Eur J Pediatr (2011) 170:1369–1376
DOI 10.1007/s00431-011-1584-5

REVIEW

Educational paper

Defects in number and function of neutrophilic granulocytes causing primary immunodeficiency

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Received: 21 June 2011 / Accepted: 13 September 2011 / Published online: 4 October 2011
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Abstract The neutrophilic granulocyte (neutrophil) is the most important cellular component of the innate immune system. A total absence of neutrophils or a significant decrease in their number leads to severe immunodeficiency. A mature neutrophil, released from the bone marrow, should be able to migrate from the blood towards the tissues, following a chemotactic gradient to a pathogen. In order to be neutralized, this pathogen has to be recognized, phagocytosed, and destroyed by lytic enzymes contained in the neutrophil's granules and reactive oxygen species formed by the enzyme complex NADPH oxidase. Rare genetic defects leading to the loss of each one of these biological properties of the neutrophil have been described and are associated with immunodeficiency. This review provides a summary of the normal development and biological functions of neutrophils and describes the diseases caused by defects in neutrophil number and function.

Keywords Neutrophilic granulocyte · Severe congenital neutropenia (SCN) · Leukocyte adhesion deficiency (LAD) · Chronic granulomatous disease (CGD) · NADPH oxidase · Immunodeficiency

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Introduction

Fulminant infections in infants and young children are dramatic events and should be evaluated thoroughly in countries were hygienic standards have made such diseases exceptional in children. An unusual severe course of infection or an unusual pathogen, recurrent pyogenic infections, and recurrent infections of the upper and lower respiratory tract may be caused by immunodeficiency due to an inborn absence or significantly decreased number of neutrophilic granulocytes (neutrophils) or functional defects of these cells [9] (Table 1).

Similar to all primary immunodeficiencies, congenital neutropenia and functional disorders of neutrophils are rare. The lack of exposure to these diseases for the average clinician renders it difficult to decide whether a work-up of neutrophil function ought to be included in the diagnostic process. This often leads to delay in the diagnosis of these life-threatening diseases.

In general, a lack of functionally normal neutrophils in an individual may lead to infections with *Staphylococcus aureus*, gram-negative organisms such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*, the gram-positive bacterium *Nocardia asteroides*, and infections with fungi and yeasts such as *Aspergillus* spp. and *Candida* spp. (Fig. 1). In the case of *S. aureus*, the severity of the disease or the location of the infection (e.g., liver abscess) distinguishes an immunocompromised patient from a healthy individual. The other above-mentioned pathogens, including fungi, do not cause disease in an immunocompetent child. Hence, once suspected or identified, such infections should always lead to scrutinizing neutrophil number and functions.

Immunodeficiencies caused by lack of neutrophils due to an inborn error are rare, but the initial step in diagnosing

Table 1 Most important diseases caused by low numbers or dysfunction of neutrophils

Category	Subgroup	Diagnosis	Treatment
1. Lack of neutrophils (neutropenia)	Iatrogenic/toxic	Effect of cessation of drug therapy	Removal of toxic agent
	Immune mediated	Detection of auto- or alloantibodies	None, usually transient
	Infection related	Virological tests	None, usually transient
	Severe congenital neutropenia	Bone marrow aspiration	G-CSF
		Gene analysis	Stem cell transplantation
	Syndrome associated	Genetic counselling Dependent of presumed cause	Dependent of cause
2. Decreased motility	Leukocyte adhesion deficiencies (LAD1, LAD2, LAD1 variant/LAD3)	Functional adhesion tests Gene analysis	Stem cell transplantation
3. Decreased "danger sensing"	Toll-like receptor deficiencies (IRAK4 deficiency, MyD88 deficiency)	Functional tests Gene analysis	None, mild clinical course from late childhood on
4. Impaired killing mechanisms	NADPHoxidase dysfunction (chronic granulomatous disease)	Functional tests Gene analysis	Stem cell transplantation prophylactic (antibiotics, antifungals, γ-interferon)
	Impaired granule formation (Chédiak–Higashi syndrome, specific granule deficiency)	Microscopic (electron) evaluation Gene analysis	Stem cell transplantation

such a disorder is easily taken, since neutropenia will be detected by a routine blood count with differential. When neutropenia persists and more common causes are excluded, further work-up can reveal an underlying genetic cause. Several gene defects have been identified that are responsible for an inadequate maturation of neutrophil precursors in the bone marrow [27]. Alternatively, in myelokathexis, the release from the bone marrow of otherwise normally developed neutrophils is hampered, leading to neutropenia in the blood and tissues [10].

