Jana P. Schmid
Marjorie Côte
Mickaël M. Ménager
Agathe Burgess
Nadine Nehme
Gaël Ménasché
Alain Fischer
Geneviève de Saint Basile

Inherited defects in lymphocyte cytotoxic activity

Authors' addresses
Jana P. Schmid[1,2], Marjorie Côte[1,2], Mickaël M. Ménager[1,2], Agathe Burgess[1,2], Nadine Nehme[1,2], Gaël Ménasché[1,2], Alain Fischer[1,2,3], Geneviève de Saint Basile[1,2,4]
[1]Institut National de la Santé et de la Recherche Médicale (INSERM), U768, Paris, France.
[2]Faculté de Médecine, Université Paris Descartes, Paris, France.
[3]AP-HP, Hôpital Necker-Enfants Malades, Service d'Immunologie et d'Hématologie Pédiatrique, Paris, France.
[4]Centre d'Etude des Déficits Immunitaires, Assistance Publique-Hôpitaux de Paris, Hôpital Necker-Enfants Malades, Paris, France.

Correspondence to:
Geneviève de Saint Basile
U768, Institut National de la Santé et de la Recherche Médicale (INSERM)
Hôpital Necker Enfants Malades, 149 rue de Sèvres
75015 Paris, France
Tel.: +33 1 44 49 50 80
Fax: +33 1 42 73 06 40
e-mail: genevieve.de-saint-basile@inserm.fr

Acknowledgements
This work was supported by grants from the French National Institute for Health and Medical Research (INSERM), the French National Research Agency (ANR) and the Fondation pour la Recherche Médicale (FRM). M.C, A.B, M.M.M, and NN. were supported by doctoral or postdoctoral fellowships from the Ministère de l'Education Nationale de la Recherche et de la Technologie and l'Association de Recherche contre le cancer (ARC).

Immunological Reviews 2010
Vol. 235: 10–23
Printed in Singapore. All rights reserved

Immunological Reviews
0105-2896

Summary: The granule-dependent cytotoxic activity of lymphocytes plays a critical role in the defense against virally infected cells and tumor cells. The importance of this cytotoxic pathway in immune regulation is evidenced by the severe and often fatal condition, known as hemophagocytic lymphohistiocytic syndrome (HLH) that occurs in mice and humans with genetically determined impaired lymphocyte cytotoxic function. HLH manifests as the occurrence of uncontrolled activation of T lymphocytes and macrophages infiltrating multiple organs. In this review, we focus on recent advances in the characterization of effectors regulating the release of cytotoxic granules, and on the role of this cytotoxic pathway in lymphocyte homeostasis and immune surveillance. Analysis of the mechanisms leading to the occurrence of hemophagocytic syndrome designates γ-interferon as an attractive therapeutic target to downregulate uncontrolled macrophage activation, which sustains clinical and biological features of HLH.

Keywords: *cytotoxicity, hemophagocytic lymphohistiocytic syndrome, IFN-γ, immunodeficiency disorders, natural mutants, granule exocytosis*

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (collectively known as cytotoxic lymphocytes) contribute to immune protection from viral infections and cell transformation. CTLs are activated by specific antigen recognition, whereas the cytotoxic activity of NK cells is initiated by specific activating receptors or combinations thereof and is inhibited by self-major histocompatibility complex class I recognition (1, 2). Cytotoxic lymphocytes kill target cells through polarized release of the content of cytotoxic granules toward the target cell (3). Cytotoxicity proceeds through a number of steps, including formation of an immunological synapse (IS) at the cell–cell contact, the movement of cytotoxic granules toward the microtubule organizing center (MTOC), followed by a reorientation of the MTOC toward the target cell (3, 4). Cytotoxic granules concentrate in a specific zone in the center of the synapse that is separated from the area where T-cell receptor and signaling molecules are localized (5–7). Effector molecules, which include perforin and the serine proteases granzymes, are stored complexed

within chondroitin sulfate serglycin proteoglycan matrix in the cytotoxic granules, recognized as secretory lysosomes (8, 9). Following IS formation, matured cytotoxic granules dock at the IS and release their content upon fusion with plasma membrane through a degranulation process. Effectors are released into an intercellular cleft transiently formed between the two cells (10, 11). Perforin is required for the delivery of granzymes into the cytosol of the target cell, even though the precise site of perforin action in the target is still controversial (11). Granzyme B induces caspase-dependent apoptosis (12), whereas granzyme A can act in a caspase-independent manner (13).

