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MULTI-AUTHOR REVIEW

Familial hemophagocytic lymphohistiocytosis: a model for understanding the human machinery of cellular cytotoxicity

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Abstract Cytotoxic T lymphocytes, natural killer cells, and NKT cells are effector cells able to kill infected cells. In some inherited human disorders, a defect in selected proteins involved in the cellular cytotoxicity mechanism results in specific clinical syndromes, grouped under the name of familial hemophagocytic lymphohistiocytosis. Recent advances in genetic studies of these patients has allowed the identification of different genetic subsets. Additional genetic immune deficiencies may also induce a similar clinical picture. International cooperation and prospective trials resulted in refining the diagnostic and therapeutic approach to these rare diseases with improved outcome but also with improved knowledge of the mechanisms underlying granule-mediated cellular cytotoxicity in humans.

Keywords Cellular cytotoxicity · Natural killer · Hemophagocytosis · Mutation analysis

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Introduction

Different cell types of the human immune system are devoted to defending the organism against pathogens. Cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and NKT cells are able to kill infected cells. Although NK, CTL, and NKT cells may use different receptors to recognize their targets, they all contribute to delivering a lethal hit able to destroy the target cell that has been recognized. All three cytolytic cell types contain secretory lysosomes containing the pore-forming protein perforin but also granzymes, a series of serine proteases. Once released from the cytolytic cell, perforin is able to form transmembrane pores that allow granzymes to enter the cytoplasm of the target cell, thus cleaving the substrates that trigger apoptosis. As a final result, cell death will occur within minutes [1].

NK cells function as important sentinels of the immune system, working as primary responders and alerting the host about the presence of infectious organisms. They represent a subset of cytotoxic lymphocytes, able to recognize and lyse tumor cells and virus-infected cells without previous sensitization. NK cells are of bone marrow origin, circulate in the blood, and become activated by cytokines, pathogen-derived substances, or interaction with target cells that express NK cell receptor ligand [2]. In contrast to B and T cell receptors, NK cell receptors are encoded in the germ line and do not undergo somatic recombination; the balance of signals between activating and inhibitory receptors determines the outcome of NK cell function. Some inhibitory receptors recognize MHC class I molecules, which are present on virtually all healthy cells, thus preventing NK cell attack. Loss of MHC class I from cells owing to infection or tumor transformation can lead to NK cell activation, as proposed by the "missing self hypothesis," provided that an activating

receptor is engaged. The activating NK receptors bind to host-derived or pathogen-encoded ligands that are upregulated on "stressed" or infected cells. Upon activation, NK cells directly lyse target cells by exocytosis of perforin and granzymes. Moreover, NK cells also display regulatory capabilities mediated by various cytokines released upon engagement of different triggering NK receptors or upon signaling by other cytokines. This is particularly relevant during the early phases of inflammatory responses. Several data have recently highlighted the role of the interactions between NK cells and other cells of the innate immune system that occur during the early phases of acute inflammation, secondary to infection [2]. Various studies have focused on the crosstalk between NK cells and monocyte-derived dendritic cells (DCs) and more recently on the involvement of plasmacytoid dendritic cells (PDC), mast cells, basophils, eosinophils, and neutrophils [3]. In view of these observations it appears that a complicated network of interactions can take place after the recruitment of these different cells to inflammatory sites in response to tissue damage resulting from invasion by pathogens (or tumor cells).

The discovery of human leukocyte antigen (HLA) class I specific inhibitory receptors (including the allotype-specific killer immunoglobulin-like receptors) and of various activating receptors and their ligands provided the basis for understanding the molecular mechanism of NK-cell activation and function that results from the balance between activating and inhibitory signals. In an allogeneic setting NK cells may express inhibitory killer immunoglobulin-like receptors that are not engaged by any of the HLA class I alleles present on allogeneic cells. Such "alloreactive" NK cells turned out to be pivotal for eradication of residual leukemia blasts in patients undergoing haploidentical hematopoietic stem cell transplantation [4, 5].