In recent years, numerous *functional defects* of neutrophils caused by genetic defects have been identified. As cell counts and standard blood tests are normal in these cases, the diagnosis is difficult. The suspicion of a functional defect has to be substantiated by highly specialized tests, which are expensive and performed in few laboratories, and is thus not always easily accessible in every country. It is therefore suspected that many cases remain undiagnosed, and patients may possibly die without diagnosis, even in Europe.

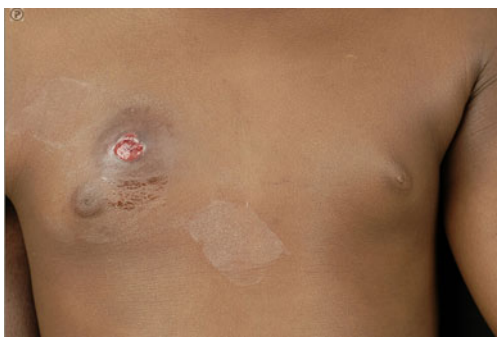

Fig. 1 An ulcerative lesion at the right chest of a formerly healthy 2-year-old boy. The rare fungus *Rhizopus oryzae* caused this infection which led to the diagnosis auosomal recessive chronic granulomatous disease

Fundamental research over the past decades has led to increased knowledge on neutrophil biology, in particular the elucidaton of functional defects. In patients that present with a clinical phenotype suggestive of a neutrophil defect, careful analysis will over time reveal other proteins that are essential for normal neutrophil development and function. This was recently illustrated by the finding of our group and others that mutations in *FERMT3*, the gene encoding kindlin-3, are responsible for the disease described as leukocyte adhesion deficiency 1 variant (LAD1 variant) or LAD3 [18, 22].

As to date, defects in adhesion molecules, essential for migration of neutrophils, defects in granule formation, and an inability to undergo a respiratory burst are examples of well-defined functional disorders of neutrophils. This article will discuss the most important primary immunodeficiencies caused by defective numbers or function of neutrophils, as compared to the normal biology of these cells.

Neutrophil development and neutropenia

Neutropenia can be mild (1,000–1,500/μL), moderate (500–1,000/μL), or severe (<500/μL). Iatrogenic neutropenia dwarfs all other causes. Otherwise, neutropenia is most frequently caused by auto- or alloantibodies or is associated with bone marrow depression in the course of viral infections such as EBV, CMV, or parvovirus. Because

these causes are transient, neutrophil numbers usually recover spontaneously. However, these causes may also lead to persistent neutropenia for a considerable period of time and should thus be excluded before the exceptional genetic causes are considered.

Neutrophils mature in the bone marrow in about 2 weeks, a process in which the myeloid-specific growth factors granulocyte colony-stimulating factor (G-CSF) and granulocyte–monocyte CSF play an important role. In the first half of this period, the neutrophil precursors differentiate from myeloblasts through promyelocytes to neutrophilic myelocytes. In the promyelocyte stage, the azurophil granules are formed, and the specific granules are formed in the myelocytic stage. Later stages of neutrophil differentiation comprise metamyelocytes, band forms, and segmented cells, characterized by the typical appearance of the nucleus (Fig. 2). Further divisions do not take place during this period.