Phenotypic consequences of natural mutants that affect effectors of the granule-dependent cytotoxic function of lymphocytes provide evidence of a critical role played by this cytotoxic pathway in the regulation of immune homeostasis. In humans, defects in lymphocyte cytotoxic function result in a severe and often fatal immunopathological condition referred as the hemophagocytic lymphohistiocytosis (HLH) (14, 15). Similar natural or engineered murine models, when challenged with lymphocytic choriomeningitis virus (LCMV) faithfully reproduce features of HLH. These murine models have been instrumental to better understand the pathophysiology of HLH. The importance of perforin in immune surveillance against cancer has been previously demonstrated in perforin-deficient mice. Recent studies in humans support a similar role as based on the more frequent occurrence of lymphoma in patients carrying hypomorphic perforin mutations.

In this review, we summarize what is known about the inborn errors that cause HLH as well as the pathophysiology of HLH. An alternative therapeutic option that is based on our current understanding of the pathophysiology of this severe condition is also presented.

Phenotypic expression of HLH

HLH consists of the occurrence of a high non-remitting fever associated with hepatosplenomegaly and neurological manifestations ranging from confusion, seizures, to coma (16). These clinical manifestations are associated with pancytopenia, notably anemia and thrombocytopenia, hepatitis with elevated liver enzymes, hypertriglyceridemia, hypofibrinogenemia, hyponatremia, and elevated ferritin (17). In addition, unremitting expansion and activation of polyclonal $CD8^+$ T cells and macrophages that phagocytose blood cells (hemophagocytosis) are the hallmarks of this syndrome. Activated $CD8^+$ T lymphocytes and macrophages infiltrate multiple organs, including the bone marrow, the lymph nodes, the spleen, the liver, and the brain (18–20). Activated lymphocytes and macrophages can be found in the cerebrospinal fluid. The infiltrating lymphocytes and macrophages are the most prominent in the interstitial and perivascular spaces of the organs, where macrophages are found to frequently contain blood cells. When noted at an early stage of HLH, the white pulp of the spleen is often reduced in size and depleted of lymphocytes, whereas the red pulp is expanded as a result of the mononuclear cell infiltration. In the liver, portal tracts are the place of T lymphocyte and macrophage infiltration. In the lymph nodes, sinuses are frequently involved and dilated (19). Central nervous system (CNS) infiltration begins generally in the meninges, then perivascular changes occur, leading to diffuse infiltration of the tissue and multifocal necrosis at a later stage (21). Activated cells overproduce multiple inflammatory cytokines including γ-interferon (IFN-γ), interleukin-6 (IL-6), IL-18, and tumor necrosis factor-α (TNF-α) (17, 22). It is likely that this 'hypercytokinemia' plays a key role in the onset of clinical and biological manifestations typical of HLH. In infiltrated tissues, these products induce cell necrosis and organ failure. HLH can be triggered by infection, notably by viruses from the herpes group, especially Epstein–Barr virus (EBV), in humans. Altogether, manifestations of HLH can be explained largely as a consequence of high cytokine production by macrophages, in response to T-lymphocyte sustained activation.

Inherited diseases causing HLH

Familial hemophagocytic lymphohistiocytosis

Familial hemophagocytic lymphohistiocytosis (FHL) is inherited as an autosomal recessive disease (20) with an incidence estimated to be 1:50 000 births (23). Overwhelming HLH is the distinguishing and isolated feature of this condition with no other associated signs, as opposed to other inherited conditions causing HLH (see below). Symptoms of HLH are usually evident within the first 6 months of age and can even, in rare cases, develop *in utero* (GSB, unpublished observation) or at birth (24). However, familial forms with a later onset can also be seen, up to adulthood. Generally, age at onset is fairly similar in a given kindred (19). A characteristic of FHL is a defective NK cell cytotoxicity (25). T-cell cytotoxicity induced by anti-CD3 antibody is also defective in a large number of cases.

Molecular basis of FHL

Linkage analysis using homozygosity mapping in four inbred FHL families of Pakistani descent identified a locus (FHL1) on chromosome 9q21.3-22 (26). However, no causative gene

has been found associated with this locus. Using genome-wide linkage analysis, four additional loci have subsequently been identified on chromosomes 10q21-22 (FHL2) (27), 17q25 (FHL3) (28), 6q24 (FHL4) (29), and very recently on chromosome 19p13 (FHL5) (30, 31). There is still further evidence of additional genetic heterogeneity and thus of yet undefined gene(s) associated with FHL (GSB, unpublished observation) (*Table 1*).