In the granule-dependent exocytosis pathway, target cell recognition by CTL and NK cells is followed by the polarized release of preformed cytolytic granules into the synaptic cleft formed between the effector and the target (Fig. 1). These granules contain lytic molecules including perforin, granzymes, granulysin, and other lysosomal enzymes, and also contain a proteoglycan matrix (serglycan) that maintains protease enzymes in an inactive stage, and FAS ligand [6]. At the acidic pH of the granules lytic molecules are inactive; the killer cell is thus protected from itself [6]. Exocytosis of mature cytolytic granules is a complex phenomenon that can be divided into different steps: polarization, docking, priming, and fusion. Several molecules are involved in this process: lysosomal trafficking regulator (LYST); adaptor protein 3 (AP3), which regulates the transport of proteins from Golgi to the cytotoxic granules and polarization; Rab27a, which is relevant in the docking step; syntaxin 11, which is a SNARE protein and appears to bind to Munc18-2 (or syntaxin-binding protein-2, STXBP2), possibly regulating granule docking and membrane fusion; and Munc13-4, which is crucial for priming the cytotoxic granules required for the fusion with the plasma membrane [7]. The role and relevance of these various molecules have been mainly understood by studying the loss of function in the absence of the normal protein. The exact role of perforin in exocytic cytolysis is controversial. According to the most accepted hypothesis, once it is anchored through the binding of the calcium-dependent C2 domain to lipid in the target membrane, perforin begins polymerization to form cylindrical pores that allow granzymes and granulolysin to diffuse into the target cell and will eventually cause an ionic exchange resulting in an osmotic imbalance. Whatever the mechanism, the presence of perforin is essential for granzymes to induce apoptosis by caspase-independent or caspase-dependent pathways. The various granzymes have different proteolytic specificities. The most abundant are granzyme A and B. Granzyme A cleaves basic residues in a caspase-independent pathway while granzyme B activates caspase 3 directly or induces a change in mitochondrial permeability causing the release of various proteins that lead to caspase activation [8, 9].

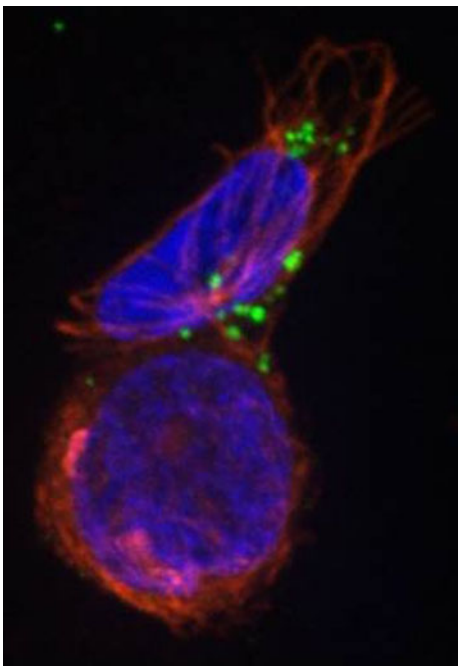

Fig. 1 NK cell recognizing a target and polarizing its cytoskeleton (tubulin, *red*) and lytic granules (perforin, *green*) towards the target. Nuclei are stained *blue* with Hoechst

Familial hemophagocytic lymphohistiocytosis (FHL)

Hemophagocytic lymphohistiocytosis (HLH) is a severe hyperinflammatory syndrome caused by uncontrolled but ineffective immune response. Cardinal signs and symptoms are prolonged and unexplained fever unresponsive to antibiotics, hepatosplenomegaly, pancytopenia, and hemophagocytosis. Crucial in the pathophysiology of HLH is a defect in cytotoxic activity that prevents efficient removal of antigens and downregulation of immune response resulting in sustained activation and proliferation of CTLs and NK cells [10–12]. Persistently activated CTLs and NK cells produce large amounts of cytokines including IFN-γ, TNF-α, and GM-CSF leading to activation of histiocytes (macrophages and dendritic cells). These cells in turn home to sites of CTLs and NK cells triggering activation, resulting in tissue infiltration and secretion of high levels of inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, and IL-18 [13, 14]. Organ infiltration and hypercytokinemia by activated lymphocytes and histiocytes lead to the clinical picture of HLH.

In 1952 Farquhar and Claireaux [15] reported in two siblings a disease causing fever, cytopenia, and hepatosplenomegaly with rapidly fatal outcome, despite treatment with antibiotics and steroids. From this first report an autosomal recessive inheritance was proposed and then confirmed as the common mode of inheritance for the familial form of HLH (FHL). The incidence is estimated as 1:50,000 births. Symptoms are usually present within the first months after birth; yet, later onset was soon reported, with 20% of cases presenting when older than 5 years. More recently FHL has been diagnosed in adults with a similar phenotype [16–18].

FHL is a genetically heterogeneous disorder caused by mutations in genes involved in the granule-dependent exocytosis pathway [19]. To date five independent loci implicated in FHL have been identified and the underlying genetic defect described for four of these.

Familial hemophagocytic lymphohistiocytosis type 1 (FHL1)

A first gene mapping approach applied to four consanguineous families of Pakistani origin identified a 7.8 cM region on chromosome 9q21.3-22 [20]. Unfortunately, the underlying genetic defect responsible for the disease has not yet been identified.

Familial hemophagocytic lymphohistiocytosis type 2 (FHL2)

The first gene reported to be a cause of FHL is *PRF1*, which encodes for the perforin protein [21]. Lack of perforin leads to loss of cytolytic effect, as demonstrated in a perforin knock-out mouse model [22]. In patients with FHL2, PRF1 mutations induce a complete or partial reduction of the synthesis of the perforin protein, resulting in an impairment of the granule-dependent exocytosis pathway of NK and CD8+ T cells [8, 23].