Once in the blood, neutrophils are short-living cells with a circulating half life of 6–8 h. Then, they leave the blood and move to the tissues, where they live for about two more days, after which they presumably die by apoptosis. To maintain equilibrium they are produced at a rate of 5–10$\times 10^{10}$ a day in the bone marrow. The bone marrow contains an amount of mature neutrophils 10 times the number of circulating neutrophils. During infections, release of neutrophils and band forms from the marrow is accelerated, leading to neutrophilia.

The presence of maternal alloantibodies which have passed the placenta and are directed against epitopes on the child's neutrophils is a common cause of neutropenia in the first months of life. Also in early infancy, autoantibodies against epitopes NA1 and NA2 can lead to destruction of neutrophil precursors and thus neutropenia. Because of the often mild and transient course of this disease, it is called benign neutropenia of infancy (BNC). BNC most often recovers spontaneously around 3 years of age [6]. Autoantibodies against neutrophil epitopes may also appear in the course of autoimmune disease.

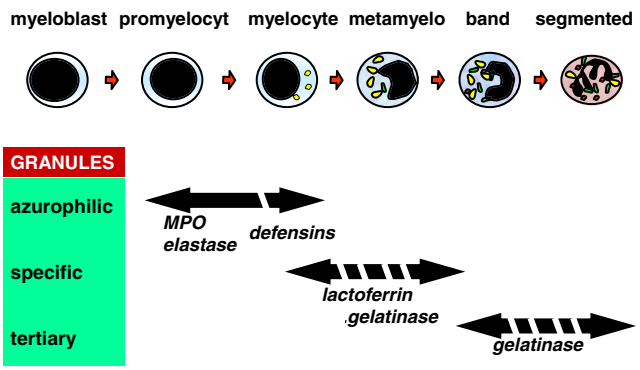

Fig. 2 Myelopoiesis and neutrophil differentiation in the bone marrow, showing the consecutive appearance of granules and their contents

When neutropenia is present and common causes as described above are excluded, a first step is bone marrow aspiration to perform a microscopical examination and a myeloid cell culture to assess the production, maturation, and differentiation of neutrophil precursors. Subsequently, analysis of the most common genes that cause neutropenia might be performed.

The most common cause for severe congenital neutropenia (SCN) are mutations in the gene *ELA2* or *ELANE* [8]. The same gene is affected in cyclic neutropenia in which severe neutropenia occurs with a relatively strict periodicity of 3 weeks. There is no clear genotype–phenotype relationship. We have recently described a multigenerational family with SCN, indicating a clear autosomal dominant inheritance of the *ELANE* mutation. This family displayed considerable variability in the clinical penetrance of neutropenia [26]. The pathophysiology of *ELANE* mutations in SCN is not completely understood, but evidence points towards a role for elastase in maintaining a normal function of the endoplasmic reticulum (ER) in myeloid precursor cells, leading to early apoptosis.

The classical form of SCN described by Kostmann (hence the name “Kostmann's syndrome”) is caused by autosomal recessive mutations in the *HAX1* gene [2, 3].The Hax-1 protein plays a role in the function of mitochondria and possibly accelerates apoptosis. Other identified genetic causes for neutropenia are mutations in *CSFR3* (encoding the G-CSF receptor) and *GFI1* (encoding a hematopoietic transcriptional repressor). Both disorders are autosomal dominant [17, 27].

Recently, autosomal recessive mutations in the glucose-6-phosphatase catalytic subunit 3 (*G6PC3*) gene have highlighted the importance of glucose homeostasis for the survival of neutrophils. Patients with G6PC3 deficiency also suffer from cardiac and urogenital malformations [5]. Recent data from the European Severe Chronic Neutropenia Registry (SCNER) showed that 56% of patients with SCN have heterozygous mutations in *ELANE*, 18% have biallelic mutations in *HAX-1*, and 4% show mutations in *G6PC3*. Around one third of patients (29%) are genetically unclassified [17, 27].

Patients with neutropenia frequently present with persistent oral ulcerations, apart from infections with the above-mentioned microorganisms (Fig. 3). Severe neutropenic patients can be treated with G-CSF to reduce the number of infections and increase survival. Unfortunately, long-term treatment with recombinant G-CSF is associated with an increased incidence of leukemia. It has been suggested that the increase in leukemia may be due to the G-CSF treatment. However, it now appears more likely to be due to the increased survival of preleukemic patients [27].

