LymphoSign Journal

The journal of inherited immune disorders

Volume 6, Number 1, 2019 EISSN 2292-5945

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Chronic granulomatous disease 2018: advances in pathophysiology and clinical management

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ABSTRACT

Chronic granulomatous disease (CGD) is a rare immunodeficiency disorder of phagocytic cells resulting in failure to kill a characteristic spectrum of bacteria and fungi and to resolve inflammation. The last few years have witnessed major advances in pathogenesis and clinical management of the disease:

1. Better understanding of 3 physiologic anti-inflammatory functions of NADPH oxidase-derived reactive oxygen species: Promotion of the clearance of dying host cells, suppression of inflammasomes, and regulation of type I interferon signalling. This insight is opening new avenues for targeted drug interventions.
2. Advances in reduced intensity conditioning (RIC) for allogeneic hematopoietic stem cell transplantation (HSCT) make it a promising and safe procedure even for fragile patients with ongoing severe infection or hyperinflammation.
3. Encouraging early data of a multicenter trial of gene-replacement therapy using a self-inactivated lentiviral vector.

Combining targeted anti-infectious/anti-inflammatory measures and considering extended indications for curative HSCT are key to improving patient outcome further. Gene therapy will likely become a viable option for disease correction, but long-term assessment is not yet possible.

Statement of novelty: We discuss important advances in pathogenesis and treatment of CGD that will change our approach to clinical management.

Introduction to chronic granulomatous disease (CGD)

CGD is a rare inherited deficiency of the innate immune system affecting 4–5 per million live births in North America. The disease is characterized both by severe recurrent bacterial and fungal infections as well as hyperinflammatory complications (most evident in the pulmonary, gastrointestinal, and urinary tracts). CGD results from the absence or malfunction of any of the 5 NADPH oxidase subunits in phagocytic cells. Physiologically, after stimulation of neutrophils, monocytes, and macrophages, the activated oxidase transfers electrons from NADPH to molecular oxygen generating short-lived superoxide (O_2^-), the so-called oxidative burst, which is converted into microbicidal reactive oxygen species (ROS, e.g., highly diffusible hydrogen peroxide; H_2O_2) (Figure 1). In CGD, generation of O_2^- and formation of H_2O_2 are defective. The quantitative dihydrorhodamine 123 (DHR) flow cytometry assay (detecting H_2O_2) is today's most accurate diagnostic test for CGD (Roos et al. 2017), while the qualitative (microscopical) and less discriminant nitroblue tetrazolium (NBT) dye test (detecting O_2^-) is still in clinical use.

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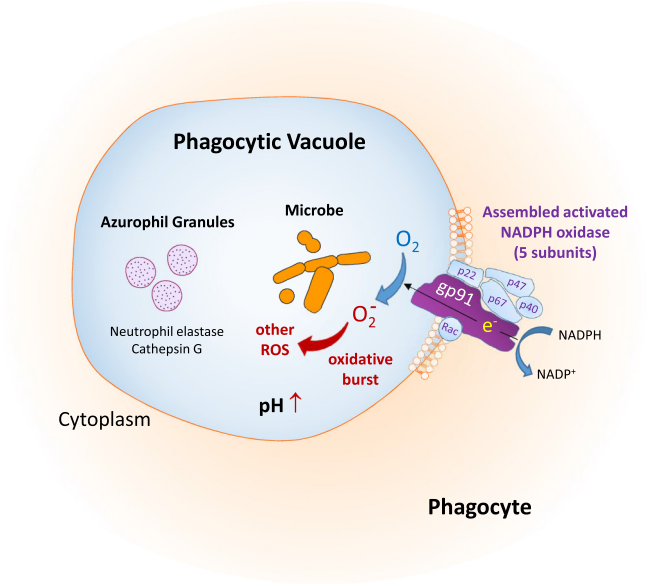

Figure 1: Phagocyte NADPH oxidase-dependent intracellular microbial killing by ROS and proteases released from azurophil granules into the phagocytic vacuole.

Etiology and pathophysioloy of the disease

New knowledge of the multiple physiologic functions of the phagocyte NADPH oxidase (phox) complex can now define CGD as a dual genetic defect of pathogen clearance by neutrophils and of clearance of dying host cells by macrophages. This insight is important for understanding the seemingly paradoxical association between primary immunodeficiency and autoinflammation and is opening new opportunities for targeted drug treatments.

Genetic defects

In North America, 70% of CGD cases are caused by loss-of-function mutations in the X-linked gene encoding gp91^{phox}, the electron transferase subunit of the NADPH complex (Figure 1). 30% of cases are caused by biallelic mutations in either of 3 autosomal recessive (A/R) genes encoding cytosolic proteins (p47, p67, p40^{phox}) which regulate gp91^{phox} after their translocation to the plasma membrane, or in the gene coding for the membrane-bound p22^{phox} protein that stabilizes gp91^{phox}. A/R mutations in the gene encoding p47^{phox} are the second most common cause of CGD, observed in 20% of cases in North America.

In regions with high rates of consanguinity (e.g., North Africa, the Arab world, Israel, and Turkey) A/R forms of CGD predominate over the X-linked recessive (XR) form. An additional factor contributing to the A/R CGD predominance are founder effects in specific populations. Altogether the mutations identified in A/R CGD patients in these regions are mostly homozygous, indicating that both parents contributed an identical, mutated allele that causes the disease (Wolach et al. 2017).

To the above list of 5 genetic NADPH oxidase defects, a novel sixth cause of CGD has to be added, namely A/R loss-of-function mutations in the *CYBC1* gene coding for EROS. This hitherto undescribed transmembrane protein is highly expressed in neutrophils, monocytes, and macrophages. It plays a central role as chaperone, critical for expression of the gp91^{phox}-p22^{phox} heterodimer in cell and phagosomal membranes, and is thereby essential for ROS generation. EROS-deficient mice quickly succumb to infection (Thomas et al. 2017), and in a consanguineous family an EROS-deficient A/R CGD patient was found with both infectious and autoinflammatory manifestations, who subsequently underwent successful hematopoietic stem cell transplantation (HSCT) (Thomas et al. 2019). Eight additional homozygous CYBC1 deficient individuals with signs of CGD were identified in the Icelandic population likely reflecting a founder effect (Arnadottir et al. 2018).

The disease-causing gene mutation should be determined in every CGD patient. This is essential for genetic counseling and important for prognostication in CGD (Kuhns et al. 2010): Residual O_2^-/H_2O_2 generation (as in most p47^{phox} and p40^{phox} deficiencies (van de Geer et al. 2018)) presents a lower risk for infections and mortality compared to absent O_2^-/H_2O_2 formation (as in most cases of gp91^{phox} deficiency).

In gp91^{phox} deficiency, a deletion extending into the telomeric *XK* gene should be excluded when patients manifest red blood cell acanthocytosis and absent expression of the Kx blood group antigen. In patients with this McLeod neuroacanthosis syndrome, transfusions of ubiquitous Kell antigen positive blood will cause strong reactions due to generation of anti-Kx antibodies (Jung et al. 2007).

Impaired microbial killing

In healthy individuals, the NADPH oxidase in neutrophils, apart from being directly responsible for production of microbicidal ROS, is indirectly responsible for liberation and activation of complementary

microbicidal proteases (e.g., elastase and cathepsin G) from primary (azurophilic) granules (Figure 1). Both mechanisms collaborate in killing/digestion of pathogens entrapped by neutrophils in phagocytic vacuoles, a process deficient in CGD patients (Reeves et al. 2002).

A fraction of neutrophils producing O_2^- finally disintegrate their own intracellular membranes and release decondensed chromatin (DNA/histones) together with microbicidal granule proteins into the extracellular space, forming web-like neutrophil extracellular traps (NETs) (Figure 2) (Sollberger et al. 2018). We now know that pus consists mostly of neutrophils surrounded by NETs. Released NETs continue the killing process for hours and can also trap and kill microbes that are too big to be phagocytosed (e.g., bacterial aggregates and fungal hyphae). This ancient antimicrobial defense is deficient in CGD patients (Fuchs et al. 2007) and can be reversed by gene therapy (Bianchi et al. 2011).

Excessive inflammation

In healthy individuals, dying (apoptotic) cells are rapidly cleared by macrophages in an "immunologically silent" way therefore preventing hyperinflammation by the debris. Apoptotic neutrophils externalize oxidized phosphatidyl serine (oxPS) on their surface membrane which is then recognized through oxPS receptors on macrophages, triggering neutrophil uptake into phagocytic vacuoles (Figure 3) (Matsura 2014).

The subsequent clearance of apoptotic neutrophils within macrophages, also called efferocytosis (Latin effere = "to carry to the grave"), ultimately resolves inflammation. Following uptake, LC3-proteins of the autophagy system are recruited to phagosomes in an NADPH-oxidase dependent manner, thereby enhancing their fusion with lysosomes and facilitating controlled degradation of the cargo (Figure 4) (Heckmann et al. 2017). LC3-associated phagocytosis is accompanied by release of anti-inflammatory cytokines (e.g., interleukin (IL)-10).

In CGD patients infectious foci stimulate granuloma formation. Chronic granulomatous inflammation may compromise vital organs and account for additional morbidity. Apoptosis of neutrophils (Sanford et al. 2006) and their clearance by macrophages are delayed in CGD (Bagaitkar et al. 2018). Defective clearance of dying cells leads to excess generation of pro-inflammatory cytokines (e.g., IL-1 beta and TNF alpha) and autoinflammation (Figure 5). In CGD patients, defective efferocytosis of human monocytes can be restored during experimental short term treatment with pioglitazone (PIO), a licensed anti-diabetic drug mediating resolution of inflammation. This treatment is accompanied by enhanced phagocyte mitochondrial ROS production, which bypasses the NADPH oxidase defect (Fernandez-Boyanapalli et al. 2015*a*). Since PIO also restores bactericidal capacity in murine CGD (Fernandez-Boyanapalli et al. 2015*b*), it was used in an infant with CGD to overcome refractory bacterial lung abscesses before succesful HSCT (Migliavacca et al. 2016). Efficacy and safety of this intervention require further exploration of PIO in therapy-refractory CGD patients with an indication for HSCT.

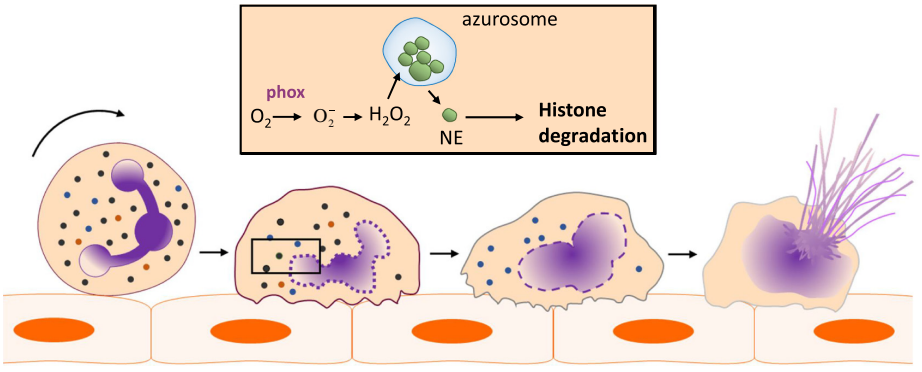

Figure 2: ROS-dependent formation of neutrophil extracellular traps (NETs): H_2O_2 triggers dissociation of the "azurosome" protein complex (residing in azurophil granules) causing leakage of neutrophil elastase (NE) into the cytoplasm. This protease moves to the nucleus and clips histones facilitating chromatin decondensation. After nuclear membrane vesiculation chromatin comes into contact with cytoplasmic and granule proteins and starts forming NETs, released after rupture of the cytoplasmic membrane.