Over 70 different mutations have been identified so far throughout the perforin gene [16, 21, 23–30] with some showing restriction to specific ethnic groups: mutation c.1122G > A (p.W374X) was found with a high incidence in Turkish patients [31], the c.50delT(L17FsX) was predominant in patients of African-American origin [29], and the c.1090-1091delCT(L364fsX) has never been identified in non-Japanese patients [28].

In a genotype-phenotype study performed on 124 patients with FHL2 collected by an international consortium, the median age at disease onset was 3 months, but it was significantly delayed in patients with at least one missense mutation, allowing some residual perforin protein and function. NK activity was absent or reduced in all evaluable patients [30].

PRF1 mutations account for 20–50% of FHL, depending on the cohort studied [24, 27, 29]. Our personal observations on a large population of over 100 cases suggest that FHL2 accounts for about 40% of total cases of FHL [19] (Aricò, 2011, unpublished data).

Familial hemophagocytic lymphohistiocytosis type 3 (FHL3)

In 2003 a third locus, 17q25, was reported in linkage with familial hemophagocytic lymphohistiocytosis. The involved gene *UNC13D* encodes for the protein Munc13-4, which is thought to contribute to the priming of the secretory granules and their fusion into the plasma cell membrane [32]. Munc13-4 deficiency impairs the delivery of the effector proteins, perforin and granzyme, into the target cells resulting in defective cellular cytotoxicity and a clinical picture that was not discernible from FHL2. It has been demonstrated that upon co-culture with susceptible target cells, FHL3 NK cells displayed low levels of surface CD107a (LAMP-1) staining, in contrast to healthy controls or perforin-defective patients. Therefore this kind of cytofluorimetric analysis was reported as a rapid tool for identification of Munc13-4 defects, characterized by impaired degranulation [14].

The frequency of *UNC13D* mutations in FHL was estimated to be between 30 and 40% based on different groups [13, 33, 34] (Aricò, 2011, unpublished data).

To date over 50 different mutations of *UNC13D* have been reported as the cause of FHL3 [32, 35–38]. These mutations are scattered over the gene with up to 15% of them affecting mRNA splicing [39]. No correlation

between ethnic groups and specific mutations was observed with the exception of the c.1596 + 1G > C reported to be the most common *UNC13D* mutation in Japan [40] and the c.754−1G > C, which accounts for the majority of *UNC13D* mutations in FHL3 Korean patients [41].

In the largest series of patients with FHL3 reported so far, the median age at onset was 4 months, with a wide range up to 18 years. Similarly to FHL2, patients with at least one missense mutation had a later onset of symptoms. Age at diagnosis was significantly higher in FHL3 versus FHL2 patients when only disruptive mutations were considered. The same study showed reduced or absent NK cytolytic activity in 44 of the 45 patients analyzed (98%) with quantitative evaluation of lytic units (when available) significantly inferior to that of healthy controls [38].

Familial hemophagocytic lymphohistiocytosis type 4 (FHL4)

Genome-wide homozygosity mapping in Turkish/Kurdish FHL consanguineous families found linkage to a region on chromosome 6q24, which maps to the *STX11* gene encoding for syntaxin 11 protein [42]. Syntaxin 11 is a member of the family of soluble *N*-ethylmaleimide sensitive factor attachment protein receptors present on target membranes (t-SNARE) [42]. SNARE proteins play a role in regulating intracellular protein transport between donor and target membranes. This docking and fusion process involves the interaction of specific vesicle-SNARE (v-SNARE) with specific t-SNARE. It is not yet clear which exact step is regulated by syntaxin 11 and which other partners are involved in forming a SNARE complex necessary for membrane fusion [43].

Very few patients with FHL4 have been reported so far, with a large prevalence of families of Turkish/Kurdish origin [37, 42–45]. Recently, one novel homozygous nonsense mutation in Hispanic siblings, and two novel heterozygous missense mutations in a Caucasian patient were also reported [46], suggesting that FHL4 may occur outside this original ethnic group.

Patients with FHL4 seem to have a later onset of the disease compared to FHL2 and FHL3 [47]. Yet, the total number of reported patients does not allow a wider genotype-phenotype study, as performed for FHL2 and FHL3.

NK cells from patients with FHL4 fail to degranulate when encountering susceptible target cells, as also reported in patients with FHL3 [14, 43]. However, differently from FHL3, this defect was only detectable in resting NK cells while interleukin-2 stimulation could restore NK-cell degranulation and cytotoxicity. These data can explain why disease progression was observed to be less severe with a later onset in FHL4 patients than in FHL2 or FHL3 [43].