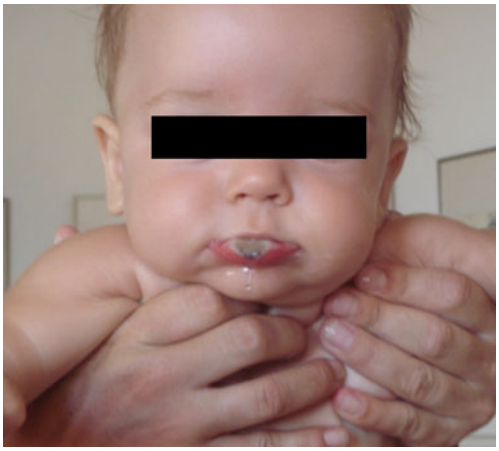

Fig. 3 Oral ulceration in a child with severe congenital neutropenia, who had also suffered from pneumonia and recurrent otitis

The pathophysiology of another disorder characterized by neutropenia sets it apart from the above-mentioned diseases. A rare combined immunodeficiency disorder, referred to as WHIM syndrome, is characterized by disseminated human papillomavirus-induced warts, hypogammaglobulinemia, recurrent bacterial infections (immunodeficiency), and myelokathexis, a form of neutropenia caused by an abnormal retention (i.e., *kathexis* in old Greek) of mature neutrophils in the bone marrow. Analysis of the bone marrow aspirate distinguishes myelokathexis from other causes of neutropenia.

It is not known which factors exactly cause egress of blood cells from the bone marrow under normal conditions. Evidence suggests that protease release by neutrophils in the bone marrow may play a role. Cases of WHIM syndrome have been linked to autosomal dominant gain of function mutations in the gene encoding CX chemokine receptor 4, resulting in hyperresponsiveness to its ligand stromal-derived factor 1 leading to decreased motility [10].

Neutropenia in childhood can also be one of the symptoms of various syndromes. Then, it is usually not the presenting or dominant symptom, but it may lead to severe infections, against which precautions should be taken.

Functional defects:

1. *Neutrophil motility and adhesion*

   Leukocytes recognize concentration differences in a gradient of chemotaxins and thus direct their movement towards the source of these agents, i.e., towards the inflammatory site. Apart from the ability to move, binding to endothelial cells is essential for neutrophils to leave the bloodstream. Movement between or through endothelial cells is mediated by adhesion molecules. Further on, neutrophils bind to tissue cells and extracellular matrix components (Fig. 4). These interactions are dynamic because in order to move, after first binding their ligands, adhesion molecules at "the tail of the movement" subsequently must be able to detach. By then binding to other ligands at "the front of the movement," the neutrophil is able to move on. Of course, these adhesion molecules and their controlled ability to switch on and off (i.e., get activated and deactivated) are tightly regulated [19].