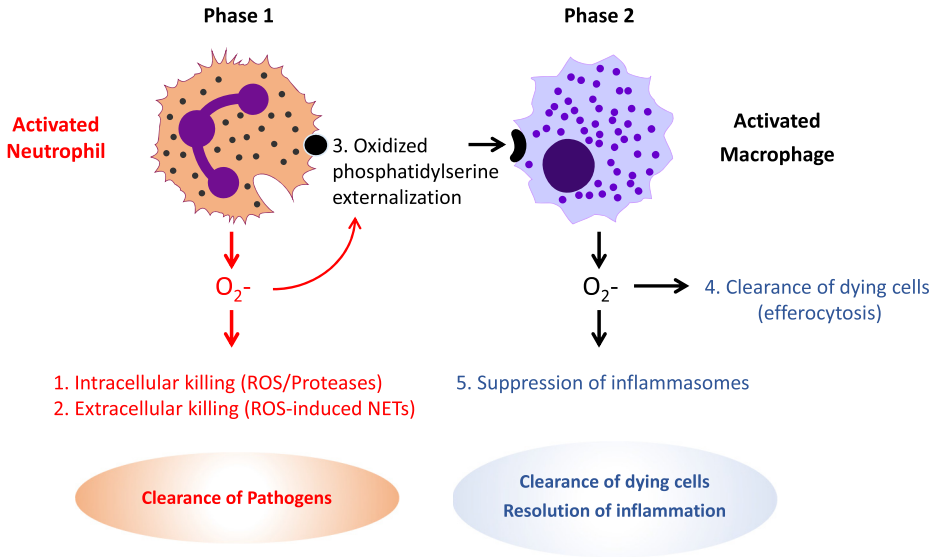

Figure 3: Summary of physiologic NADPH oxidase functions: Clearance of pathogens by activated neutrophils and clearance of dying neutrophils by activated macrophages resulting in resolution of infection and inflammation.

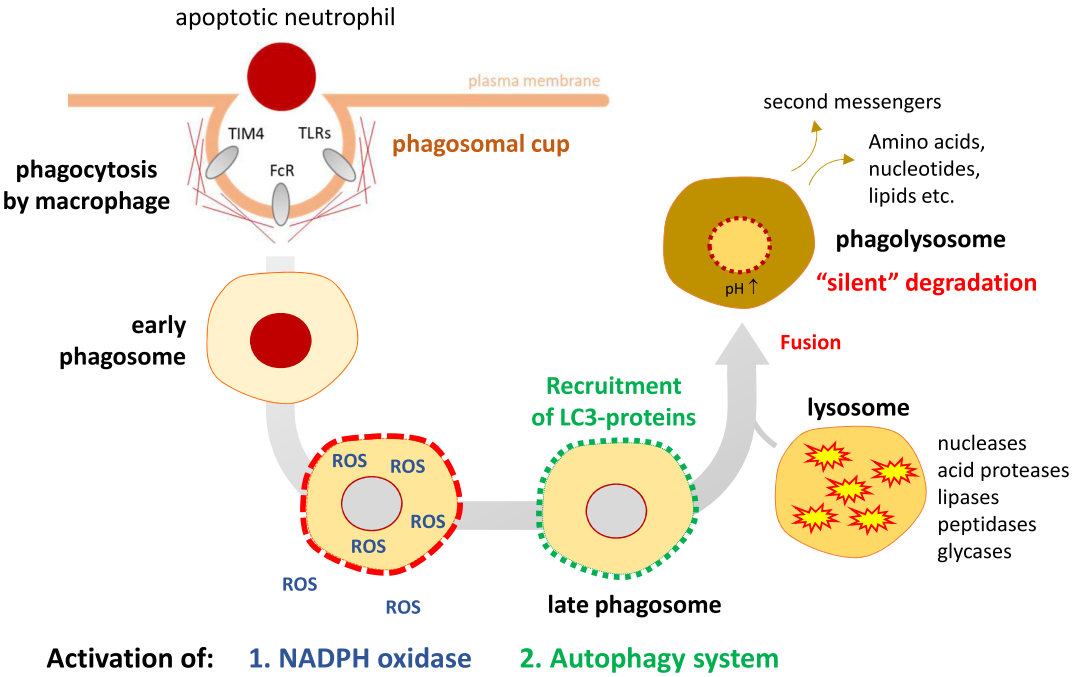

Figure 4: Silent clearance of dying cells by normal macrophages via LC3-associated phagocytosis (efferocytosis).

Clinical manifestations of CGD

CGD is characterized both by severe infections and hyperinflammatory manifestations. During the course of CGD the first infection occurs much earlier than the first inflammatory manifestation (at median ages of 0.9 versus 11.3 years) (Dunogué et al. 2017). While infection remains a concern in adults as the primary cause of death, inflammatory events emerge and predominate during adolescence and adulthood.

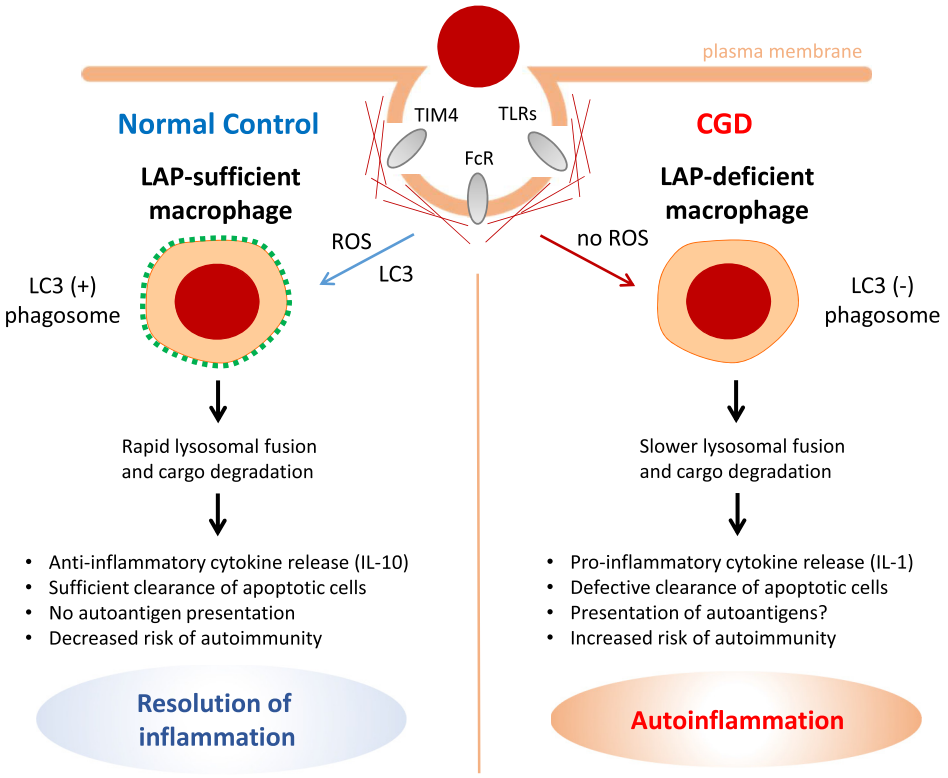

Figure 5: LC3-associated phagocytosis (LAP) and inflammation: Consequences of defective clearance of dying cells in CGD.

Bacterial and fungal infections

In North America (Winkelstein et al. 2000) and Europe (van den Berg et al. 2009) typical infections in CGD arise from a limited number of 5 organisms: *Staphylococcus aureus* (lymphadenitis, liver abscess, pneumonia rarely), *Burkholderia cepacia* (necrotizing pneumonia, sepsis), *Serratia marcescens* (sepsis, osteomyelitis), *Nocardia*, and *Aspergillus* spp. (subacute pneumonia, dissemination to brain and bone).

Invasive filamentous fungal infections are acquired through inhalation of spores, resulting in pneumonia that can spread to ribs, spine, and brain. They remain the most frequent cause of mortality in CGD. *Aspergillus fumigatus* is the most frequent species isolated, while *Aspergillus nidulans* causes more inflammatory and refractory disease (Henriet et al. 2012). Mortality is reduced by treating with azoles (voriconazole or posaconazole). Resection of infected tissue may be required in case of extension to chest wall or vertebrae.

Several emerging pathogens endemic in other regions of the world have to be added to this list and may infect also travelling CGD patients from Western countries:

- Tuberculosis (TB) constitutes a high risk for children with CGD andresults in a complicated clinical course (Lee et al. 2008). Routine *Bacillus Calmette-Guérin* (*BCG*) vaccination in CGD causes severe disease, e.g., draining skin lesions at the vacination site, regional lymphadenitis ("BCGitis"), and more rarely disseminated disease ("BCG sepsis") (Conti et al. 2016).
- Non-typhoidal *Salmonella* is a leading cause of blood-stream infection in CGD patients in endemic regions world-wide (Lee and Lau 2017).
- *Chromobacterium violaceum*, a gram negative bacillus, confined to (sub)tropical climates and encountered in brackish waters, causes sepsis in exposed CGD patients (Sirinavin et al. 2005).
- *Burkholderia pseudomallei*, a water/soil bacterium found in (sub)tropical regions causes systemic melioidosis. It has been identified in CGD children of South East Asia. They acquire the infection during work in rice-fields via skin wounds, inhalation, drowning, or after heavy monsoon rains (Lee and Lau 2017).
- *Leishmania infantum*, an intracellular protozoon endemic on the Mediterranian coast, has caused

visceral leishmaniasis-associated hemophagocytic syndrome in Portuguese and Spanish CGD patients (Martín et al. 2009).

Inflammatory manifestations

The classic inflammatory complications of CGD are most prominent in the pulmonary, gastrointestinal, and urinary tracts (e.g., as interstitial lung disease, granulomatous colitis, and granulomatous cystitis). Granulomatous colitis in CGD mimicks Crohn's disease and affects up to one half of CGD patients (Marciano et al. 2004). Colonoscopy permits diagnostic biopsies revealing epitheloid granulomas and pigment-laden macrophages. Empiric initial therapy is based on corticosteroids.

To this list, 2 emerging complications have to be added:

- Fulminant mulch pneumonitis, an emergency caused by massive inhalation of aerosols from mulch, compost, or dead leaves containing *Aspergillus* spores. The excessive inflammatory response to the spores results in miliary infiltrates and hypoxia requiring ventilation. An intravenous combination of voriconazole and methylprednisolone is life-saving (Siddiqui et al. 2007).
- Non-cirrhotic portal hypertension following liver abscesses: obliteration of central/portal veins and nodular regenerative hyperplasia can ultimately lead to portal hypertension (Hussain et al. 2007). Liver abscesses, mostly by *S. aureus*, in the context of prolonged fever should be treated by antibiotics in combination with steroids to avoid excess surgical intervention (Straughan et al. 2018).

Surgical sites in CGD often become infected and heal very slowly with fistulas. Sutures should not be removed early and drains be left for a prolonged period. Excessive wound granulation with dehiscence responds to steroids.

