in a subset of IEI have also been correlated with inflammatory bowel disease [88,89] indicating it as a possible therapeutic target for these more common diseases; moreover IL-18 inhibitor is already under Phase II clinical trial for diabetes mellitus, with good safety and tolerability results but failures to efficacy [90].

JAK/STAT signaling pathway

Therapies targeting JAK/STAT signaling pathway, downstream cytokine signaling including interferons, and IL-6 are currently being used or undergoing clinical research (Figure 4). Two JAK inhibitors are approved for clinical use, ruxolitinib and tofacitinib targeting the deleterious effects of exacerbated cytokine-derived inflammation [91]. Ruxolitinib is a JAK1/JAK2 inhibitor, inhibiting downstream of the interferon response. They are used in IEI caused by GOF mutations in *STAT1* and *STAT3*. Another JAK inhibitor, baricitinib, has been used in interferonopathies. Studies in patients with PIDDs in which this pathway is exacerbated, have provided the grounds for using such therapies in more common diseases including, arthritis, psoriasis and more recently clinical trials are studying ruxolitinib as therapy against COVID-19 as elevated levels of cytokine levels have been shown to play an important role in disease severity [92–94]. JAK/STAT pathway is also relevant in cancer, in this context, tofacitinib is being proposed as antimyeloma therapy since it could reverse the growth-promoting effect of tumor microenvironment [95].

PI3K/mTOR signaling

Patients with activated PI3Kδ syndrome are caused by a heterozygous mutation resulting in a GOF and patients present lymphoproliferation and viremia with Epstein-Barr virus (EBV) and/or cytomegalovirus (CMV) [96]. Patients present an increase of downstream PI3K regulator of mTOR signaling, thereby the use of rapamycin is proposed as a therapy. Alteration of cytoskeleton organization has been found to be involved in the pathogenesis of this syndrome, showing susceptibility to skin infections [97]. Another example of targeted therapy in these patients is the inhibitor of PI3Kδ (Leniolisib), which has shown promising results in clinical trial to use in activated PI3Kδ syndrome immune-deficiency patients [98].

Other targeted therapies

CTLA-4 participates in the attenuation of the immune response. Patients with immune dysregulation caused by CTLA-4 haploinsufficiency can be treated with abatacept, a CTLA-4 agonist that binds to CD80/CD86 from antigen-presenting cells, specifically restoring this disturbed pathway in these patients. CTLA-4 is recycled after endocytosis in T cells, helped by the lipopolysaccharide-response beige-like anchor (LRBA) protein [99]. Also, homozygous mutations in LRBA, which cause LOF of LRBA protein, increase the degradation of CTLA-4. Attenuation of inflammatory response and restoration of Treg cells can also be achieved with abatacept [100].

Gene therapy

Gene therapy has appeared as a new approach to cure a subset of primary immunodeficiencies [101]. Importantly, gene editing of $CD34^+$ cells has achieved immune reconstitution of patients with SCID due to loss of adenosine deaminase activity and Wiskott–Aldrich syndrome [102]. Furthermore, the treatment with gammaretroviral vector transduced-autologous $CD34^+$ hematopoietic stem and progenitor cells of ten children with mutations in the cytokine receptor γ chain resulted high survival and restoration of functional polyclonal T-cell repertoire [103]. Such approach has also been used for X-linked chronic granulomatous disease with mutations in *CYBB* in two patients, resulting in clearance of chronic infections and correction of neutrophils [104]. In this trial, they used busulfan conditioning and reported a progression of acute myeloid leukemia in both patients. However, this genotoxicity was not found in other trial of this gene editing for X-linked chronic granulomatous disease [105]. Such genotoxicity has been resolved focusing on correct the mutation in a way that an endogenous control of expression is achieved [106,107]. Indeed, recent advances with CRISPR-Cas9 technology appear as promising safe and efficient tool to advance gene therapy for these and other IEI [108].

Conclusion

Establishing a molecular diagnosis in IEI is crucial to guide clinical management. High-throughput sequencing has provided the better molecular understanding of primary immunodeficiencies but still, many patients with severe immunodeficient phenotypes remain without a molecular diagnosis. Given continued price drop in NGS, it is likely that the list of genes associated to PIDD will continue to expand. In this setting, thorough phenotypic characterization and functional testing is key to identify novel genes associated to PIDD. Identifying a genetic