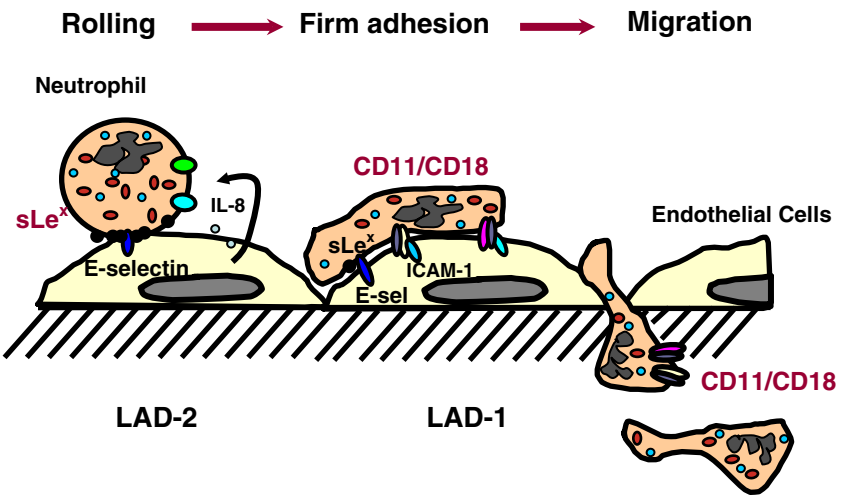

Fig. 4 Neutrophil motility. Activation of endothelial cells leads to rolling of neutrophils in the blood circulation along the endothelial cell lining, mediated by adhesion molecules called selectins, and their sugar ligands. This results in firm adhesion, mediated by the family of adhesion molecules called integrins. These steps are essential for the subsequent migration of neutrophils, between the endothelial cells, towards the inflammatory focus in the tissues beneath. Absence of these adhesion molecules in LAD2 (selectin ligands) and LAD1 (β2 integrins) leads to immunodeficiency because otherwise normal neutrophils are unable to leave the blood stream and reach the inflammatory site

Chemotactic peptides are released by infecting microorganisms and the host complement system [the split product of activated C5 (C5a)]. In addition, lipid mediators, such as leukotriene B_4 and platelet-activating factor, are also strong chemoattractants. For each of these agents, receptors are present on neutrophils. Furthermore, interleukin-8, a member of chemokine superfamily family of leukocyte activators, is a powerful chemoattractant and activator for neutrophils.

Small GTPases of the Rho family, in particular Rac-2 in neutrophils, have been shown to be crucial in the signaling leading to cytoskeletal remodeling and cellular motility. This is exemplified by an extremely rare defect in chemotaxis, ROS production, and degranulation in neutrophils of patients with Rac2 deficiency [1].

Firm adhesion is severely compromised in neutrophils lacking expression of the β2 integrin chain, leading to complete absence of an important family of adhesion molecules, the β2 integrins. The classical adhesion deficiency is called LAD1, caused by mutations in the gene encoding the common β2 subunit. In this disease, otherwise structurally normal neutrophils are not able to attach to endothelial cells and are thus retained in the bloodstream. It is characterized by delayed separation of the umbilical cord followed by an often fatal omphalitis. The blood count shows an extremely high number of neutrophils, sometimes prompting physicians to first consider infant leukemia [11].

Whereas β2 integrins are essential for firm binding and attachment, other adhesion molecules are needed to initiate contact between neutrophils and endothelial cells, leading to "rolling" on the endothelial cell lining. This is an essential step prior to firm adhesion. In LAD2, this process is disturbed. It is caused by absence of the SLeX oligosaccharide, the ligand for interaction with adhesion molecules of the selectin family on the leukocyte. This causes a marked decrease in chemotaxis accompanied by pronounced neutrophilia. Apart from the leukocyte defect, these patients suffer from severe mental retardation and stunted growth and exhibit the rare Bombay blood group type. Infections are but one of the prominent characteristics of this syndrome [12].

LAD3 or LAD1 variant is characterized by the combination of a relatively mild LAD phenotype and platelet dysfunction in the presence of normal integrin expression. An extensive study of several families with individuals presenting with this phenotype showed mutations in the gene *FERMT3*, encoding a hematopoietic structural protein called Kindlin-3 that acts as a switch to activate integrins [18, 22].

2. *Sensing danger*

Toll-like receptors (TLRs) are proteins that play a key role in the innate immune system. They are receptors for structurally conserved molecules derived from microbes. Once these microbes have entered the body, they are recognized by TLRs which activate immune cell responses.

Neutrophils express all TLRs, except TLR3 and TLR7 [14, 23]. Most TLR family members share a signaling motif called TIR domain, which is responsible for activation and/or translocation of a pivotal nuclear transcription factor NF-κB. This is an essential signaling pathway leading to secretion of IL-1β and TNFα thus initiating the inflammatory response. Further downstream, all TLRs signal through MyD88 and Mal, which interact with various IL-1 receptor-associated kinases (IRAKs).