In addition to autoinflammation, there is an increased risk of autoimmune disease in CGD with a focus on syndromes that resemble lupus. In the national US registry of more than 350 CGD patients, 0.5% of patients met diagnostic criteria for SLE and 2.7% criteria for discoid lupus (Winkelstein et al. 2000).

Classic SLE is characterised by overexpression of type I interferon pathway transcripts that are also highly expressed in CGD patients and in $p47^{phox}$ deficient mice (Holmdahl et al. 2016; Thomas 2017). This finding would implicate NADPH oxidase-derived ROS as a feedback mechanism dampening activation of the type I interferon pathway and possibly preventing autoimmunity.

Clinical presentation in adults

In the first nation-wide retrospective study focusing on the long-term outcome of a French pediatric CGD cohort of 80 patients, the grown-up CGD patients displayed similar rates and characteristics of severe infections and inflammatory episodes as in childhood (Dunogué et al. 2017). Main sequelae of pediatric CGD observed in adulthood were:

1. Growth failure as a consequence of serious infections, inflammation, and of repeated steroid treatments.
2. Chronic dyspnea from restrictive respiratory failure after repeated lung infections.
3. Chronic digestive complications from episodes of inflammatory colitis, bowel stenosis, and perianal fistulas with substantial impact on quality of life.

The social consequences of the above complications and repeated hospital admissions were serious with poor educational achievement in half of the patients. Proper surveillance and follow up are required for adult CGD patients, especially during the crucial transition from pediatric to adult medical departments. Regular follow up should be performed to detect and properly manage occult infections, effectively treat inflammatory events, and prevent long-term complications (Thomsen et al. 2016).

Clinical presentation in carriers of X-linked CGD

While many female carriers of X-linked CGD are asymptomatic, some become symptomatic with severe or recurrent infections, or autoimmune (e.g., discoid lupus erythematosus) or inflammatory (e.g., inflammatory bowel disease) manifestations. In female carriers of XR-CGD the *CYBB* gene, encoding $gp91^{phox}$, is silenced randomly in each cell early in development allowing expression of only 1 X-chromosome (so-called lyonization). Neutrophils with inactivation of the *CYBB*-mutated X-chromosome will have normal O_2^- production whereas cells with inactivation of the normal X-chromosome will have a CGD phenotype.

In a large study of 93 females with XR-CGD carrier state (Marciano et al. 2018), carriers with CGD type infections showed a strong correlation between risk of infections and skewed inactivation of the wild type allele (with <20% O_2^- forming cells). From these data it seems prudent to consider trimethoprim/sulfamethoxazole prophylaxis in carriers when the %DHR value is <20%. In contrast, susceptibility to autoimmune/inflammatory manifestations in the XR-CGD carrier state was unrelated to the amount of O_2^- generation. The association of autoimmune phenomena with the carrier state per se, nevertheless, suggests that XR-CGD carriers in general are not ideal donors for HSCT.

Conventional treatment of CGD

Clinical management of CGD has been reviewed before (Leiding and Holland 2016; Seger et al. 2017).

General health care

Common sense measures are sometimes neglected and have to be re-emphasized to patients and parents:

- Wounds should be washed well and rinsed with antiseptic solutions (e.g., 2% H_2O_2 or Betadine).
- Extensive dental work/surgery associated with bacteremia should be covered with additional antibiotics, e.g., amoxicillin/clavulanic acid.
- Pulmonary infections can be prevented by refraining from smoking, not using bedside humidifiers and avoiding sources of *Aspergillus* spores (animal stables, garden work, and constructions sites).
- Risk of perirectal abscesses can be diminished by avoiding rectal manipulations, e.g., suppositories or taking rectal temperature.
- All routine immunizations should be given to CGD patients except live bacterial vaccines (*BCG* and *Salmonella typhi* vaccines, contraindicated in CGD). Measles and varicella live vaccines as well as yearly influenza vaccine are safe and indicated to prevent potentially lethal bacterial superinfections.
- CGD patients traveling to or residing in endemic regions should know about potential infections and adopt preventive measures as advised by specialists in travel medicine (Lee and Lau 2017).

Antimicrobial prophylaxis

The cornerstone of clinical care is mainly based on retrospective studies and consists of lifelong antibacterial and antifungal prophylaxis with intracellularly active microbicidal agents. Lipophilic co-trimoxazole (trimethoprim/sulfamethoxazole, TMP/SMX) results in a marked reduction of serious bacterial infections and abscess drainages (Margolis et al. 1990). It is recommended at 5 mg/kg/day, TMP up to 160 mg daily.

For antifungal prophylaxis the lipophilic itraconazole is the drug of choice with high activity against *Aspergillus* spp. (Gallin et al. 2003). Itraconazole is recommended up to a maximum of 200 mg once daily. For optimal bioavailability, itraconazole capsules should be taken at 5 mg/kg/day with food, while itraconazole solution should be taken at 2.5 mg/kg/day in fasting condition.

In addition to the above antimicrobials, interferon gamma (IFNγ) is part of the routine prophylaxis regimen in US centers, while most European experts use IFNγ only in selected cases. The dose is 50 ug/m^2 s.c. 3×/week. This prophylactic regimen is based on a prospective multicenter randomized placebo-controlled trial of IFNγ in 128 CGD patients performed before the advent of antifungal prophylaxis with itraconazole. The trial resulted in reduction of the frequency of mainly severe bacterial infections >70% (The International Chronic Granulomatous Disease Cooperative Study Group 1991). There were no improvements in NADPH oxidase function nor a significant efficacy in preventing *Aspergillus* infections during the limited study period. A later prospective non-randomized long term (lasting 5 years) Italian multicenter study of 35 CGD patients, comparing treatment with TMP/SMX and itraconazole alone versus addition of IFNγ, showed no difference in the rates of severe infection (Martire et al. 2008). The exact mechanism of how IFNγ exerts its effect in CGD is still unknown, adding to the debate over its utility. Since IFNγ upregulates human leukocyte antigen (HLA) expression, IFNγ prophylaxis has to be stopped at least 4 weeks before HSCT.

Treatment of serious infections

Significant rises in C-reactive protein (CRP) should prompt evaluation for infection, including appropriate imaging and pathogen identification. In the case of fever

and (or) persistent cough, a liver abscess, *Salmonella* and *Burkholderia* septicemia as well as *Aspergillus* pneumonia (inhalational miliary or focal invasive) have to be excluded first. CT or MRI imaging should be followed until resolution of infections. A definitive microbiological diagnosis by tissue biopsies is essential for proper treatment. An appropriate sample may require fine needle aspiration or percutaneous drainage of liquid pus.

Recommended empiric initial therapy is based on limited clinical data because of the rarity of CGD. Consultation with an experienced infectious disease specialist is strongly advised. Antibiotics chosen for initial therapy should cover a broad spectrum of gram negative bacteria (including *Burkholderia* spp. and *Serratia marcescens*) as well as gram-positive organisms (including *S. aureus* and *Nocardia* spp.). Useful first-line agents with an appropriate antimicrobial spectrum are meropenem PLUS vancomycin added in regions with methicillin-resistant *S. aureus*. In case of failure to respond within 24–48 h, an antimycotic drug (e.g., voriconazole) may be needed. Initial therapy is tailored once culture/susceptibility and histopathology are known. As infections often respond slowly, intravenous treatment must be followed by prolonged oral treatment, sometimes continued over months.

Consider addition of steroids in case of fulminant *Aspergillosis* or severe *Nocardia* infection not responsive to appropriate antibiotic therapy (Siddiqui et al. 2007; Freeman et al. 2011).

Treatment of inflammatory complications

Antimicrobial prophylaxis decreases the risk of severe infections, but not the risk of inflammatory manifestations which can affect >50% of patients (especially those with XR-CGD). Management of inflammatory complications is challenging, as treatment with anti-inflammatory/immunosuppressive agents increase the risk of infection. Treatment of inflammatory manifestations in CGD has been reviewed before (Magnani and Mahlaoui 2016).

Short courses of corticosteroids followed by gradual tapering are required in acute granulomatous exacerbations of the bowel (e.g., gastric outlet obstruction), the urinary tract (e.g., ureteral obstruction) and the lung (inhalative acute miliary pneumonia). Long-term treatment of granulomatous colitis follows treatment options for Crohn's disease under the cover of the routine antimicrobial prophylaxis in CGD. Recommended first-line therapy in severe CGD-colitis cases is prednisone (1 mg/kg/day) for 1–2 weeks with slow tapering over 1–2 months to 0.1–0.25 mg/kg/day (Leiding and Holland 2016). Steroids inhibit granuloma formation by suppressing proinflammatory cytokine production and TNFα-dependent fusion of macrophages into multinucleated giant cells (Maltesen et al. 2010). Steroid dosage during taper can be adjusted to the severity of colonic inflammation by following fecal levels of calprotectin released from apoptotic neutrophils (Nakazawa et al. 2017).

Steroids are effective in severe CGD-colitis, but when taken off treatment relapse is high. Some patients become steroid-dependent requiring second line therapies (including immunosuppressants and biologic agents). Patients with refractory colitis necessitate a multi-step approach and may become eligible for allogeneic HSCT.

Azathioprine, an immunosuppressant, is used as second line therapy to maintain remission in steroid-dependent cases and to improve outcomes in patients with fistulating disease.

Thalidomide blocks nuclear localization of NFκB, thus inhibiting the production of inflammatory cytokines. It has been used by the Paris group for treatment of refractory colitis with complete clinical responses in 4/6 patients after 6 months (Noel et al. 2013) and was not associated with increased risk of infections.

Infliximab, a monoclonal antibody to TNFα, was used by the NIH group in 5 CGD patients with steroid-refractory colitis. It is effective, but resulted in severe intercurrent infections with CGD pathogens in all 5 and death in 2, precluding long-term therapy in CGD (Uzel et al. 2010).

Use of Anakinra, an IL-1-receptor antagonist, has also been reported in CGD patients with refractory colitis. Its efficacy has been contested by the NIH group, when treatment of 5 CGD patients with severe colitis led only to marginal or no benefit (Hahn et al. 2015).

Allogeneic HSCT is the only curative treatment for refractory colitis in CGD. HSCT can save patients from extensive colon surgery, risky because of poor wound

healing, anastomosis complications and fistula formation. Inflammatory manifestations rapidly regress post-HSCT permitting withdrawel of steroids with a subsequent growth spurt into predetermined percentile channels in children (Seger 2010).

Curative treatments

The above prophylactic and routine therapeutic approaches are mostly supportive and depend on lifelong patient compliance. CGD thus remains a lethal disease, nowadays at an adult age. The ultimate goal is to develop safe curative approaches, e.g., allogeneic HSCT regimens with reduced-intensity conditioning (RIC) and autologous stem cell gene therapies (GT) for CGD patients without a suitable HLA-compatible donor.

Allogeneic HSCT

CGD is a proinflammatory disease with a high risk of alloreactivity (rejection and GvHD) after myeloablative cytotoxic marrow conditioning. Therefore, antithymocyte globulin in HLA-matched sibling transplantation and alemtuzumab in matched unrelated (MUD) transplantation were introduced for balanced *in vivo* depletion of alloreactive recipient and donor T cells. In addition, toxic myeloablative conditioning was replaced by a sub-myeloablative RIC regimen to decrease tissue damage and reduce release of proinflammatory cytokines. These 2 measures allowed safe transplantation even in high-risk CGD patients with ongoing infection/inflammation and in younger adults. Pre-existing infections and chronic inflammatory lesions cleared in all engrafted survivors. Even children with severe lung restriction improved their lung function slowly, normalizing decreased oxygen saturation, reversing clubbed fingers and toes, and manifesting a growth spurt (reviewed in Seger 2010).