Perforin deficiency in FHL2

Deficiency of the cytolytic effector perforin (PRF1), which is present in cytotoxic granules, was the first genetic defect identified as causing FHL (32) (*Table 1*, *Fig. 1*). Perforin deficiency accounts for about 30–35% of FHL cases. Perforin is critical for the regulated access of granzymes to the cytosol of the target cell, where the latter cleave key substrates to initiate

Table 1. Autosomal recessive disorders associated with occurrence of HLH

	FHL1	FHL2	FHL3	FHL4	FHL5	GS2	CHS	HPSII
Gene	Unknown	*PRF1*	*UNC13D*	*STX11*	*STXBP2*	*RAB27A*	*CHS1/LYST*	*AP3B1*
Locus	(9q21.3-22)	(10q21-22)	(17q25)	(6q24)	(19p13)	(15q21)	(1q42-43)	(5q14-1)
Protein/ Function	?	Perforin/ pore-forming protein	Munc13-14/ priming factor	Syntaxin 11/ membrane fusion	Munc18-2/ membrane fusion	Rab27a/ tethening	Lyst/lysosomal fission- protein sorting	Ap3β1/sorting of lysosomal protein
Murine model	?	*prf1−/−*	*Jinx*	Not done	Not done	*ashen*	*beige*	*pearl*
HLH	+	+	+	+	+	+	+	±*
Cytotoxic activity	?	−	−	±	±	−	−	−
Hypopigmentation	−	−	−	−	−	+	+	+
Specific keys features							Giant granules, primary neurological disease	Neutropenia

HLH, hemophagocytic lymphohistiocytic syndrome; FHL, familial hemophagocytic lymphohistiocytosis; GS, Griscelli syndrome; CHS, Chediak-Higashi syndrome; HPS, Hermansky-Pudlak syndrome.
*Only one case have developed HLH, who also carries an heterozygous Rab27a mutation.

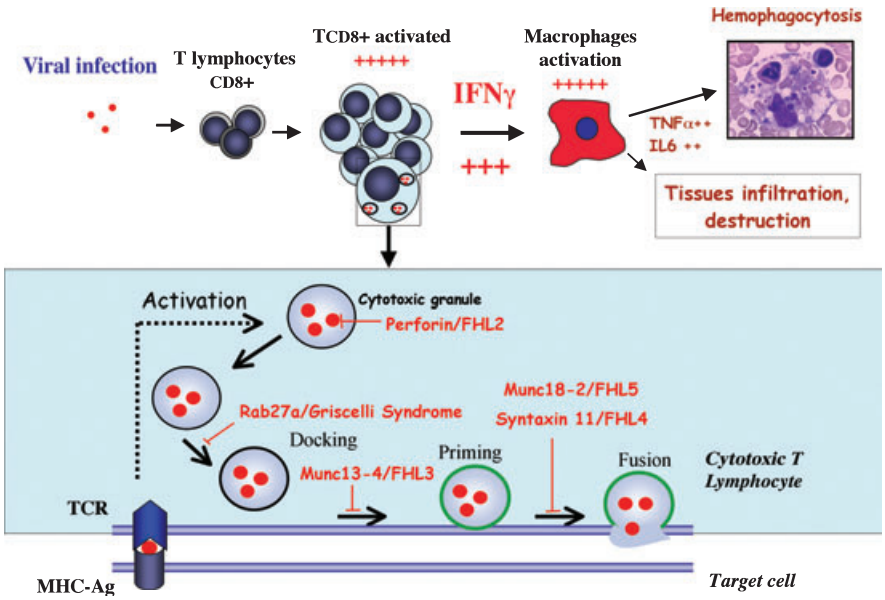

***Fig. 1.* Inborn errors in the cytotoxic activity of lymphocytes.** Top: Schematic diagram of the immune mechanisms leading to the occurrence of a hemophagocytic syndrome. Following a viral infection, antigen-specific $CD8^+$ T lymphocytes undergo massive expansion and activation and secrete high levels of interferon (IFN)-γ. The overwhelming activated effector cells induce excessive macrophage activation and pro-inflammatory cytokine production including tumor necrosis factor (TNF)-α and interleukin-6 (IL-6). Macrophages spontaneously phagocytose blood elements (here shown platelets, red blood cells, and a polymorphonuclear cell). Activated lymphocytes and macrophages infiltrate various organs, resulting in massive tissue necrosis and organ failure. Bottom: The genetic defects causing hemophagocytic lymphohistiocytic syndrome (HLH) affect a precise step of the cytotoxic machinery, i.e. granule content, docking, priming, or fusion. Only the defects causing Griscelli syndrome (GS) and familial hemophagocytic lymphohistiocytosis (FHL) are shown.

apoptotic death. The perforin protein comprises several regions, i.e. a cleavable amino-terminal leader peptide, a central region similar to membrane-attack complex-like proteins (MACPF), a calcium-binding domain (C2), and a cleavable N-glycosylated carboxy terminus (33, 34). Following the release of perforin from cytotoxic granules, binding of calcium on its C2 domain allows its interaction with the target membrane, a first key step in cytotoxic activity (35). Then, the MACPF domain of the protein mediates oligomerization of the protein and conformational change, a step that is critical for pore formation and lytic activity. This domain was recently shown to share high similarity with pore forming toxins (36). Structural analysis indeed supports a common mechanism in pore formation and cell membrane disruption.