These data have been substantiated in neutrophils from children with recurrent pyogenic infections, especially by *Streptocccus pneumoniae*, caused by an inherited IRAK4 deficiency [23]. These patients, once they have survived early childhood, tend to recover during adolescence. It is hypothesized that this is related to maturation of the humoral response which renders the innate response to streptococcal infections mediated by IRAK4 redundant. A clinically identical phenotype as reported for IRAK4 deficiency is seen in rare patients with autosomal recessively inherited mutations in the gene encoding MyD88 [4].

3. *Killing mechanisms*

Phagocytosis is the initial step which allows neutrophils to destroy an invasive pathogen. In order to be phagocytosed, a pathogen needs to be opsonised. Immunoglobulins, which bind specifically to their antigens, and complement factors, which bind aspecifically to conserved microbial motifs, provide an anchor for their respective receptors on the neutrophil surface. After phagocytosis, the process of pathogen killing starts immediately. The combined activity of an active enzyme complex that is able to produce toxic oxygen radicals as well as the release of various lytic proteins which are stored in the granules (elastase, cathepsin G, defensins) are required to eliminate invading pathogens [13] (Fig. 5).

Chronic granulomatous disease (CGD) is the most frequent disorder of neutrophil function. In CGD, components of an enzyme complex named NADPH oxidase are absent, which leads to an inability to kill phagocytosed microorganisms. Apart from fulminant infections by bacteria and fungi, this also leads to chronic, low-grade inflammatory reactions characterized by granuloma formation. In the gut, this process is indistinguishable from Crohn's disease, in the lung CGD that can mimic sarcoidosis [25] (Fig. 6).

The NADPH oxidase complex is composed of two subunits in the plasma membrane (flavocytochrome

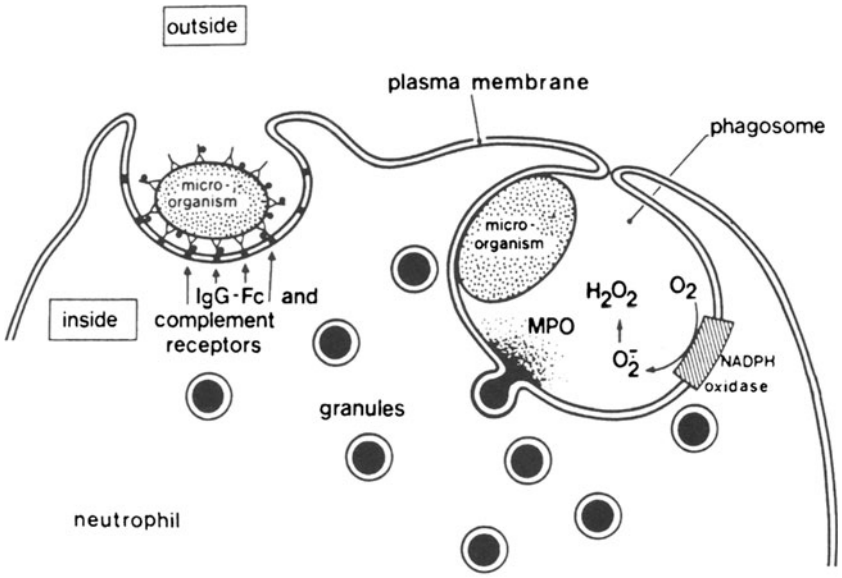

Fig. 5 Neutrophil killing. A microorganism is opsonized with immunoglobulins and complement and thus recognized by a neutrophil. The pathogen is then engulfed (phagocytosis) and is digested within the phagosome by lytic enzymes stored in the neutrophil's granules and toxic oxygen radicals formed by the NADPH oxidase

b_{558} subunits p22-*phox* and gp91-*phox*), and three activity-regulating proteins (p40-*phox*, p47-*phox*, and p67-*phox*) which form a complex in the cytosol of resting cells that unfolds upon neutrophil activation [20]. At the same time, the small Rac GTPase is also activated [1].