To further optimize the RIC protocol, targeted drug monitoring (TDM) was introduced. Low-dose busulfan is administered, serum busulfan levels are measured, and the cumulative area-under-the-concentration curve was individually adjusted real-time to a sub-myeloablative target range of 45–65 mg/Lxh. An international prospective CGD/HSCT trial using this Zürich RIC protocol was performed at 16 centers in 10 countries (Güngör et al. 2014). 56 CGD patients aged 0–40 years were enrolled of whom 42 patients had high-risk features and 25 were adolescents or young adults. 2-year probability of overall survival (OS) was 96% and event-free survival (EFS) was 91%. Equivalent outcomes between matched siblings and MUDs were observed. Incidences of acute graft versus host disease (GvHD) and limited chronic GvHD (cGvHD), as well as an incidence of 5% of graft failure were low. Excellent myeloid donor chimerism (>90%) was documented in 93% of surviving patients.

Because of its efficacy and favorable toxicity the low busulfan RIC regimen is a promising treatment modality for CGD, however, requires good lab facilities for real-time TDM. In the absence of TDM facilities, the Zürich RIC protocol may still be used by aiming at a higher submyeloablative target of 65 mg/Lxh with the help of a recent busulfan dosing nomogram (Bartelink et al. 2012).

Another reduced toxicity conditioning (RTC) regimen based on myeloablative treosulfan (available in Europe) was used by the Newcastle group and has also achieved good survival rates in a large retrospective CGD study (Morillo-Gutierrez et al. 2016). Secondary graft failure in 12% of the 70 patients requires further investigation, especially with regard to the durability of myeloid chimerism.

The decision for or against allogeneic HSCT should be made early in childhood. Patients with $gp91^{phox}$ mutations and absent O_2^- production have a survival of only 50% beyond 40 years of age compared to patients with $p47^{phox}$ mutations and residual H_2O_2 generation (over 80% survival beyond 40 years) (Kuhns et al. 2010). Our algorithm for patient selection and HSCT timing in CGD (Figure 6) is based on 4 parameters, early HLA-typing, quantitative O_2^-/H_2O_2 production, mutation analysis, and the individual clinical course. Provided a suitable HLA-compatible MSD/MUD donor has been found, 3 situations have to be analysed:

1. If there is completely absent O_2^-/H_20_2 production early HSCT can be recommended for any type of CGD (XR-CGD or A/R CGD)(because of the high long-term risk of therapy-refractory infection/inflammation and death in any CGD patient with no oxidase function).
2. In case of residual O_2^-/H_20_2 generation with better long-term outcome watchful waiting on medical therapy may be recommended for any type of CGD while observing the individual clinical course for a clinical HSCT indication.

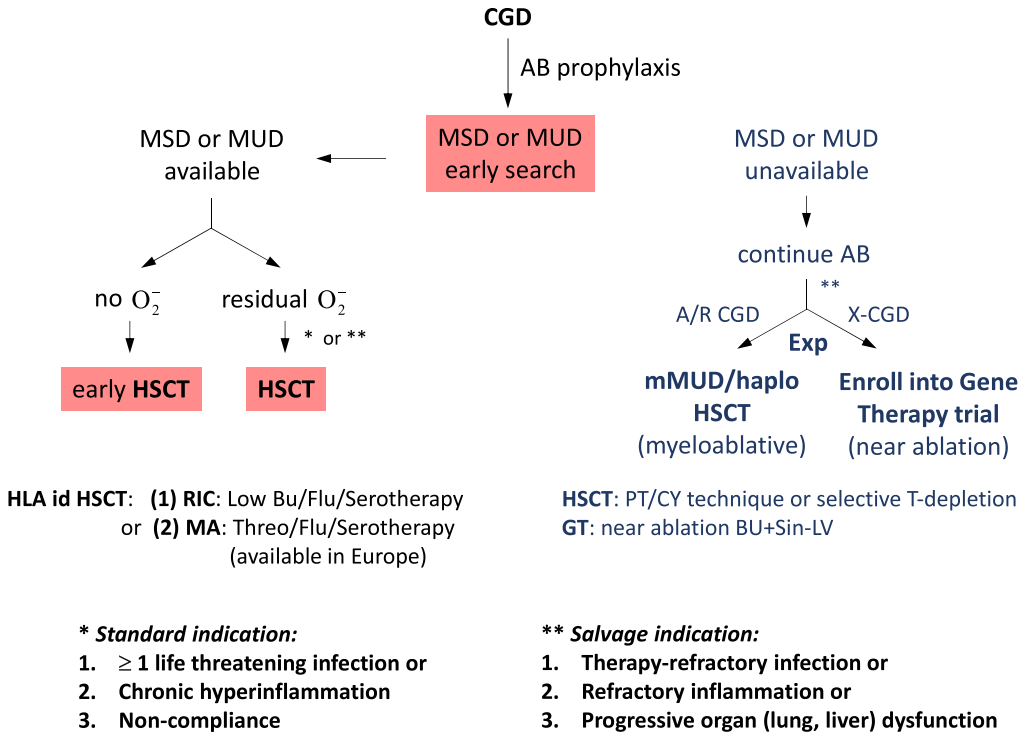

Figure 6: HSCT in CGD: Algorithm for patient selection and timing.

3. In case of late presentation for HSCT after an eventful therapy-refractory clinical course with important organ sequelae, salvage HSCT can still be successful but needs to be discussed carefully by a board of experienced transplanters.

Two recent reports from Sweden (Åhlin et al. 2013) and UK (Cole et al. 2013) comparing HSCT versus conventional treatment support HSCT as being the preferable treatment for severe CGD. Children not undergoing transplantation have more serious infections, surgical interventions, hospital admissions and lower height for age compared with post-HSCT children. Long-term post-HSCT survival and quality of life data in adult CGD are not yet available.

Therapies under investigation

HLA-haploidentical HSCT

Transplants with haploidentical donors present greater risk of graft rejection and GvHD. The current use of CD34+ selected stem cell grafts (with 4–5 log donor T cell depletion from the transplant to avoid severe GvHD) results in 4–6 months of profound T cell deficiency. This non-selective, global T cell depletion technique is accompanied by risks of graft failure/rejection as well as severe viral reactivation until thymopoiesis is established. Three novel approaches to "graft engineering" have been developed in order to minimize these 2 risks and are now improving outcomes for PID patients with haploidentical parent donor grafts.

1. A new protocol of selective depletion of alloreactive T-cells *in vivo*, on days +3/+4 post-HSCT by high-dose cyclophosphamide (at 50 mg/kg/day) presents a promising alternative (Luznik et al. 2010). Cyclophosphamide (Cy) is non-toxic to stem cells and kills rapidly deviding alloreactive T cells post transplantation (PT), while sparing resting T cells with specificity for infectious agents. PT/Cy controls GvHD without affecting engraftment and allows more rapid reconstitution of donor immunity to opportunistic infections. This promising new technique has been successfully applied in a few CGD patients (Parta et al. 2015; Regueiro-García et al. 2018) and should now be investigated in a prospective multicenter study. The easy logistics of PT/Cy are an asset in countries with no access to an unrelated donor registry and lacking a sophisticated laboratory for graft manipulations required for conventional T cell depletion of haploidentical grafts.

2. In countries with available laboratory technology another refined *in vitro* method of selective T cell depletion is increasingly used for removal of GvHD-inducing CD3+TCR alpha/beta+ T cells from HLA-haploidentical grafts. This new depletion technique retains TCR gamma/delta+ T cells which can promote engraftment without causing GvHD and might alleviate viral reactivation. In 3 landmark PID studies using CD3+TCR alpha/beta+/CD19+ depletion a combined overall transplantation survival of 90% was seen, including 4 successfully transplanted CGD patients (e.g., Shah et al. 2018). However, a significant rate of viral infections was still observed, despite the presence of TCR gamma/delta+ T lymphocytes.
3. A solution to viral disease is the adoptive transfer of virus-specific memory T cells from an antigen-experienced donor. Prior to infusion the donor T cells have to be depleted of GvHD-inducing CD45 RA+ naive T cells, while retaining their anti-infectious memory T cell repertoire. CD45 RA+ depleted donor lymphocyte infusions (DLIs) can safely be administered at low T cell doses in monthly intervals post HSCT, escalating to 100×10^3 T cells/kg in haploidentical recipients. Among 31 patients with absent CMV-specific immune reactivity at baseline significant expansion of CMV-specific T cells after 3 DLIs was demonstrated in 20 within 100 days (Maschan et al. 2018). CD45 RA-depleted memory DLIs thus constitute a simple and likely efficient measure to prevent viral reactivation in the setting of TCR alpha/beta T cell depleted grafts.

Stem cell gene therapy

CGD remains an attractive though difficult target for autologous stem cell gene therapy (GT). Functional correction of as few as 10%–20% of neutrophils should be sufficient to prevent CGD typical infections based on the experience in X-linked CGD carriers (Marciano et al. 2018). The major obstacle however is the lack of selective growth advantage of gene-transduced stem cells. Previous protocols of unregulated gene addition mediated by retroviral vectors have either resulted in graft failure or development of a myelodysplastic syndrome due to transactivation of the MDS1/EVI1 protooncogene by gamma-retroviral insertions (Stein et al. 2010). Early data of an ongoing prospective clinical trial in XR-CGD (based on a new self-inactivating lentiviral vector with an internal myelospecific chimeric promoter driving $gp91^{phox}$ expression (Santilli et al. 2011)) indicate a safer integration profile with no evidence of transactivation of nearby cellular genes/clonal expansion and stable oxidase activity in >20% of neutrophils in the first treated CGD patients (Thrasher 2018).

Following the excellent outcome of recent clinical trials of viral-mediated gene therapy of HSCs for several PIDs resulting in long-term constitutive gene expression, search efforts are now directed to site-specific gene editing allowing expression of corrective DNA sequences at the native site under physiologic regulation or in a genomic "safe harbour" (Keller et al. 2018). Taking the advances in GT and HSCT together, one can be cautiously optimistic about further important advances in the next 5–10 years on the long road to optimal curative therapies for CGD patients lacking a suitable transplantation donor.

Abbreviations

AR	autosomal recessive
CGD	chronic granulomatous disease
GT	gene therapy
GvHD	Graft-versus-host disease
HSCT	hematopoietic stem cell transplantation
IFNγ	interferon gamma
IL-1β	interleukin 1 beta
MA	myeloablation
NET	neutrophil extracellular trap
O_2^-	superoxide anion
oxPS	oxidized phosphatidylserine
PHOX	phagocyte NADPH oxidase
PIO	pioglitazone
PT/CY	post-transplant cyclophosphamide
RIC	reduced intensity conditioning
ROS	reactive oxygen species
TDM	targeted drug monitoring
TMP-SMX	trimethoprim-sulfamethoxazole
TNFα	tumor necrosis factor alpha
XR	X-linked recessive

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Coronin 1A deficiency identified by newborn screening for severe combined immunodeficiency

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ABSTRACT

Introduction: Coronin 1A belongs to a large family of actin regulatory proteins with a role in T cell homeostasis. A role for coronin 1A was also observed in macrophages, NK, and neuronal cells. To date, coronin 1A deficiency has been described in relatively few patients with combined immunodeficiency.