Over 70 different recessive mutations in the perforin gene have so far been found in FHL2 patients. They consist in either microdeletion, nonsense, or missense mutations. Some perforin mutations are recurrent in a given populations, suggesting common ancestors. For example, the Trp374 stop (G1122A) occurs at high frequency in Turkish families, the L364 frameshift (1090delCT) in the Japanese population, and the L17 frameshift (50delT) change in the African population. As a consequence of most perforin gene mutations, perforin protein expression is diminished or barely detectable in cytotoxic granules (11, 32, 37), leading to defective cytotoxic activity. In addition, some peculiar missense mutations of the perforin gene have been observed that specifically affect the proteolytic cleavage and thus the maturation of the protein or its calcium-binding ability (38, 39). In these cases, the mutated protein can be synthesized, packed, and released at normal levels, but is devoid of cytotoxic function in the target cell. Missense mutations only partially impairing perforin expression and function have also been reported. They are frequently associated with atypical (mostly late onset) HLH disease. Among them, the substitution Ala91Val found with high frequency in healthy individuals (around 4–8%) was first considered as a neutral polymorphism (40). Nevertheless, further studies demonstrated that Ala91Val variant results in a partial loss (50%) of PRF1-dependent cytotoxicity, thus strongly suggesting that the Ala91Val allele can predispose to late onset disease expression or susceptibility to lymphoma when present on both genes and can be HLH causing if it is inherited with a second perforin allele with 'null' activity (41–43). Recent reports also provide convincing evidence that temperature-sensitive mutations in PFR1 may be associated with delayed FHL onset and predisposition to hematological malignancy (44) (discussed below). Thus, it is now fairly clear that some genotype/phenotype correlation exists in perforin deficiency, with a correlation between the age of onset and the residual functional activity of the protein. Phenotypes range from very early HLH onset to predisposition to hematological cancer (44). Some variability in expression can however persist as a function of environment (i.e. infection with virus) or potential modifier genes.

Given the restricted function of perforin in cytotoxic cells, the identification of perforin deficiency as a cause of HLH provided direct evidence of the importance of cytotoxic function of T and NK cells in the control of lymphocyte homeostasis during an immune response. In addition, it was decisive in the identification of additional causes of genetically HLH that were all found to also impair the granule-dependent cytotoxic function of lymphocytes.

Munc13-4 deficiency in FHL3

The second identified cause of FHL (FHL3), results from mutations in *UNC13D* that encodes Munc13-4, a member of the Munc13-UNC13 family (28) (*Table 1*, *Fig. 1*). Munc13-4 deficiency accounts for 30–35% of FHL cases. Structurally, Munc13-4 comprises two calcium-binding (C2) domains that are separated by long sequences containing two Munc13-homology domains (MHD1 and MHD2). Most of the mutations so far identified in *UNC13D* are deletions, splice site mutation, or nonsense mutations predicted to result in major changes in the protein. Few missense mutations in this gene have also been reported, although little is known about their functional consequences (45–47). The exocytosis of cytotoxic granules from Munc13-4-deficient T and NK lymphocytes was found impaired, whereas other secretory pathways including the polarized secretion of IFN-γ normally occurs (28, 45, 48). Study of Munc13-4-deficient CTLs shows that at least two different steps require Munc13-4. (i) Munc13-4-deficient lymphocytes can normally make stable conjugates with target cells and polarize the lytic machinery. However, while cytotoxic granules can dock at the IS, they cannot release their content. Thus, Munc13-4 is required to prime lytic granules that are docked at the plasma membrane, likely by regulating the interaction between the vesicle (v)- and target (t)- SNARE required for the fusion of the granule with the plasma membrane (28, 49). Indeed, at this step, Munc13-4 is likely to play a similar role as other members of the Munc13 family expressed at the neurological synapse (NS). Munc13-1, has been shown to act as priming factors for synaptic vesicle secretion (50, 51). (ii) An additional role of Munc13-4, located upstream of priming at the IS, was recently shown (52). In CTLs, Munc13-4 is required for the formation of a

pool of endosomal vesicles that are required to regulate the cytotoxic granule secretory pathway. This pool of vesicles carries effectors of the exocytic machinery, and, following target cell recognition, these vesicles polarize and coalesce with perforin-containing granules, which have simultaneously but independently polarized toward the IS (52). Similar findings were recently made for NK cells, further supporting this second role of Munc13-4 in the maturation of cytotoxic granules (53). In NK cells, depending on the way cells are triggered, either by natural cytotoxicity or Fc-receptor engagement, there is preferential recruitment of either Rab27a- or Munc13-4- associated vesicles to perforin-containing granules. These studies reveal that the process of lytic granule maturation and exocytosis is more complex than previously appreciated and that several maturation steps are required before a cytotoxic granule is ready to release its contents. The requirement of these multiple maturation steps may also prevent inappropriate release of granule content and its undue bystander deleterious consequences. Limiting the quantum of cytotoxic granules ready to release their content endows cytotoxic cells with an iterative cytotoxic activity, a well-known characteristic of these serial killers.