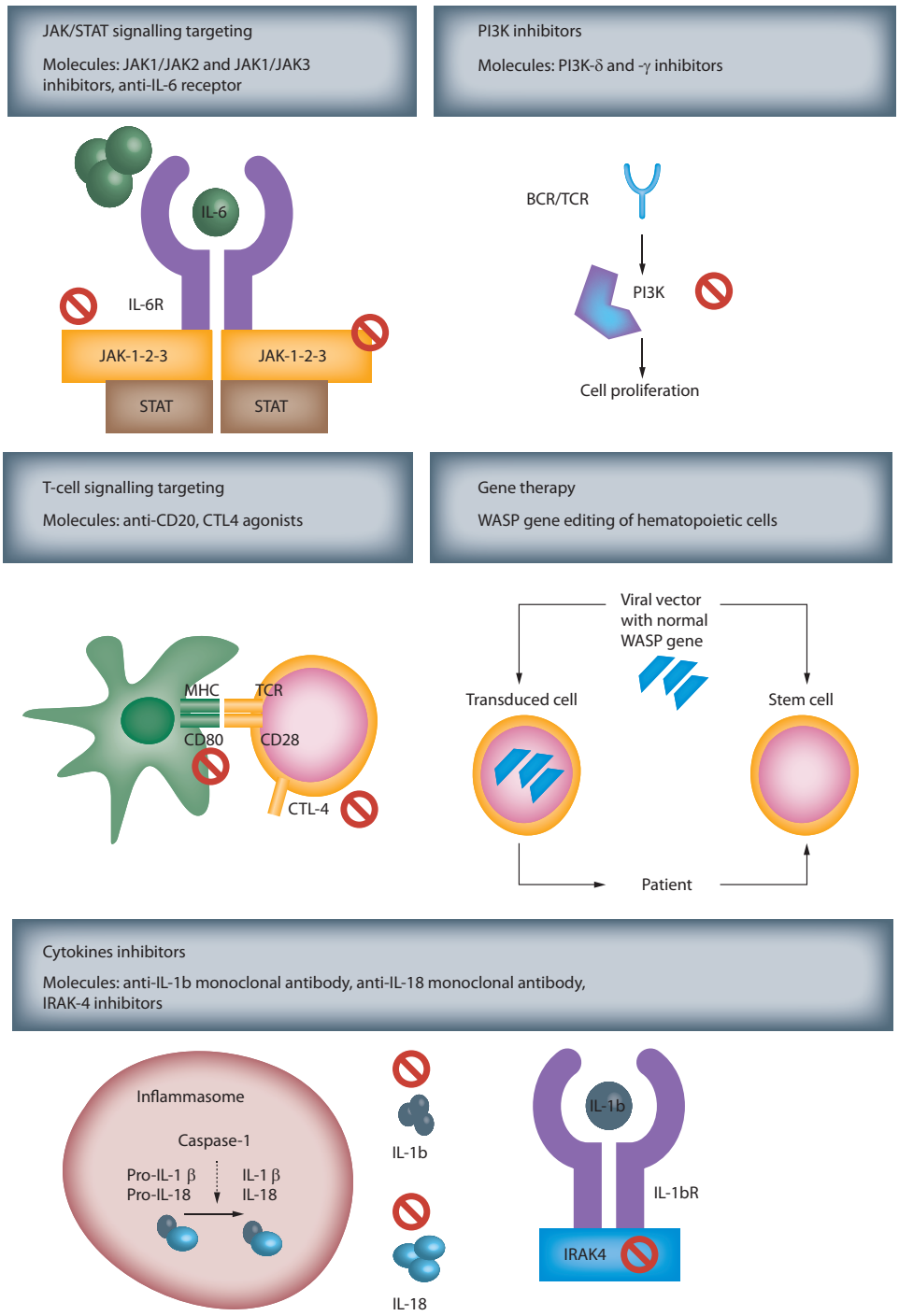

Figure 4. Precision medicine in primary immunodeficiency and immune dysregulation. Targeted therapies currently used or under development for primary immunodeficiency and immune dysregulation. Molecules targeting Jak1 and Jak2 (ruxolitinib), Jak1 and Jak 3 (tofacitinib) and anti-IL-6 receptor (tocilizumab) are used for inflammatory-associated diseases and rheumatoid arthritis. For PI3Kδ syndrome, immune deficiency PI3K-δ inhibitor (leniolisib) is under development, whereas selective PI3K-δ (seletalisib) is under development for therapy of psoriasis and SpA patients. Targeting T-cell signaling is being used for multiple sclerosis, haploinsufficiency in CTL-4 or immune dysregulation diseases with autoimmunity. Gene therapy is promising technology for patients with severe combined immunodeficiency using gene editing of hematopoietic cells. One example is the viral gene therapy with *WASP* normal gene in hematopoietic cells from WASP-deficient patients. Molecules targeting cytokines or inflammation including monoclonal antibodies against IL-1b or IL-18 and IRAK4 inhibitors are used or under development for patients with immune dysregulation.
SpA: Spondyloarthritis.

cause and disrupted pathways is important to apply and develop targeted therapies for IEI and establish precision medicine for IEI.

Future perspective

Considering all advances of last years in NGS and immunological testing, several PIDD will be discovered. Further innovative and optimization of gene-editing technologies will enable to improve gene therapy efficacy and elucidate PID-associated biological mechanisms.

Executive summary

Inborn errors of immunity are phenotypically & genetically heterogeneous

- Inborn errors of immunity (IEI) are a heterogeneous group of diseases caused by defects in genes encoding proteins that participate in the immune response. They can present with increased susceptibility to infection and/or immune dysregulation and early-onset autoimmunity.

Genetic diagnosis & functional validation of novel IEI

- Next-generation sequencing has improved the capacity of diagnosis and discovery in primary immunodeficiencies. However, there are still 60–70% of patients with primary immunodeficiencies without a molecular diagnosis suggesting there is ample room for discovery.
- Functional studies are paramount to determine pathogenicity of novel variants and candidate genes as underlying causes of IEI.

Precision medicine

- Genome-editing technologies have provided a novel and efficient tool for gene discovery and gene therapy.
- The discovery of novel variants associated to immunodeficiency enables further understanding of immune pathways involved in disease-facilitating targeted treatments and implementation of precision medicine.

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