Aim: We studied here the molecular and genetic basis of immunodeficiency detected by newborn screening for severe combined immunodeficiency.

Methods: Patient data was collected in accordance with REB approved protocols. Immune work up, including T and B cell proliferative responses and serum concentrations of immunoglobulins, was performed. Next generation sequencing techniques and cellular analyses were also utilized.

Results: The patient presented with T cell lymphopenia, reduction in $CD4^+CD45Ra^+$ cells and hypogammaglobulinemia. Uniquely, she also had persistent severe neutropenia. Whole exome sequencing and Sanger confirmation revealed a novel homozygous mutation in coronin 1A.

Conclusion: Coronin 1A deficiency can be detected after birth by T cell receptor excision circle-based newborn screening.

Statement of novelty: We report here a patient with a novel mutation in coronin 1A, identified during newborn screening with low T cell receptor excision circle levels and neutropenia, which is a unique finding in this condition.

Background

Coronin 1A belongs to a large family of actin regulatory proteins which are highly conserved, from yeast to humans (Xavier et al. 2008). The human coronin 1A gene (*CORO1A*) maps to chromosome 16p11.2 and consists of 11 exons. It encodes a 461 amino acid, 57 kDa actin-binding protein (Suzuki et al. 1995), and is expressed in various hematopoietic cells (Oku et al. 2003), as well as in the brain (Ahmed et al. 2007). Coronin 1A is believed to associate with polymerized actin and the actin branching facilitator actin-related protein 2/3 complex (Arp2/3) (Rybakin and Clemen 2005; Föger et al. 2006). This protein has a clear role in T cell homeostasis both in mice and human (Shiow et al. 2008), although the exact mechanism is yet to be clarified. A role for coronin 1A was also observed in macrophages (Jayachandran et al. 2007), NK (Mace and Orange 2014), and neuronal cells (Martorella et al. 2017).

Human coronin 1A deficiency was first described in a patient who presented with oral thrush, repeated

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respiratory infections and disseminated chicken pox after varicella vaccine. The patient was found to have 2 null mutations in *CORO1A*. She had T cell lymphopenia and poor T cell responses to mitogens and antigens as well as poor humoral responses (Shiow et al. 2008, 2009).

Later on, several reported cases extended the spectrum of disease associated with mutations in *CORO1A* (Moshous et al. 2013; Mace and Orange 2014; Stray-Pedersen et al. 2014): hypomorphic mutations in 3 siblings were associated with a predisposition to EBV-mediated B cell lymphoproliferation and transformation into lymphoma at 12, 7.5, and 14 months, respectively (Moshous et al. 2013).

An additional kindred with a compound heterozygous mutation resulting in complete loss of protein expression (Stray-Pedersen et al. 2014) presented with a late onset disease at 7 years of age, with epidermodysplasia-verruciformis-human-papilloma-virus (EV-HPV), molluscum contagiosum and mucocutaneous herpetic ulcers, as well as granulomatous tuberculoid leprosy. Abnormalities in NK cell cytotoxic function in one of these patients were identified, and shown to be related to defects in regulation of the F-actin microenvironment, thus impeding lytic granule secretion (Mace and Orange 2014). Interestingly, both asymptomatic carrier parents were found to have immune abnormalities, including $CD4^+$ and NK cell lymphopenia.

The newborn screen (NBS) program for severe combined immunodeficiency (SCID) was initially implemented in Wisconsin in 2008 (Baker et al. 2010). Since then, similar programs were initiated in multiple states and world-wide (Kwan et al. 2014; Kanegae et al. 2016; Blom et al. 2017). The NBS program in Ontario, Canada, has been successfully implemented since 2013 (Cross 2013). The basis behind the successful NBS for SCID is the T cell receptor excision circles (TREC) assay (Accetta Pedersen et al. 2011). This assay involves an RT-PCR reaction to detect a TREC, which is a physiological byproduct of the V(D)J recombination process occurring in maturing thymocytes. Since TRECs do not replicate, they are a good biomarker of thymic output.

Several studies have retrospectively examined the yield of the TREC assay as a NBS for classical SCID as well as other conditions associated with T cell lymphopenia (Kwan et al. 2014; van der Spek et al. 2015). Low or undetectable TREC levels were found to have an excellent yield in the diagnosis of "typical SCID" (defined as autologous T cell count <500 cells/μL and response to PHA proliferation of <10% of normal (Roifman et al. 2012)), as well as some cases of combined immunodeficiency (CID) (autologous T cell count of >500 cells/μL and reduced response to PHA proliferation). While prematurity is a common reason for low TREC numbers, repeating the test at a later age prevents unnecessary investigations. Pre- and perinatal medication use can also result in a false positive test, thus requiring prudence in interpretation of the test. Other causes for T cell lymphopenia included various syndromes, such as Di George syndrome/22q11.2 chromosome deletion, trisomy 21, trisomy 18, ataxia telangiectasia, CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital and ear abnormalities) syndrome and various less common entities. Importantly, identification of these syndromes, including CID, by NBS programs is not uniform. Depending of the cutoff for reporting of low TREC levels, variable numbers of infants are recognized as having idiopathic T cell lymphopenia, and required continued follow up and investigations for combined immunodeficiency.

We report here a case of coronin 1A deficiency caused by a novel homozygous mutation. The infant was detected to have profound T cell deficiency by TREC-based NBS.

Methods

Patient

Patient information was collected prospectively and retrospectively from medical records and entered to the Canadian Centre for Primary Immunodeficiency Registry (REB Protocol No. 100005598, The Hospital for Sick Children).

Serum concentration of immunoglobulin and specific antibodies

Serum concentrations of immunoglobulins were measured by nephelometry. Levels of serum antibodies to tetanus were measured by ELISA.

T and B cell proliferative response

Lymphocyte proliferative responses to mitogens including phytohemagglutinin (PHA) and anti-CD3. All assays were performed in triplicate and were compared with simultaneously stimulated normal controls, as previously described (Sharfe et al. 2014).

Exome sequencing and variant calling

DNA from blood was submitted to The Centre for Applied Genomics (TCAG), Toronto, ON, Canada for exome library preparation and sequencing. DNA was quantified by Qubit DNA HS assay (Life Technologies, Carlsbad, CA, USA) and 100 ng of input DNA was used for library preparation using the Ion AmpliSeq Exome Kit (Life Technologies) according to the manufacturer's recommendations. The Ampliseq Exome library was immobilized on Ion PI™ Ion Sphere™ particles using the Ion PI Template OT2 200 Kit v3. Sequencing was performed with the Ion PI Sequencing 200 Kit v3 and Ion PI Chip v2 in the Ion Proton™ semiconductor sequencing system following the manufacturer's recommendation.

Alignment and variant calling were performed using Torrent Suite (v4.0) on the Ion Proton Server, using the Ion Proton Ampliseq germline low stringency setting and the hg19 reference genome. The variants were annotated using an in-house annotation pipeline (Stavropoulos et al. 2016) based on Annovar (November 2014 version) (Wang et al. 2010) and RefSeq gene models (downloaded from UCSC 1 August 2015).

Sequencing analysis

Patient's genomic DNA was extracted from peripheral blood lymphocytes using the Geneaid Genomic DNA Mini Kit. Genomic DNA was amplified by PCR with specific primers designed upstream and downstream of the coronin 1A gene. Sequencing was done using GenomeLab Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter) and analyzed on CEQ 8000 Genetic Analysis System (Beckman Coulter).

Western blotting

Whole-cell lysates were prepared in a 1% Triton X100 buffer and analyzed by Western blotting. Anti-coronin 1A antibodies were purchased from Creative Diagnostics Inc. and Giα3 were purchased from Santa Cruz Biotechnology Inc.

Case presentation

The patient is a 14 month old female, born at term to a single mother of African descent. Perinatal history was unremarkable. There is no known consanguinity in the family. The patient's mother has a history of recurrent vaginal yeast infections and neutropenia. The patient was found to have low TREC values during NBS.

Clinical course

The patient's infectious history includes a coronavirus upper respiratory tract infection at 7 months of age, and recurrent thrush requiring oral fluconazole

Table 1: **Patient immune work up.**

	Patient	Normal range
WBC ($\times 10^9$/L)	1.83	$6.0–13.0 \times 10^9$/L
Neutrophils ($\times 10^9$/L)	0.34	$1.27–7.18 \times 10^9$/L
Lymphocyte ($\times 10^9$/L)	1.13	$1.5–8 \times 10^9$/L
Markers (cells/μL)		
$CD3^+$	367	1600–6700
$CD4^+$	267	1000–4600
$CD8^+$	40	400–2100
TCRγδ	75	—
$CD19^+$	183	600–2700
$CD16/56^+$	187	200–1200
TRECs (copies/0.5 μg DNA)	382	>400
Mitogenic response	(Stimulation index/control)	
PHA	642/659	>350
Immunoglobulins (g/L)		
IgG	2.9	3.2–11.5
IgM	0.1	0.5–1.9
IgA	0.1	0.0–0.9
Anti-tetanus toxoid IgG (IU/mL)	>7.0	>0.1
	(% lymphocytes/control)	
$CD3^+/CD45\ RO^+$	51.0	—
$CD3^+/CD45\ RA^+$	29.6	—
$CD4^+/CD45\ RO^+$	38.2	—
$CD4^+/CD45\ RA^+$	6.3	—

treatment. She is otherwise well and is growing and developing appropriately for her age. She is currently given *Pneumocystis Jiroveci* (PJP) prophylaxis, and hematopoietic stem cell transplantation (HSCT) has been offered.

Immune evaluation

Since birth, the patient has had persistent lymphopenia as well as neutropenia. Immune work up further revealed a reduction in $CD19^+$, $CD4^+$, and $CD8^+$ cells, with a relatively more profound $CD8^+$ cell lymphopenia and an increased CD4:CD8 ratio (Table 1). TCRVβ analysis for clonality revealed mild expansion of 2 $CD4^+$ clones, while analysis of $CD8^+$ clones was insignificant due to CD8 lymphopenia (Figure 1). Over time, the patient developed a mild reduction in NK cell counts as well. She also had reduced number of $CD4^+CD45Ra^+$ naïve T cells. T cell responses to mitogens were normal. Her humoral work up showed hypogammaglobulinemia with good specific response to tetanus vaccine. An initial TREC value was recorded at 42 copies/3 μL. A repeat test from the same dried blood sample was abnormal at 17 copies/3 μL (cut off values > 75 copies/3 μL). Whole blood TREC were also low at 382 copies/0.5 μg DNA (Table 1). Screening for TBX deletion and purine profile was normal. Maternal immune work up was done and revealed consistent neutropenia and B cell lymphopenia.