Familial hemophagocytic lymphohistiocytosis type 5 (FHL5)

In 2009 zur Stadt et al. reported a novel FHL-related gene located at chromosome 19p in consanguineous families of Saudi Arabian or Turkish origin. Based on this finding they identified mutations in *STXBP2* encoding for Munc18-2. This protein is involved in the regulation of vesicle transport to the plasma membrane by the interaction with syntaxin 11. This interaction is eliminated by the mutations of *STXBP2* found in patients with FHL5, which lead to a decreased stability of both proteins [48]. Almost simultaneously a similar report was provided by Côte et al. [49].

To date 14 different mutations of *STXBP2* have been described [34, 48, 50]. Apparently only a minority of patients with FHL, ranging from 6 to 14% [34, 49, 50], belong to the FHL5 subset. Similarly to those with FHL4, they appear to have a later onset compared to FHL2 and FHL3 [51]. Although current information on the clinical picture of FHL5 patients remains limited, most of them appear to fall within the most common HLH syndrome described by the diagnostic criteria [50, 52]. However an atypical presentation of the disease with gastrointestinal disorders, bleeding disorders, and hypogammaglobulinemia was recently reported [51]. In FHL5 patients, NK and cytotoxic T cell activity is absent or markedly reduced. Thus it appears that Munc18-2 is essential in the late step of the secretory pathway for the release of cytotoxic granules by binding syntaxin 11 (Fig. 2).

The defective exocytosis of NK-cell cytotoxic granules can be overcome by ectopic expression of wild-type *STXBP2* [49]. Defective results in the CD107 assay have been documented in resting NK cells, while the behaviour of activated NK cells needs further evaluation; variable results on CTL cytotoxicity were documented [48–50].

Genetic immune deficiencies associated with HLH

In addition to FHL, which presents with HLH as the primary and only manifestation, other genetic conditions may cause a clinical syndrome that largely overlaps HLH although they have some additional, distinctive clinical features.

Chédiak-Higashi syndrome (CHS)

First mentioned by Beguez Cesar [53], the syndrome received its name from Chediak and Higashi [54, 55] who described its characteristic diagnostic findings. CHS is a rare, autosomal recessive disorder characterized by variable degrees of oculocutaneous albinism, mild bleeding tendency, recurrent bacterial infections, and progressive

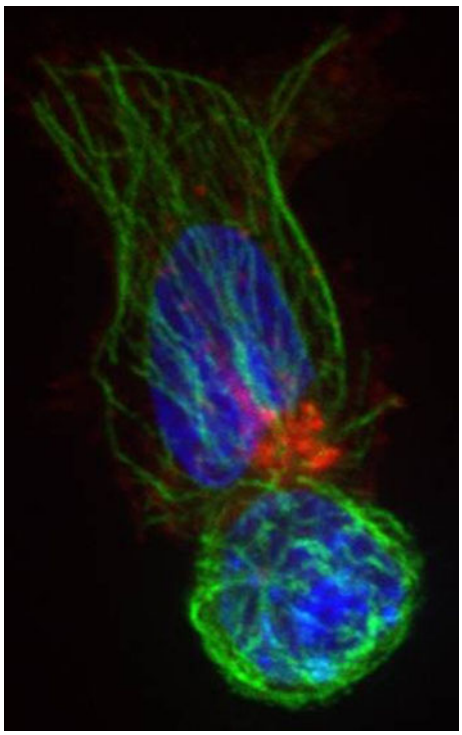

Fig. 2 Munc18-2-deficient CTL (FM) with microtubules (*green*) and perforin granules (*red*) polarized towards the target. Nuclei are labelled in *blue* with Hoechst

neurologic dysfunction, in addition to sporadic occurrence of HLH [56–59]. The disease is caused by mutations in *LYST* (lysosomal trafficking regulator gene), mapped to 1q42.1-q42.2, that encode for the 429-KD, ubiquitously expressed LYST protein [60, 61]. This protein is involved in intracellular trafficking, and it is thought to participate in the sorting of lysosomal proteins to late endosomes [62] or in the regulation of fusion or fission of lysosomes [63]. Loss of function mutations of LYST, in CHS patients and in the *beige* mouse model, result in the enlargement of lysosomes and lysosome-related organelles including melanosomes, platelet-dense bodies, and cytolytic granules [64]. The presence of giant inclusion bodies of lysosome origin in a variety of granule-containing cells, including hematopoietic cells and melanocytes, has thus become the hallmark of the disease [57]. As a consequence, enlarged vesicles fail to undergo normal movements, and lysosomal exocytosis is impaired leading to presenting features of CHS [65]. In particular, in CTLs and NK cells, the giant cytotoxic granules polarize at the immunological synapse, but their secretion is impaired leading to a defective cytotoxic activity. Aberrant formation of melanosomes, indeed, is responsible for hypopigmentation that typically affects CHS patients [66]. Various CHS clinical phenotypes have been correlated with molecular genotypes. Nonsense or frameshift mutations with subsequent early truncation of the protein are associated with the severe, early onset form of the disease that affects more than two-thirds of patients and is characterized by fatal infections and HLH, which occurs during the so-called accelerated phase. Indeed, missense mutations are associated with the milder, late-onset CHS, with slowly progressive neurological impairment and infections but not with HLH [67].