Although Munc13-4 expression is detected in multiple tissues, the phenotype of FHL3 patients does not differ from the one of perforin-deficient patients. In particular, FHL3 patients display no respiratory tract disease, despite the fact that a high level of Munc13-4 expression is detected in goblet cells of the lung epithelium. Similarly, Munc13-4 was shown *in vitro* to regulate granule secretion in platelets or polymorphonuclear cells, but there is no evidence of associated *in vivo* phenotype. One cannot fully exclude that subtle defects in these tissues could induce mild clinical manifestation that have so far been missed, or alternatively, the functional characteristics of the Munc13-4-dependent exocytic pathway may be strictly limited to the regulated secretory function of CTLs and NK cells.

Syntaxin 11 deficiency in FHL4

Patients with FHL4 carry mutations in the syntaxin 11 gene (*STX11*) (29), a member of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (t-SNARE) family involved in membrane fusion events (*Table 1*, *Fig. 1*). Syntaxin 11 is phylogenetically related to the target membrane SNARE proteins (t-SNARE) syntaxin 1–4 (49, 54). The selective pairing of t-SNARE, v-SNARE, and synaptosomal-associated proteins (SNAP) from opposing membranes forms a very stable parallel four helical bundle regulating membrane fusion during intracellular trafficking (55) (*Fig. 2*). Syntaxin 11 is highly expressed in hematological cells, mostly in CTLs, NK cells, and myeloid cells (29, 47, 56). There are still controversial data regarding the subcellular localization of STX11. Syntaxin 11 was reported to be either cytosolic, enriched in late endosomes, in trans-Golgi network, or present at the plasma membrane. The various cell types used in subcellular experiments may account for these discrepancies. So far, all of the deleterious mutations in STX11 reported in FHL4 patients are null mutations, most of them were identified in the Turkish and Kurdish population, where they account for approximately

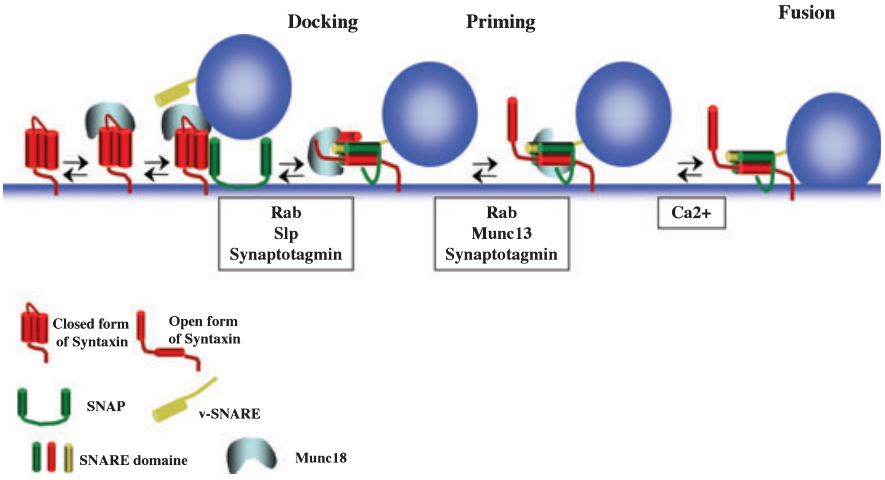

Fig. 2. Model of vesicle docking, priming, and fusion at the plasma membrane. Vesicles are transported and docked at the target membrane. At the plasma membrane, Munc18 in a first-step binds to the close conformation of the syntaxin, creating a docking platform, which gates initiation of the reaction. Docked vesicles are then primed by Munc13, which regulates the switch of syntaxin from a close conformation to an open conformation. SNARE complex forms between the SNARE domains of a v-SNARE on the vesicle and of a syntaxin and of a synaptosomal-associated protein (SNAP) present on the target membrane. Completion of the fusion reaction occurs when Munc18 clasps across the zippering four-helix assembled SNARE complex. Formation of a SNARE complex precedes membrane fusion.