Genetic diagnosis

Whole exome sequencing identified a novel homozygous mutation in *CORO1A*: c.601C>T p.Arg201Cys (Figure 2), and was further confirmed by Sanger sequencing. This mutation is located in the tryptophan-aspartate (WD) repeat-containing element of *CORO1A* (Figure 3), which is thought to facilitate

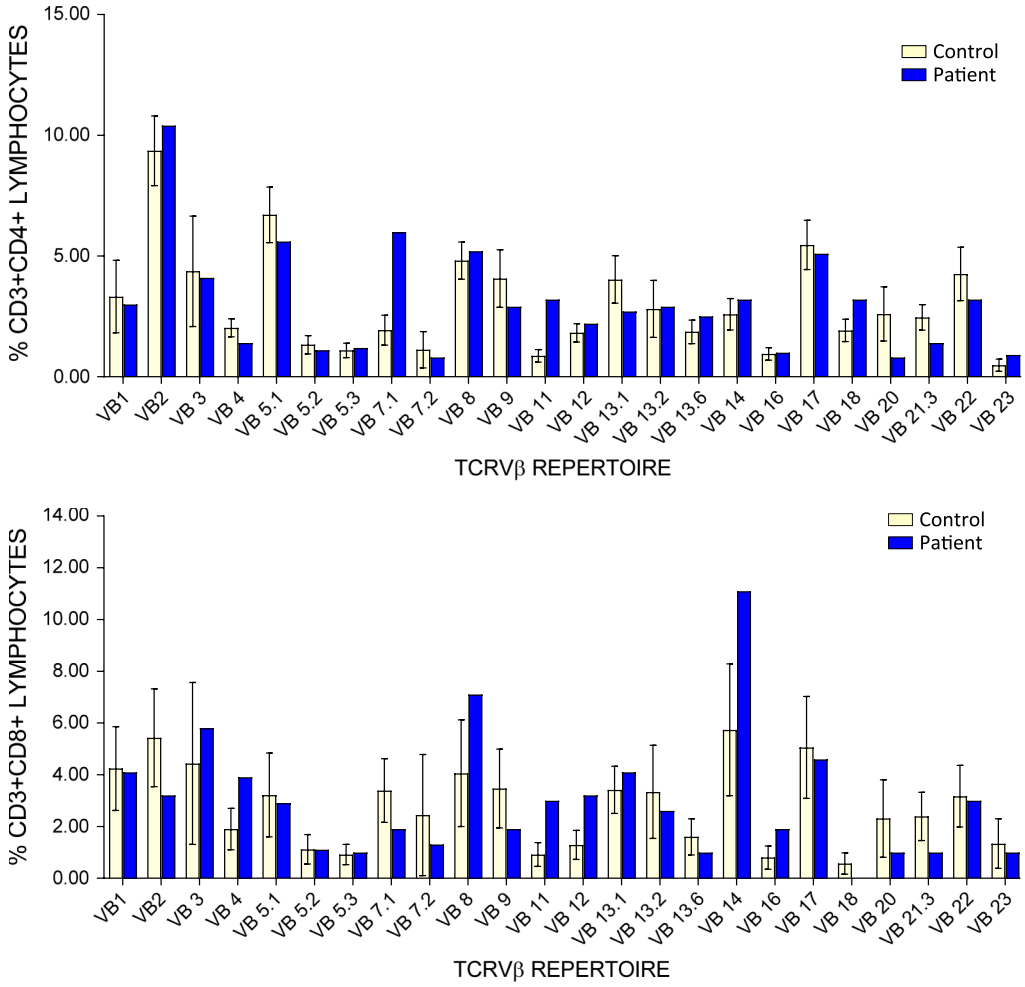

Figure 1: TCRVβ repertoire. TCRVβ analysis for clonality revealed mild expansion of 2 $CD4^+$ clones as well as 2 $CD8^+$ clones.

the formation of heterotrimeric or multiprotein complexes. The patient's mother was found to be a heterozygous carrier. Western blotting confirmed the complete lack of coronin 1A protein expression in the patient (Figure 4).

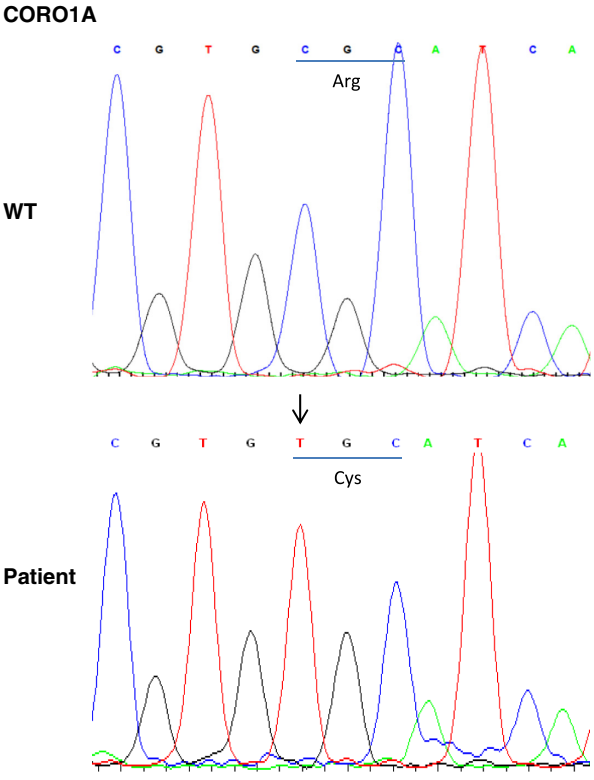

Figure 2: Homozygous mutation in *CORO1A*. Electropherogram of the wild-type (WT; upper panel) sequence and novel homozygous mutation detected in the patient, c.601C>T (lower panel), in the *CORO1A* gene.

Discussion

We report here the first coronin 1A deficient patient detected by positive NBS for SCID. This patient presented with leukopenia and neutropenia, but is currently doing clinically well at 14 months of age with no severe or recurrent infections. Previous reports show great variability in clinical presentations, ranging from patients presenting as severe combined immunodeficiency and severe vaccine-associated varicella infection (Shiow et al. 2008), through patients presenting with EBV related lymphoproliferation at a young age (Moshous et al. 2013) and a yet later onset of disease, at 7 years of age (Stray-Pedersen et al. 2014).

All patients reported thus far had T cell lymphopenia (Shiow et al. 2008; Moshous et al. 2013; Stray-Pedersen et al. 2014). Another feature common to most patients is the severe reduction in CD45Ra^{+} naïve T cells (Moshous et al. 2013; Moshous and de Villartay 2014; Stray-Pedersen et al. 2014) suggesting a role of coronin 1A in mature T cell survival or thymocyte development. However, B and NK cell counts, T cell responses to mitogen and antigens, as well as humoral function are all variable among patients (Shiow et al. 2008; Moshous et al. 2013; Mace and Orange 2014; Stray-Pedersen et al. 2014). One patient (Mace and

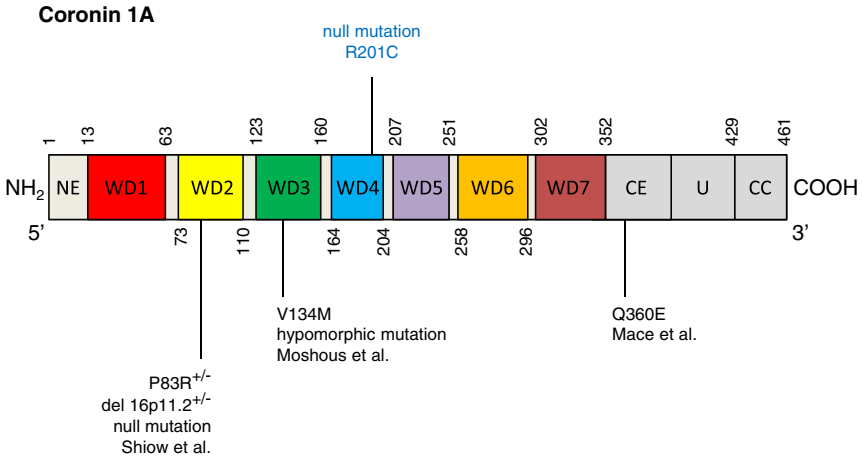

Figure 3: Localization of R201C mutation in WD repeat of Coronin 1A. Schematic representation of Coronin 1A protein. The major structural domains are depicted: CC, coiled coil domain; CE, C-terminal extension; NE, N-terminal extension; U, unique region. The 7 WD (tryptophan-aspartate) repeat domains forms a 7-bladed propeller. The novel c.601C>T homozygous mutation resulting in amino acid change p.Arg201Cys (R201C) is a loss of function mutation.

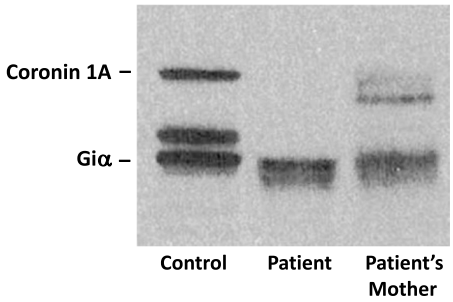

Figure 4: Analysis of Coronin 1A protein expression by western blotting. Complete absence of Coronin 1A protein expression is apparent in the patient. The patient's mother, a heterozygous carrier of the same mutation, has lower expression of protein compared to an unrelated control. Giα is shown as loading control.

Orange 2014) was described to have abnormalities in NK cytotoxic function.

A genotype–phenotype correlation was postulated previously (Moshous and de Villartay 2014). However, the finding of a kindred with late onset disease and a null homozygous mutation (Stray-Pedersen et al. 2014) make any assumptions on such a correlation difficult.

Early studies on coronin 1A in phagocytes indicated an important role in the rearrangement of actin and regulation of $p40^{phox}$, a component of the NADPH oxidase (Grogan et al. 1997). Defective NADPH oxidase function, which is responsible for production of microbicidal superoxide and reactive oxygen species, leads to chronic granulomatous disease. Binding studies show that coronin 1A associates with the C-terminal region of $p40^{phox}$. In the absence of its binding partner, coronin 1A and F-actin fail to rearrange during phagocytosis. Indeed, coronin 1A has also been shown to regulate other aspects of neutrophil biology, including trafficking and apoptosis. In murine models of coronin 1A deficiency, aberrant accumulation of lymphocyte-function associated antigen 1, needed to transition neutrophils from being firmly adherent to migratory, impairs leukocyte movement to sites of inflammation (Pick et al. 2017). Coronin 1A has also been identified by proteomic analysis to be involved in neutrophil apoptosis (Moriceau et al. 2009). Overexpression was associated with a lower rate of apoptosis due in part to delayed phosphatidylserine externalization, as shown in patients with cystic fibrosis. Functional analysis of neutrophils was not performed in our patient, however, one could hypothesize that the loss of coronin 1A expression may have resulted in dysregulated apoptosis and innate defense against infections.

We report here the first case of coronin 1A deficiency presenting as a clinically asymptomatic positive NBS for SCID. As the newborn TREC assay for screening for SCID is gaining worldwide prevalence, we might see an increase in diagnoses of coronin 1A as well as other combined immunodeficiencies and a broadening of the clinical spectrum of these conditions. Most combined immunodeficiencies are not diagnosed by the various NBS programs, whose purpose is to provide timely diagnosis and treatment only to the most severe cases of T cell deficiencies. As combined immunodeficiencies have a wide spectrum of disease severity, often within the same genetic entity, one would expect for the more severe presentations within the spectrum of disease to be diagnosed by low newborn TREC results. Whether this early recognition will result in an improved prognosis remains to be proven. The cutoff of a specific NBS program for reporting abnormal results, and the algorithm for investigating these results, also affect the capture rate of non-SCID T cell lymphopenias. This must be weighed against the resources in the specific program for follow up and investigation of these newborns.

Our patient presented with severe neutropenia along with her lymphopenia. This finding, which is also consistent in her asymptomatic mother, has not been previously reported in coronin 1A deficiency. Although coronin 1A is known to function as a regulator of cytoskeleton, it was not reported to affect neutrophil function (Combaluzier and Pieters 2009).