Hermansky-Pudlak type 2 (HPS2, OMIM #608233)

Hermansky-Pudlak syndrome (HPS) [68] defines a group of at least eight human autosomal recessive genetic disorders characterized by partial oculocutaneous albinism and bleeding disorders [69, 70]. HPS2 is also associated with increased susceptibility to infections due to congenital neutropenia and impaired cytotoxic activity [71]. This form is caused by mutations of the gene encoding to the β-3A subunit of *adaptor protein-3* (AP3) complex [72]. Defects in the β-3A subunit disrupt the complex leading to lysosomal protein missorting in certain cell types including melanosomes, platelets, CTLs, and NK cells. Misdirected sorting of tyrosinase, the substrate for melanin synthesis in melanosomes, is responsible for hypopigmentation, whereas aberrant targeting of neutrophil elastase contributes to the observed neutropenia [70, 73]. Cytotoxicity impairment is attributed to the presence of enlarged cytotoxic granules unable to move along microtubules and thus to polarize to the immunological synapse [71]. Although all patients with HPS2 analyzed to date have defective cytotoxic activity, only one case of HLH has been reported so far [74], in contrast to all other genetic disorders associated with defective cytotoxicity activity (Table 1). Thus whether the cytotoxic defect in HPS2 predisposes to HLH or not remains to be clarified.

Griscelli syndrome type 2 (GS-2)

GS2 is a rare autosomal recessive disease characterized by partial oculocutaneous albinism and the "accelerated phase" with HLH [57, 75]. Differential diagnosis with FHL is difficult as the main differential clinical feature is the silvery hair, which can be extremely subtle [76]. GS2 can be distinguished from CHS by the lack of giant inclusion bodies and the typical microscopic pattern of uneven distribution of large pigment granules detected in GS2 [56].

GS2 results from the mutation of *RAB27a* [33] encoding a member of small GTPase family protein. Mutations in *RAB27a* have been described in more than 100 independent patients; most are nonsense or frameshift mutations leading to truncation of the protein [33, 77, 78], although missense mutations have also been reported [79]. CTL and NK cell activity defects result from the inability of cytotoxic granules to dock to the plasma membrane (Fig. 3), whereas hypopigmentation is accounted for by a defective release of

Table 1 Overview of some characteristics of genetic disorders associated with occurrence of hemophagocytic lymphohistiocytosis

Subtype	OMIM number	Gene map	Protein affected	Cytotoxicity defect	Functional screening	Animal model	Notes
FHL 1	603552	9q21.3-22	Unknown	Unknown	Unknown	None	
FHL2	267700	9q21.3-q22	Perforin	Complete	Defective perforin expression	Pfn1 −/−	
FHL3	608898	17q25.1	Munc13-4	Complete	Defective degranulation	Jinx	
FHL4	603552	6q24	Syntaxin 11	Moderate	Defective degranulation	None	
FHL5	613101	19p	Munc18-2	Moderate	Defective degranulation	None	
Griscelli syndrome type 2	607624	15q21	RAB27a	Complete	Defective degranulation	Ashen	Hypopigmentation
Chediak-Higashi syndrome	214500	1q42.1-q42.2	LYST	Complete	Defective degranulation	Beige	Hypopigmentation, abnormal granule size
Hermansky-Pudlak syndrome type 2	608233	5q14.1	Adaptor protein-3 (AP3)	Complete	Defective degranulation	Pearl	Hypopigmentation, abnormal granule size
XLP 1	308240	Xp25	SH2D1A	Partial	Defective SAP expression	–	
XLP 2	300635	Xp25	XIAP or BIRC4		Defective XIAP expression	–	

melanosomes from melanocyte dendrites [69, 80]. A direct interaction between Munc13-4 and Rab27α has recently been demonstrated, suggesting that the complex is an essential regulator of priming step in the secretory pathway [81].