20% of the FHL cases (47). The identification of STX11 deficiency as a cause of FHL prompted investigators to search for a role of this member of the SNARE family in cytotoxic granules exocytosis. Although a defective cytotoxic activity of FHL4 patients' NK cells was found, it appears more difficult to demonstrate, at least by using standard techniques, that the cytotoxic activity of STX11-deficient CTLs is significantly impaired. IL-2 stimulation was found to partially restore the cytotoxic NK cell defect (30, 56). In addition to being expressed in lymphocytes, STX11 is highly expressed in monocytes and macrophages. Therefore, an additional role of STX11 in the negative regulation of the phagocytic activity of macrophages was recently suggested (57). Further studies are required to assess the significance of this observation and to clarify the potential contribution of STX11-deficient monocytes in the development of the immune deregulation in FHL4 patients. Of note, however, the FHL4 phenotype does not really differ from FHL2 or FHL3.

Thus, STX11 is thought to be another key effector of the cytotoxic machinery required for the release of cytotoxic granules contents, likely by participating to the regulation of membrane fusion events (58). The precise step of the cytotoxic pathway regulated by STX11 remains to be characterized, even though its role in the fusions step of cytotoxic granules to the plasma membrane at the IS is the most likely step.

Munc18-2 deficiency in FHL5

The most recently identified cause of FHL, referred as FHL5, results from deficiency in the syntaxin-binding-protein-2 (*STXBP2*) encoding for Munc18-2 (30, 31). STXBP2/Munc18-2 (SM) belongs to the SM family of fusion accessory proteins. They are SNARE partners and play, together with SNARE, complementary roles in membrane fusion (59, 60) (*Table 1*, *Fig. 1*). Similarly to STX11, STXBP2/Munc18-2 is widely expressed. The highest levels of expression are observed in the spleen, lung, kidney, and testis. Both missense and nonsense mutations in *STXBP2* were characterized in FHL5 patients from diverse geographic origin (Europe, Turkish, Arabic, African). These mutations differentially affect protein stability (30, 31). There appears to be a phenotypic correlation, based on the age of onset and the severity of the disease, with severity of mutations (30). For example, the transition of a highly conserved proline to a leucine (P477L) in STXBP2/Munc18-2 leads to undetectable protein expression associated with early disease onset, most of the patients having died rapidly from HLH in the absence of hematopoietic stem cell transplantation (HSCT). In contrast, an in frame-splice site mutation of STXBP2/Munc18-2 (IVS14-1G>C) was identified in several other FHL5 cases. It preserves residual expression of a likely active protein, resulting in milder disease and delayed onset of HLH, beyond 5–8 years of age. STXBP2/Munc18-2 protein was previously reported to principally interact with syntaxin 3 and to a lesser extent with syntaxin 2. There is no change in the expression of syntaxin 3 in FHL5 patients' lymphoblasts (30). In contrast, expression of STX11 is dramatically impaired in STXBP2/Munc18-2-deficient lymphoblasts, and, when co-expressed, the proteins co-immunoprecipitate. Thus, STX11 protein is likely the main partner of Munc18-2 in lymphocytes and requires the presence of Munc18-2 for its stable expression. In accordance with the pathophysiology of FHL, Munc18-2 deficient NK cells show impaired cytotoxic activity, which can be partially restored upon IL2 stimulation, as previously reported for STX11 deficient NK cells (30, 31). In Munc18-2 deficient CTL, the cytotoxic granule exocytosis was not found consistently impaired. Types of mutations, and experimental conditions used are likely to be critical parameters in this setting, so that, resulting cytotoxic activity of Munc18-2 deficient CTL may vary *in vitro*. Similar observations were made with STX11 deficient CTL.

A role of Munc18-2 at a late step of the exocytic pathway is supported by the observation that perforin-containing granules of Munc18-2-deficient NK cells normally polarize toward cognate target cells, even though they are impaired in their ability to exocytose their content (30). Thus, the same defective cytotoxic phenotype characterizes both STX11 and STXBP2 deficiencies, an observation that further suggests functional interaction between these two proteins in the degranulation process.

The role of SM proteins in vesicle exocytosis has mainly been deduced by studying STXBP1/Munc18-1 in neurons, where its deletion leads to a complete loss of neurotransmitter secretion in mice (61, 62). As in the process of cytotoxic granule exocytosis, a sequential docking–priming fusion pathway is thought to orchestrate synaptic vesicle exocytosis at the NS. STXBP1/Mun18-1 was shown in a first step to bind to the closed conformation of its specific syntaxin, i.e. syntaxin 1, and thus creates a docking platform which gates the initiation of the reaction (*Fig. 2*). Docked vesicles are then primed for fusion by Munc13-1 (the homolog of Munc13-4 in cytotoxic cells). Completion of the fusion reaction occurs when STXBP1 clasps across the zippering four-helix assembled trans-SNARE complex (60). Identification of Munc18-2 deficiency as the cause of FHL5 strongly suggests that at the IS of a cytotoxic cell conjugated with a target, the STXBP2–STX11 complex,

could exert a role similar to that of STXBP1–syntaxin 1 complex at the NS, by regulating granule docking and initiation of SNARE complex formation upstream of the priming step.