Summary

We hereby report the first case of coronin 1A deficiency presenting in a well newborn as part of the NBS program. Coronin 1A is a rare combined immunodeficiency, and the few cases reported in the literature had a variable although detrimental clinical course. As our patient is currently well, we are confronted with one of the challenges posed by early diagnosis of rare diseases, i.e., the inability of predicting prognosis, and

thus the difficulty in recommending a potentially risky procedure, such as HSCT.

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Establishing reference ranges for lymphocyte proliferation responses to phytohemagglutinin in patients with T cell dysfunction

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ABSTRACT

Introduction: The evaluation of lymphocyte proliferation responses is a critical component of the clinical work up for patients with suspected immunodeficiencies. Those with severe combined immunodeficiency (SCID) have consistently low to absent responses (stimulation index, SI) to the mitogen phytohemagglutinin (PHA). However, patients with combined immunodeficiency (CID) have more varied proliferative responses, and are open to a wide range of interpretations.

Aims: To establish lymphocyte proliferation response reference ranges for patients with T cell defects, especially those with CID as well as healthy controls.

Methods: Data was collected retrospectively from charts of patients with a diagnosis of SCID ($n = 39$), CID ($n = 52$), or from healthy controls ($n = 440$). Reference percentiles were calculated using the 95% of the distribution of the test results.

Results: The reference ranges for the control group ranged from 134 to 2220.5, whereas those with CID were distributed between 0.81 and 169.1. Patients with typical SCID had profound low proliferative responses, with SI <5.

Conclusion: Our results highlight the variability of lymphocyte proliferation responses to PHA in patients with CID as well as healthy controls. These reference ranges will assist with the critical interpretation of assay results, particularly when values fall on the extreme end of the range.

Statement of novelty: We provide reference ranges for lymphocyte proliferation responses to PHA from patients with CID and healthy controls.

Introduction

Mitogens, such as phytohemagglutinin (PHA), are plant lectins that are potent inducers of T cell proliferation (Nowell 1960). These non-specific stimuli activate the surface glycoprotein of T cell receptors and initiate intracellular signaling, ultimately triggering cell division (Sharon and Lis 2004).

For decades, assessment of lymphocyte proliferation has been a useful tool in the diagnosis of severe combined immunodeficiency (SCID). Patients with SCID invariably have low to absent responses to PHA (Roifman et al. 2012). However, in patients with combined immunodeficiency (CID), the results of these tests have not been standardized and are open to a wide range of interpretations (Roifman et al. 2012).

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Moreover, despite its use for over 50 years, there are currently no established reference ranges and the interpretation of test results is based upon comparison to healthy controls. This introduces multiple limitations, including variability associated with individual control samples, challenges of recruiting volunteers, and the additional labour associated with assaying control samples.

Given that the number of newly discovered CIDs continues to expand, improved interpretation of lymphocyte proliferation responses to mitogens, such as PHA, would aid in identifying such disorders.

In this study, we collected retrospective data of lymphocyte proliferation responses to PHA from 39 patients with SCID, 52 patients with CID, as well as 440 healthy controls. Our primary goal was to establish reference ranges for patients with T cell defects, especially in those with CID. Importantly, identification of the gene defect associated with these T cell disorders has allowed, for the first time, critical analysis of mitogen responses in the corresponding conditions. We have subsequently studied in a prospective manner the validity of these reference ranges in patients with SCID or CID.

Methods

Patients

Retrospective analysis

This cohort included patients who were diagnosed with SCID or CID who had a genetic diagnosis at the Hospital for Sick Children between 1990 and 2016. CID refers to patients who had lymphocyte counts of more than 500 cells/μL (Roifman et al. 2012), which is consistent with the definition provided by the International Union of Immunological Societies (Bousfiha et al. 2015). Informed consent (REB Protocol No. 1000005598) was obtained from parents and (or) patients as well as from healthy adult volunteers, and included consent for genetic analysis. Results of in vitro T lymphocyte proliferation responses to PHA were collected from charts of 39 patients with SCID, 52 patients with CID, and 440 healthy controls. The 39 patients with SCID had mutations in the following genes: ADA ($n = 14$), IL-2Rγc ($n = 13$), IL-7Ra ($n = 4$), CD3 delta ($n = 3$), DNA Ligase-4 ($n = 2$), JAK3 ($n = 1$), RAG1 ($n = 1$), and RAG2 ($n = 1$). Patients with CID had confirmed IL-2Rγc deficiency and maternal engraftment or leaky autologous cells ($n = 8$), Omenn's syndrome ($n = 10$), Zap-70 deficiency ($n = 6$), partial ADA deficiency ($n = 2$), mutations in RMRP gene ($n = 3$), MHC class II deficiency ($n = 3$), Rel-B deficiency ($n = 2$), coronin 1A deficiency ($n = 1$), IL-2Ra deficiency ($n = 1$), or unknown genetic defects ($n = 16$).

Mitogen proliferation assay

Lymphocyte proliferation responses to PHA were determined by ^{3}H-thymidine incorporation using the microtiter plate technique (Arpaia et al. 1994). In principle, the radioactive nucleotide thymidine is incorporated into DNA as cells enter the S phase of the cell cycle. The amount of ^{3}H-thymidine detected in cells stimulated with PHA is then divided by ^{3}H-thymidine detected in unstimulated cells (background), and the result is termed the stimulation index (SI) (Dean et al. 1977). In our assay, 5×10^{4} lymphocytes are plated, and subsequently activated and pulsed with 10 μg/mL PHA and 1 μCi ^{3}H-thymidine, respectively. A latent period of 3 days is observed before the lymphocytes start to proliferate (Nowell 1960). All assays were performed in triplicate and were compared with those simultaneously performed on normal controls.

Statistics

Reference percentiles for proliferation of T lymphocytes to PHA were calculated from healthy control, CID, and SCID groups, using the central 95% of the distribution of test results (lower limit: 2.5 percentile, upper limit: 97.5 percentile). 95% confidence intervals for the 2.5 and 97.5 percentiles were calculated around each of these limits.

Results

Lymphocyte proliferation responses to PHA

The SI reference percentiles (Table 1) for the control group ($n = 440$) ranged from 134 to 2220.5 (lower and upper limits, respectively), reflecting the inherent variability of samples from healthy individuals. In agreement with previous studies (Buckley et al. 1997; Roifman et al. 2012), patients with SCID ($n = 39$) had universally low T lymphocyte responses to PHA, with SI reference percentiles ranging from 0 to 4.1. Analysis of the SI from 52 patients with CID confirms the partial function of T cells, with the reference percentiles

Table 1: **Reference ranges for lymphocyte proliferation responses to PHA.**

Group	SI lower limit (p2.5)	SI upper limit (p97.5)	Sample size	Lower limit confidence interval	Upper limit confidence interval
Control	134	2220.5	440	(129, 152)	(1822, 2844)
CID	0.81	169.1	52	(0.8, 0.81)	(163.7, 241.3)
SCID	0	4.1	39	(0, 0.5)	(2.8, 4.1)

Note: Percentiles were calculated using the central 95% of the distribution of test results (lower limit: 2.5 percentile, upper limit: 97.5 percentile). 95% confidence intervals for the 2.5 and 97.5 percentiles were calculated around each of these limits (shown in the last 2 columns). CID, combined immunodeficiency; PHA, phytohemagglutinin; SCID, severe combined immunodeficiency; SI, stimulation index; p, percentile.

Table 2: **PHA stimulation index of patients with SCID.**

Mutation	Stimulation index	Sample size
ADA	0–4.1	14
CD3 delta	0.5–2.5	3
IL-2Rγc	0.47–2.8	13
IL-7Ra	0.81–1.4	4
JAK3	0.6	1
RAG1/2	1.29–1.5	2
DNA ligase-4	0.92–2.5	2

Note: Lymphocyte proliferation responses (stimulation index) to the mitogen PHA are summarized according to the patients' genetic mutation. PHA, phytohemagglutinin; SCID, severe combined immunodeficiency.

Table 3: **PHA stimulation index of patients with CID.**

Mutation	Stimulation index	Sample size
ADA (partial)	1.9–163.7	2
Coronin 1A	46	1
IL-2Ra	27.5	1
IL-2Rγc, R222C	29.2–111.3	3
IL-2Rγc, maternal engraft	0.81–17	5
MHC class II	62.5–109.1	3
Omenn's syndrome	0.8–241.3	10
Rel-B	108.5–169.1	2
RMRP	3.1–63.8	3
Zap-70	0.81–2.4	6
Unknown CID	0.86–105	16

Note: Lymphocyte proliferation responses (stimulation index) to PHA are summarized according to the patients' genetic mutation. PHA, phytohemagglutinin; SCID, severe combined immunodeficiency.

ranging between 0.81 and 169.1. A summary of the SI from patients with SCID or CID are shown in Tables 2 and 3, respectively. In the CID cohort, 51 of 52 patients had an SI of <200. Patients with IL-2Rγc deficiency and maternal engraftment had SI of 0–30, patients with Omenn's syndrome had SI of 0–20, except for 1 patient with an SI of 241. We found that 61% of CID patients had SI below 20 (71% with an SI <40, 78% with an SI <60, 86.6% SI <100). In 6 patients with Zap-70 deficiency, SI results were in the range of SCID, 0–5 (all <3). One patient with partial ADA deficiency had a SI of 1.9 and the second patient had a SI of 163. A patient with IL-2Ra deficiency had a SI of 21–30. One patient with RMRP mutation had a SI of 0–5, and the 2 others had SI of 51–100. Three patients with R222C mutations had SI: 29, 86, and 111. Three patients with MHC class II deficiency had SI: 62, 86, and 109. A patient with coronin 1A deficiency had SI of 46. Two patients with Rel-B mutations had SI of 101–150. 16 patients with CID of unknown molecular diagnoses had SI ranging from 0 to 150.

Discussion

Different centres have adopted varied methods for interpreting results of lymphocyte proliferation responses. Some have used an arbitrary threshold of counts from ^{3}H-thymidine incorporation to distinguish normal from abnormal response, with most setting up side-by-side patient and normal control samples to be used as comparators. Regardless of the method used, they were all accurate in detecting typical SCID samples which were almost invariably extremely low. However, with the expanding recognition of a wide spectrum of T cell immunodeficiencies (CID), this type of analysis has posed increasing challenges in defining abnormal responses to mitogens.

One solution adopted by many centres was to arbitrarily define a response as normal if the patients' lymphocyte stimulation index exceeded 50% of control. We found this method often times inadequate because of the great variability in single samples obtained from control and normal individuals.

In our study, SI of healthy controls ranged from 134 to 2220.5, demonstrating the wide range of the results of this test. Importantly, this highlights the issue of interpreting patients' results based on direct comparison to single samples. For example, a patient who is evaluated but doesn't have intrinsic T cell defects and has a normal SI can be interpreted as abnormal if the control is in the upper range; hence, the patient SI

might be <50% of the control. This may lead to a false positive result. The opposite is also possible, where if the control SI is very low, a patient with an intrinsic T cell defect but an SI above 50% of that control might be interpreted as normal, which could result in a false negative result.

There are groups of CIDs that could have normal lymphocyte enumeration, for example NEMO mutation with immunodeficiency (Orange et al. 2004), R222C mutations in IL-2Rγc (Somech and Roifman 2005), and Rel-B deficiency (Merico et al. 2015), but abnormal mitogen proliferation results. This test therefore becomes important in increasing the suspicion of CID. Furthermore, the results of this study help to establish the SI of healthy controls and assists in the critical interpretation of control values, especially when their SI is at the extreme end of the "normal" range.