Only when all these components interact, the specific NADPH-binding site in gp91-*phox* becomes available for NADPH from the cytosol. Then and only then, superoxide (O_2^-) is generated. NADPH donates electrons, which are passed on within gp91-*phox* to molecular oxygen in the phagosome, thus generating superoxide. Superoxide spontaneously dismutates into hydrogen peroxide (H_2O_2), which may then react with chloride ions to form hypochlorous acid (bleach), in a reaction catalyzed by myeloperoxidase. This enzyme is an abundant constituent of neutrophil azurophil granules and is released upon cell activation into the phagosome.

Genetically, we can distinguish X-linked CGD, with mutations in *CYBB*, the gene that encodes gp91-*phox*, and the less common autosomal recessive form of CGD, with mutations in any of the other NADPH oxidase components. The autosomal recessive form is characterized by better survival [25].

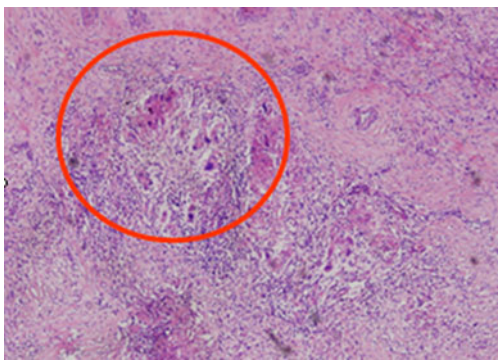

Fig. 6 Two granulomatous lesions in the thymus of an X-linked CGD patient

Killing in neutrophils is also impaired in patients with an abnormal formation of granules. This is the case in Chédiak–Higashi Syndrome (CHS), in which granule formation in many granule-containing cells is disturbed because of mutations in the *LYST* gene, which encodes a protein essential for lysosomal trafficking. One of the hallmarks of CHS is the presence of giant inclusion bodies seen in neutrophils in a blood smear, reflecting abnormal granule formation in these cells. However, neutrophil dysfunction in CHS is not a dominant feature. Partial albinism may point to the diagnosis, but abnormal function of cytotoxic T cells leading to uncontrolled inflammatory responses and lethal hemophagocytosis is by far the most important feature of this disease [15].

Another extremely rare disorder of neutrophil killing is neutrophil-specific granule deficiency (SGD). Neutrophils from SGD patients lack secondary and tertiary granules. Gene-inactivating mutations in the *C/EBPepsilon* gene have been identified in two SGD patients. The protein encoded by this gene plays an essential role in granule formation in myeloid cells [16].

Concluding remarks and treatment options

Once recruited, neutrophils have a wide range of toxic mechanisms to fight any invading microorganism. In case of severe congenital neutropenia, the clinician will face the question whether antibiotic prophylaxis and adequate treatment once the patient is infected is enough to protect

the individual patient. When infections occur too frequently and severely impair the quality of life of a patient, support of neutrophil development by G-CSF can be started by subcutaneous administration every other day or daily when the neutrophil output of the bone marrow is insufficient. In some of these syndromes, there is a risk of leukemia, often independent of the use of G-CSF itself. When the use of high G-CSF doses is necessary to get a sufficient neutrophil count, the chance of AML accumulatively increases in the long term. Careful monitoring of genetic changes may help to define the moment of (preemptive) stem cell transplantation.

Antibiotic and/or antifungal prophylaxis is a good strategy to prevent frequent bacterial infections in neutropenia and neutrophil dysfunction. The use of cotrimoxazole prophylaxis has become the mainstay in CGD and may also be sufficient in cases of neutropenia [25]. During fulminant infections that do not respond to antibiotics or antifungals, transfusion of non-HLA-matched donor neutrophils is a good option [24].

As the outcome of stem cell transplantation has considerably improved over the decades, it is increasingly performed in SCN and functional neutrophil disorders. Most experts agree that stem cell transplantation in these diseases should be seriously considered in every patient, preferably at an early age [7, 21].

Conflict of interest There is no conflict of interest since there is no financial relationship between the authors and the organization that sponsored the research.

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