Pigmentary dilution associated with the occurrence of HLH

Griscelli syndrome type 2 (GS2) is a rare autosomal recessive condition characterized by typical pigmentary dilution and occurrence of the HLH syndrome. Onset of HLH in GS2 occurs later (median age 3 years) than in FHL, suggesting that GS2-NK and CTLs exert a residual cytotoxic function *in vivo*. Bialllelic mutations in the gene encoding Rab27a, an ubiquitously expressed small GTP-binding GTPase protein, are responsible for GS2 (63) (*Table 1*, *Fig. 1*). Nonsense, frameshift, or missense mutations in Rab27a have been characterized in more than 100 independent patients. Rab27a-deficient NK cells and CTLs exhibit impaired mediated exocytosis of cytotoxic granules, although polarization is preserved. Electron microscopic studies have revealed that, unlike in control cells, cytotoxic granules in absence of Rab27 cannot reach the IS to dock to the plasma membrane (64). However, Rab27a is not directly associated with the cytotoxic granules of CTLs or NK cells (52, 56). Rab27a is enriched on endosomal structures together with Munc13-4, which we named 'exocytic vesicles'. These vesicles coalesce with perforin-containing granules before the release of granule content. Munc13-4 does interact with Rab27a in cytotoxic cells (52). This molecular interaction likely is involved in the coordination of the final step of the exocytic process, between docking and priming of granule exocytosis. Additional effectors of the GTP-bound form of Rab27a have been described. Among them, the synaptotagmin-like-protein-2a (Slp2a) expressed in lymphocytes was recently shown to drive the docking step of the Rab27a-associated vesicles at the IS (65, 66). However, cytotoxic function of CTLs from mice deficient for Slp2a are not impaired, suggesting that other effector(s) of Rab27a in cytotoxic cells, which remain(s) to be characterized, exert redundant function with Slp2 in the docking step of the Rab27a-associated vesicles. Further dissection of uncharacterized natural mutants with an HLH phenotype should help in finding additional effectors and precise mechanisms of action.

Pigmentary dilution in GS2 is similarly accounted for by a defective release of melanosome content from melanocyte dendrites. In melanocytes, Rab27a associates with melanophilin, a Slp-member specifically expressed in melanocytes, which in turn interacts with the molecular motor protein myosin-Va. This tripartite complex (Rab27a-melanophilin-myosin-Va) links melanosome to the actin network, allowing their distribution to the dendrite tips of melanocytes, from where they can be then transferred to adjacent keratinocytes (67). A deficiency in either of these three proteins was shown to impair melanosome transport resulting in the same hypopigmentation phenotype (15). They correspond to the following diseases: GS1 (myosin-Va defect), GS2 (Rab27a defect), and GS3 (melanophilin defect). Only Rab27a, however, has a critical role in the cytotoxic machinery, and thereby its defect leads to an HLH immune phenotype.

Chediak-Higashi syndrome (CHS) was the first described condition characterized by pigmentation dilution, defective T and NK lymphocyte cytotoxic activity, and occurrence of HLH, with a relatively late onset (2–10 years of age) (15, 68). A striking phenotypic feature of this autosomal recessive condition is the presence of giant intracytoplasmic lysosomal structures in all granulated cells, including hematopoietic cells and melanocytes. In cytotoxic cells, cytotoxic granules are enlarged too, and do not release their content at the IS, despite a normal polarization upon activation. A progressive primary neurological disease is another hallmark of CHS (69). Mutations in *CHS1/LYST*, which encodes LYST, a huge cytosolic protein of 425 kDa ubiquitously expressed, account for this disease (*Table 1*) (70, 71). LYST belongs to a family of proteins, called the *BEACH* (beige and Chediak-Higashi) family, which share the three same C-terminal domains, i.e. a pleckstrin homology domain (PH), a BEACH domain (70, 72), and WD40 repeats. However, the exact function of these domains remains unknown, although they are supposed to play a role in the binding of protein partners (72, 73). Most of the functional information on LYST comes from the studies of other members of the BEACH family, which define them as vesicle trafficking regulatory proteins (74). Interestingly, two identified LYST partners, HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) and LIP5, are ESCRT (endosomal sorting complex required for transport)-associated proteins, suggesting a role of LYST in the regulatory sorting of endosomal proteins into lysosomes (75, 76). Alternatively, LYST could be involved in regulating fusion or fission events of lysosomes, as HRS can also interact with a target SNARE. Recently, a lectin-like domain has been identified in the N-terminal region of LYST, which is also shared with the other BEACH proteins (77). Identification of the sugar-binding specificity of this lectin domain should aid in understanding the function of LYST. This domain could potentially be involved in oligosaccharide binding that is associated with protein traffic and sorting along the secretory pathway,

especially in relation with components of the vesicle fusion machinery.