X-linked lymphoproliferative syndrome type 1 (XLP1, Duncan disease)

XLP is an X-linked inherited immunodeficiency characterized by a severe immune dysregulation triggered, in most cases, by Epstein-Barr virus (EBV) infections [82]. Indeed, cases of XLP without evidence of EBV infection have been reported and account for almost 10% of affected patients [83, 84]. XLP results from mutations in *SH2D1A*, encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) [85, 86]. The protein seems to have an important role in the development, differentiation, and effector function of T cells [87], NK cells [88–90], NKT cells [91, 92], and possibly B cells [93]. SAP is an adaptor protein that binds to the intracellular domain of various members of the SLAM-family expressed by multiple immune cell types, promoting their activation and/or differentiation. When SAP is deficient, the SLAM family receptors switch their function and mediate inhibitory signals that suppress immune cell functions. The paradoxical behavior of the 2B4 receptor may thus be used for diagnosing XLP [94]. Patients with XLP1 show defects in the functions or development of several immune cell types, including T cells, NK-T cells, NK cells, and B cells [95, 96], which explains the clinical features of the disease. The most commonly recognized phenotypes are fulminant infectious mononucleosis or EBV-associated HLH (58% of patients), hypogammaglobulinemia (30%), lymphoproliferative disorders including malignant lymphoma (20–30%) and other less common manifestations (aplastic anaemia,

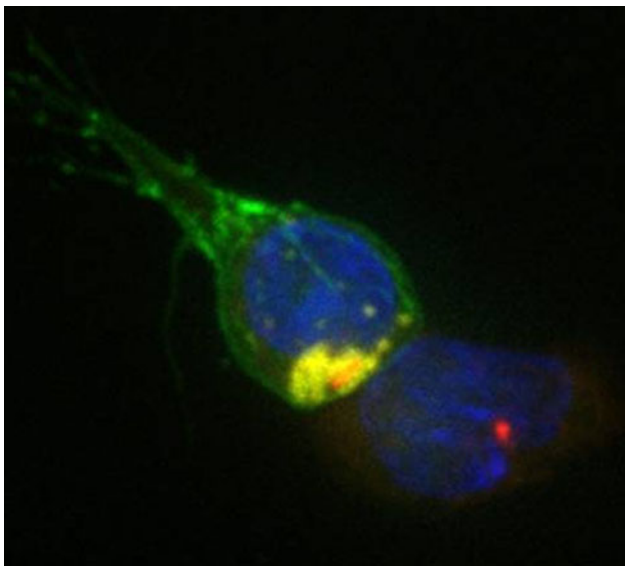

Fig. 3 Rab27a-deficient patient CTL stained with CD8 (*green*) recognizing, but not killing a target cell with granules stained with LAMP1 (*yellow*). The centrosomes and nuclei of both target and killer are stained with g-tubulin (*red*) and Hoechst (*blue*)

vasculitis, chronic gastritis) [97, 98]. Patients with XLP may be thus initially diagnosed as HLH [99].

X-linked lymphoproliferative syndrome type 2 (XLP2)

Recently, a subset of patients with an XLP-like phenotype were found to carry mutations in *BIRC4*, the gene encoding the X-linked inhibitor of apoptosis protein (XIAP) [100]. XIAP is a potent anti-apoptotic protein that directly binds to and inhibits specific caspases. In addition, it is also involved in a variety of intracellular signalling events (NF-κB pathway, the c-Jun-N-terminal kinase pathway, and the TGF-β pathway) [101]. The majority of *BIRC4* mutations lead to an absence of protein expression [100, 102]. How this results in the XLP phenotype remains to be fully explained. Previous reports postulated an increased sensitivity of XIAP-deficient lymphocytes to apoptosis and decreased populations of NKT cells [100, 103]. However, NK cell function and NKT cell number are reported to be normal in mice and humans with *BIRC4* mutations [102, 104]. The main clinical presentation of XLP2 is HLH (often associated with EBV); dysgammaglobulinemia has also been reported, although less frequently than in XLP1, but no cases of lymphoma have been described to date [102, 105]. Despite sharing a common X-linked locus of origin and the susceptibility to develop HLH, XLP1 and 2 present clinical and functional specificities [102, 106]. Thus, whether or not SAP and XIAP are part of a common signalling pathway remains to be elucidated even if a direct interaction between these two proteins appears unlikely [107]. The very limited number of patients with this defect reported so far did not allow greater clarification of the phenotype and characteristics of this subgroup of patients.

Clinical features of FHL

The initial suspicion of HLH should be based on the identification of a set of clinical signs and symptoms and laboratories abnormalities. The most common features of HLH are prolonged, unexplained fever unresponsive to antibiotics, hepatosplenomegaly, and cytopenia [17, 108]. Neurological abnormalities at diagnosis are seen in up to 30% of cases, ranging from cranial nerve palsy to seizure and a decreased level of consciousness. Cerebrospinal fluid shows pleocytosis, increased protein, or both in more than half of patients [17, 109]. Changes on neurologic imaging (e.g., parenchymal atrophy, diffuse abnormal signal intensity in the white matter on T2-weighted images, focal hyperintense lesions, delayed myelination, or parenchymal calcification) have been reported and suggested to correlate with clinical symptoms [110–112].