In our cohort, all the patients with typical SCID had profound low proliferative responses, with SI <5, consistent with previous studies (Buckley et al. 1997; Roifman et al. 2012). Patients with non-typical SCID, Omenn's syndrome or patients with maternal T cell engraftment had very low SI, yet somewhat higher than typical SCID (SI 0–30).

Remarkably, almost all patients with CID (98%) had SI of <200 and almost 85% had SI <100. Patients with Zap-70 deficiency ($n = 6$) had profoundly low mitogen responses, indistinguishable from SCID. The 2 patients with partial ADA had wide differences in their mitogen response. The first patient had a SI of 1.9 while the second had a SI of 163. PHA responses in cases with cartilage hair hypoplasia were variable, ranging from 5 to 100. Patients with MHC class II deficiency had SI well within the range of CID, unlike previous publications suggesting that these might have normal mitogen responses, but absent antigen responses (Al-Mousa et al. 2010). This is a good example of the advantage of using our analysis to define normal ranges rather than assaying single samples. The lower SI in MHC class II deficiency might be related to the decreased numbers of CD4+ cells, which contribute to about two-thirds of the T cell population as well as disturbed co-stimulatory molecules that play an important role in mitogen proliferation (Bonilla 2008). In a similar manner, patients with CID due to Rel-B, CD25, coronin 1A and others have all consistently had PHA levels below 200.

It is notable that some CIDs may present with normal proliferative responses against PHA, but not other mitogens, such as CD3/CD28. For example, loss-of-function mutations in caspase recruitment domain family, member 11 (Stepensky et al. 2013) result in aberrant NFκB signaling and hypogammaglobulinemia. However, PHA proliferative responses are preserved. Thus, it should be kept in mind that no single laboratory test can identify or define impaired cellular immunity, and all tests should be interpreted with due caution and correlated with clinical context.

Recently, alternative techniques to assess T cell proliferation have been offered. One method is to track lymphocyte division using the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE), developed by Lyons and Parish (1994). With this method, the CFSE dye is diluted by half with each cell division until it becomes undetectable (Quah et al. 2007). Another method uses 5-ethynyl-2′-deoxyuridine (EdU), a fluorescent nucleoside analogue, which directly measures de novo DNA synthesis. EdU incorporates into the DNA of dividing cells using click chemistry and the fluorescence emitted serves as an index of cell proliferation (Yu et al. 2009). The latter method seems to correlate better to ^{3}H-thymidine when studied in mice (Yu et al. 2009). The disadvantages of these assays include increased cost, dependence on expensive and complex equipment, and more importantly, the lack of proper control ranges for CID. Studies that compare flow cytometry based assays with ^{3}H-thymidine will aid in defining the advantages and limitations of these assays.

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Report of the National Immunoglobulin Replacement Expert Committee: algorithm for diagnosis of immunodeficiency requiring antibody replacement therapy

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ABSTRACT

Immunoglobulin replacement therapy is a mainstay in the treatment of immune deficiencies characterized by antibody failure. Whether the cause is primary or secondary, affected patients frequently present with a history recurrent and complicated infections of the upper and (or) lower respiratory tract. Such replacement therapy has been available since the 1980s, although treatment modalities have since been refined to provide improved protection against infections resulting in reduced morbidity and mortality. Here, we describe an algorithm for diagnosing patients with suspected primary or secondary immunodeficiency, including assessment of clinical, laboratory, and genetic information, when considering initiating immunoglobulin replacement. The increasing availability of molecular genetic techniques will likely result in decreased diagnostic delay for these patients.

Statement of novelty: We describe here an algorithm for diagnosing patients with immunodeficiency requiring immunoglobulin replacement therapy.

Immunoglobulin replacement therapy is a mainstay in the treatment of humoral immune deficiencies, including many well characterized primary immunodeficiencies (PID) (Picard et al. 2015) as well as secondary immunodeficiencies (SID). The widespread availability of intravenously infused immunoglobulin (IVIG) from the 1980s opened the door towards more effective therapeutic options for those with antibody failure, by normalizing serum IgG levels and providing improved outcomes for infections. Early studies by Roifman and colleagues

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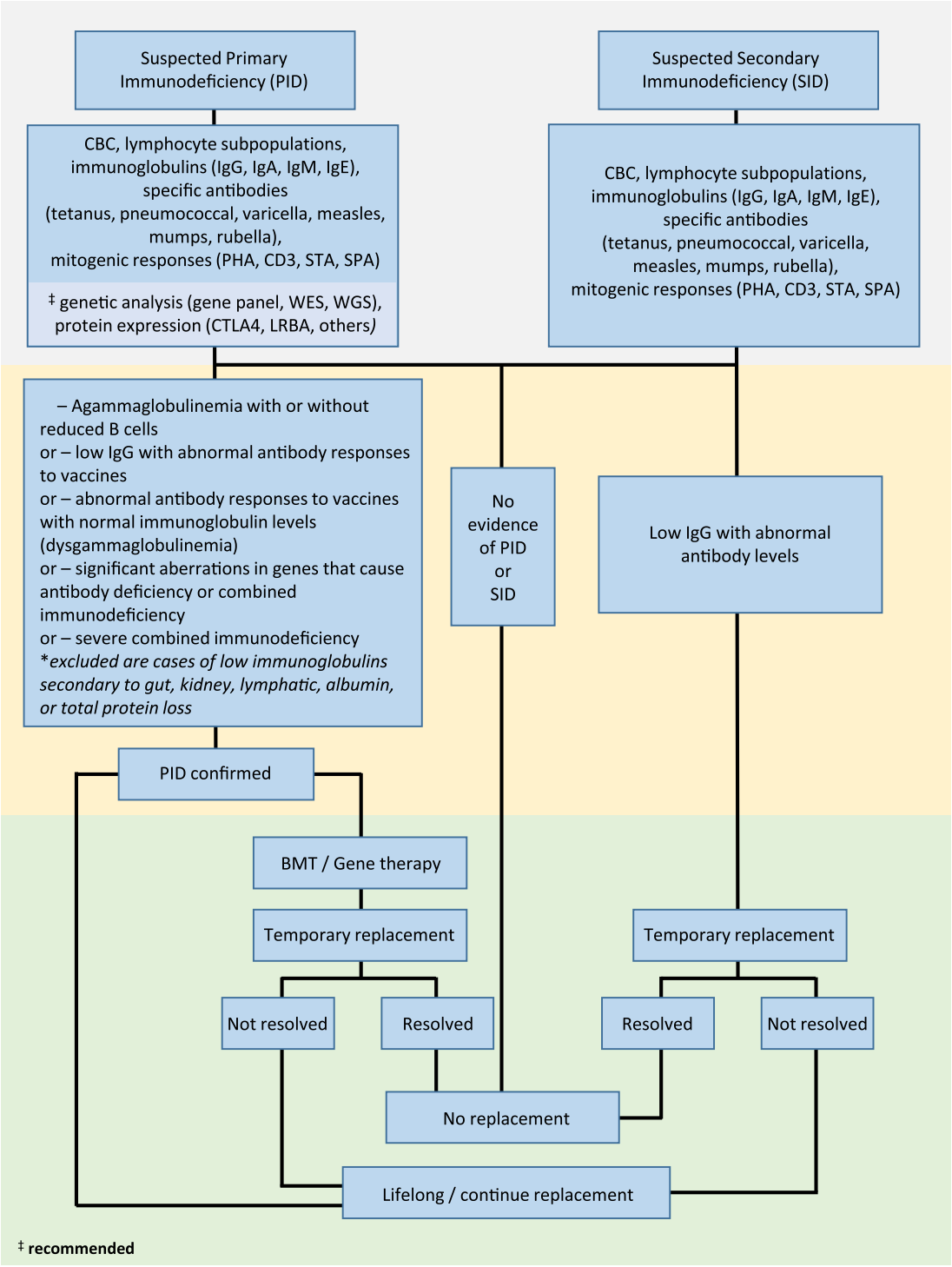

Figure 1: Algorithm for diagnosing patients with immunodeficiency requiring immunoglobulin replacement. BMT, bone marrow transplantation; CBC, complete blood count; Ig, immunoglobulin; PHA, phytohemagglutinin; STA, *Staphylococcus aureus*; SPA, staphylococcal protein A; WES, whole exome sequencing; WGS, whole genome sequencing.

demonstrated an improvement in lung function in patients receiving a higher dose of IVIG, 600 mg/kg, compared to a lower dose of 200 mg/kg (Roifman et al. 1987). In the decades since, indices of efficacious immunoglobulin replacement therapy have been refined, including a move to more individualized IgG trough levels (ranging from 500 to 1700 mg/dL) that prevent recurrent and complicated infections (Lucas et al. 2010).

While the underlying cause of PID and SID differ, the clinical manifestations are usually similar, most commonly, infections of the upper and (or) lower respiratory tract caused by encapsulated bacteria as well as infections of the gastrointestinal tract. Here, we describe the algorithm for diagnosing patients with suspected PID or SID, including assessment of clinical, laboratory, and genetic information, as well as considerations for initiation of immunoglobulin replacement therapy.

Clinical suspicion of PID (including severe combined immunodeficiency, SCID; combined immunodeficiency, CID; common variable immunodeficiency disorder, CVID; or unclassified primary antibody deficiencies) and SID (due to B cell lymphoproliferative disease, protein loss, abnormal lymphatic circulation, drug-related therapies, or increased catabolism) requires thorough patient history assessment and laboratory work up (Figure 1).

Given that antibody deficiency is characterized by recurrent infections, laboratory parameters that assess baseline and functional immune responses are essential. These include a complete blood count (CBC), flow cytometric determination of lymphocyte phenotypes (T, B, and NK cell types), serum levels of immunoglobulins (IgG, IgA, IgM, and IgE), and determination of specific antibody responses to vaccines (both protein vaccines, such as tetanus toxoid, as well as carbohydrate type vaccines, including polysaccharide pneumococcal). Assessment of lymphocyte proliferation to mitogens (phytohemagglutinin, CD3, STA, and SP3) should be performed for suspected PID. Where possible, genetic analysis (assessment by panel of known genes involved in immunodeficiency, or next generation sequencing techniques including whole exome or whole genome sequencing) can be applied to identify disease-causing variants. Similarly, flow cytometric determination of suspected cell anomalies can also be performed.

Where PID is confirmed with findings of agammaglobulinemia with or without reduced B cells, or low IgG with abnormal antibody responses to vaccines, abnormal antibody responses to vaccines with normal immunoglobulin levels (dysgammaglobulinemia), significant aberrations in genes that cause antibody deficiency, or diagnosis of SCID, immunoglobulin replacement therapy should be initiated. Where appropriate, considerations should be made for curative bone marrow transplantation or gene therapy.

In cases where SID is confirmed with evidence of low IgG in combination with abnormal antibody levels, immunoglobulin replacement should be initiated on a temporary basis until the antibody failure has resolved.

In summary, the decision to proceed with immunoglobulin replacement should be made following thorough assessment of clinical and laboratory findings. It is important to note that the true efficacy of replacement therapy should be based on the prevention of infections, rather than serum IgG levels, leading to reduced morbidity and mortality and improved quality of life. The increasing availability of molecular genetic techniques will likely result in decreased diagnostic delay for these patients.

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