Characteristic biochemical markers include elevated ferritin, triglycerides, and α-chain of the soluble interleukin-2 receptor (sCD25), and low fibrinogen. Hemophagocytosis by activated macrophages may be absent at initial bone marrow examination and thus should not preclude the diagnosis. Less common signs are lymphoadenopathy, icterus, rash, edema, high levels of transaminases, bilirubin, and lactate dehydrogenase. Atypical presentation with acute liver failure or isolated central nervous system (CNS) involvement has also been described [17, 52, 113]. Most of these presenting features can be explained by the underlying hypercytokinemia and organ infiltrations. Fever is induced by IL-1 and IL-6; pancytopenia results from high levels of IFN-γ and TNF-α and hemophagocytosis; hypertriglyceridemia is the consequence of inhibition of lipoprotein lipase by TNF-α; ferritin is secreted by activated macrophages together with high levels of plasminogen activator that result in high plasmin levels and hyperfibrinolysis.

Diagnostic strategy: where do we go from here?

In order to improve diagnosis of HLH the Histiocyte Society in 1994 defined the diagnostic criteria, which were then revised in 2004 [52] (Table 2). Yet, the diagnosis of FHL can still be challenging. Onset is at a very young age in the majority of cases, but even this cannot be taken as a rule, since about 20% of patients develop the disease when older than 2 years [17], and cases at later ages, up to young adults, are increasingly reported [16, 18, 114]. Evidence of documented or very likely consanguinity, although reported in no more than 25% of cases, may be very informative. Even more relevant has to be considered the report of a sibling with early death with undefined cause. Defective pigment of skin or hairs, although rare, is very informative.

The constellation of signs and symptoms is not specific; in most cases leukemia is suspected, to be soon ruled out by bone marrow examination, which may show, in about one-half of the cases, hemophagocytosis; none of the biochemical abnormalities is specific. It was recently proposed that these criteria might be simplified [115]. In an attempt to contribute to this debate, our group observed that the combination of fever, splenomegaly, and thrombocytopenia represents the initial clinical background to raise the suspicion of FHL; when associated with evidence of increased ferritin level, these features may be considered as a very sensitive tool to address the diagnostic work-up already during the first few hours from admission [38].

It is important to remember that an infectious trigger will be present or suspected in most cases. Yet, as in many other immune deficiencies, common pathogens, especially viruses, may represent an excessive challenge for the child

Table 2 Revised diagnostic guidelines for hemophagocytic lymphohistiocytosis (HLH)

The diagnosis of HLH can be established if either 1 or 2 below are fulfilled:
1. A molecular diagnosis consistent with HLH
2. Clinical and laboratory criteria for HLH fulfilled (5/8 criteria below):
Fever
Splenomegaly
Cytopenia (affecting ≥2 of 3 lineages in peripheral blood):
Hemoglobin <9 g/dl (in infants <4 weeks: Hb < 10 g/dl)
Platelets $<100 \times 10^9$/l
Neutrophils $<1.0 \times 10^9$/l
Hypertriglyceridemia and/or hypofibrinogenemia:
Fasting triglycerides ≥3.0 mmol/l
Fibrinogen ≤1.5 g/l
Hemophagocytosis in bone marrow or spleen or lymph nodes
Low or absent NK cell activity
Ferritin ≥500 μg/l
Soluble CD25 (i.e., soluble IL-2 receptor) ≥2,400 U/ml

Supportive evidence includes cerebral symptoms with moderate pleocytosis and/or elevated protein, elevated transaminases and bilirubin, LDH

with FHL. It is important to note that a very similar clinical picture may be observed in patients with visceral leishmaniasis, which in nonendemic areas may easily remain outside the differential diagnosis. Unfortunately, sporadic cases of undiagnosed leishmaniasis have been treated as FHL, with major consequences [116]. A search for infectious agents including EBV, cytomegalovirus (CMV), and *Leishmania* by polymerase chain reaction (PCR) is thus recommended.

When facing a child or a young adult with the clinical syndrome described by fever, splenomegaly, thrombocytopenia, and elevated ferritin, the clinician should approach an immunology laboratory to have a functional screening performed [117]. Impaired NK cell and CTL activity measured as lysis of K652 cells in a standard 4 h chromium release assay has become the hallmark of HLH [10–12, 14]. In FHL, NK cell numbers are generally normal while cellular cytotoxicity is usually defective or absent. Nevertheless, the NK cell cytotoxicity assay is laborious and thus remains a confirmatory assay restricted to some reference laboratories. Additional and more accessible tools for the screening of FHL have then been developed to provide an initial confirmation of the diagnosis and thus direct the mutation analysis [117–119]. A deficient flow-cytometry expression of perforin by cytotoxic cells can identify patients with perforin defects [26]. For the remaining majority of patients, based on the assumption that the cytotoxic machinery leads to surface expression of CD107a some of us have originally demonstrated that surface CD107a expression represents a rapid tool for identification of patients with degranulation defect [14]. This was first shown in FHL3 patients, thus becoming the standard for their identification, and later confirmed also in patients with FHL4 and FHL5 [3, 49, 54]. Intracytoplasmic staining for SAP and XIAP can provide rapid diagnosis for XLP1 and XLP2, respectively [120].

In patients with suspected genetic defects, in whom flow-cytometry screening does not detect a defect, analysis of cytotoxic activity appears mandatory. Evidence of a defective killing, especially if the defect is partial, needs a repeated assay for confirmation; when confirmed, this finding should be taken as a strong support for the diagnosis of FHL.

Mutation analysis remains the gold standard for the diagnosis of FHL and is mandatory for identification of the familial marker. Based on the current knowledge, a genetic defect may be assigned to about 80% of the familial cases, thus supporting indication for hematopoietic stem cell transplantation (HSCT), and allowing selection of familial donors, counselling, and family planning.

Treatment of FHL

In most cases the natural course of FHL is rapidly fatal within a few weeks if untreated [17, 108]. Therefore appropriate treatment should be started promptly when there is a high clinical suspicion, even if results from some diagnostic studies are still pending. The immediate aim of therapy is to suppress the hyperinflammatory state and to kill pathogen-infected antigen-presenting cells to remove the stimulus for the ongoing but ineffective activation of cytotoxic cells. Based on the large cooperative study HLH94 conducted by the Histiocyte Society, the combination of dexamethasone, etoposide, and cyclosporine has been defined as the standard of care for HLH [113]. With this strategy most patients may achieve disease control within 4–8 weeks. For those in whom a genetic defect has been documented, HSCT is strongly indicated as the only treatment approach with potential for a cure [121]. Yet, HSCT is a difficult procedure; although recent advances in transplantation procedures and supportive therapy have minimized the transplant-related mortality when a matched familial donor is available, this unfortunately occurs in no more than 20% of patients with indication. For the remaining cases, unrelated, voluntary donors are the usual opportunity for transplantation [122, 123]. Since treatment-related mortality in this setting remains nonnegligible, it is extremely important that indications for transplant are correctly defined. To avoid unneeded transplants, current treatment strategy suggests, for patients with normal

function at initial screening or even with normal NK activity, allowing a chance for treatment withdrawal after disease resolution usually achieved with the initial 8-week combined treatment. In the case of disease reactivation, which suggests that the patient is unable to maintain a disease-free condition in the absence of chemo-immunotherapy, transplantation may be considered even in the absence of a genetic marker.

Macrophage activation syndrome (MAS)

An HLH-like picture may occur in children and adults with autoimmune diseases, especially systemic onset juvenile idiopathic arthritis (s-JIA). Diagnostic guidelines for macrophage activation syndrome (MAS) complicating s-JIA have been developed, suggesting that falling platelet count, hyperferritinemia, evidence of hemophagocytosis in the bone marrow, increased liver enzymes, falling leukocyte count, persistent continuous fever $\geq$38°C, falling erythrocyte sedimentation rate, hypofibrinogenemia, and hypertriglyceridemia are the hallmarks of severe MAS [124, 125] in these patients. The incidence of MAS in patients with s-JIA is estimated at around 7%, unfortunately with a mortality between 10 and 20%, which is comparable to that of the "non-rheumatoid" patients with HLH/FHL. Treatment with cyclosporine A appears to be the current standard for MAS, but some patients may warrant a more aggressive therapy overlapping that of HLH. This is in keeping with the finding that, although most patients with MAS do not display deeply defective NK cell function, findings of reduced expression of perforin or SAP and heterozygous mutations in the FHL-related genes may launch a bridge between FHL and the pathogenic mechanisms of MAS [126–128].

Conclusion

FHL is a rare disease that needs accurate clinical, immunological, and genetic diagnostic work-up. Current standard of therapy based on chemo-immunotherapy allows rapid disease control in most patients. Due to the remaining risk of early mortality, the use of antithymocyte globuline (ATG), based on a single center experience [129], is being explored in a cooperative study. Data derived from the animal model [22] suggest that blocking the IFNγ activity may induce disease control without cytoreduction, with future therapeutic implications. Currently identified genetic defects allow a genetic marker to be assigned to over 80% of the families, although in some geographic areas this proportion may remain lower. The reactivation of the disease in patients with defective NK activity and unassigned defect, but also in a small number of families with normal NK activity, suggests that there are at least two additional FHL-related genes to be identified. In this regard, wide application of the current diagnostic standard based on functional tools may select patients in whom additional studies by confocal microscopy and protein expression appear to be of paramount importance for pointing out novel defects. These studies are expected to provide novel pieces of information, improving our knowledge of the cellular cytotoxic machinery in humans.

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