Hermansky-Pudlak syndrome (HPS) defines a group of at least eight human autosomal recessive genetic disorders (HPS-1 to -8) associating hypopigmentation and bleeding disorders (78–80). HPS type 2 is the only form of HPS also associated with immunodeficiency, mainly increased susceptibility to infections due to congenital neutropenia and defective cytotoxic activity of T and NK lymphocytes (81–83). HPS2 results from mutation in *AP3B1*, the β chain of the adapter protein-3 (AP3) complex (84) (*Table 1*). Only few missense and nonsense mutations or deletions in *AP3B1* have been reported. The AP3 is a ubiquitous cytoplasmic complex consisting in four different subunits that shuttles cargo proteins from the trans-Golgi and a tubular-endosomal compartment to endosome-lysosome-related organelles (85, 86). AP3 thus functions in protein sorting to lysosomes. Defects in the β subunit disrupt the complex, and all subunits are rapidly degraded. Misrouting of lysosomal proteins such as CD107 and CD63 to the cell membrane has been found on CTLs (81, 82). Protein missorting may explain several features of the HPSII phenotype. Aberrant subcellular targeting of neutrophil elastase likely contributes to the observed neutropenia (87, 88), while missorting of tyrosinase contributes to the pigmentation disorder (89).

In the absence of AP3, cytotoxic T and NK cell-mediated killing was shown significantly impaired, with the presence of enlarged cytotoxic granules unable to move along microtubules toward the MTOC when the cytotoxic cell recognizes a target (81–83). Although all HPSII patients tested have a defective cytotoxic activity, only one case was so far reported with the development of an HLH. A heterozygous Rab27a mutation was associated with homozygous AP3B1 mutation in this case developing HLH manifestations. It is unclear whether the additional heterozygous RAB27a mutation or an other unidentified defect in this patient contributed to this complication (82).

A key clinical question which remains regarding HPSII is whether the cytotoxicity defect in HPSII predisposes to HLH and therefore represents an indication for pre-emptive HSCT or not. Long-term follow-up will be necessary to try to answer to this question.

HLH also characterized the course of a complex disease known as X-linked lymphoproliferative syndrome (XLP) caused by mutations of the *SH2D1A* (XLP1) and *XIAP* (XLP2) genes (90, 91). *SH2D1A* encodes SAP, an adapter protein interacting with multiple receptors and *XIAP* encodes an inhibitor of apoptosis. HLH in XLP1-2 patients is most of the time associated with EBV infections. However, in both conditions, occurrence of HLH is likely a complex phenomenon that cannot be restricted to granule-dependent cytotoxic defect of lymphocytes, which is only partially (in XLP1) or even not detected (XLP2).

Pathophysiology of HLH

Studies of experimental murine models

The molecular identification of the various inherited conditions characterized by the occurrence of HLH has substantially improved our understanding of the pathophysiology of these conditions. The underlying inborn errors have revealed the importance of several crucial steps in the exocytosis of cytotoxic granules by cytotoxic lymphocytes. Analysis of animal models of HLH have contributed to the understanding of how the genetic defects of the cytotoxic machinery could be linked to the aberrant immune response observed in HLH. LCMV-infected perforin-, Unc13-d- (the mouse ortholog of human Munc13-4), and Rab27a-deficient mice developed body temperature changes, splenomegaly, pancytopenia, hypercytokinemia, and histopathological features characteristic of HLH (92–95). Thus, these LCMV-infected, cytotoxicity-deficient mice represent reliable models of human HLH. Studies in perforin-deficient mice have shown the central role of $CD8^+$ T cells and IFN-γ production in the pathogenesis of HLH, since prevention of HLH was achieved by administration of anti-CD8 antibody or neutralization of IFN-γ, whereas anti-NK, CD4 antibody, as well as antibodies neutralizing a set of other cytokines did not (93). This result is consistent with the finding of an increased number of activated $CD8^+$ T-effector cells in blood and tissues of HLH patients and substantially elevated serum IFN-γ levels in patients with inherited and acquired HLH during active disease (17, 96, authors' unpublished observations). Thus, the pathogenesis of HLH in patients with impaired cytotoxicity is most likely based on $CD8^+$ T-cytotoxic effector cells' inability to eliminate the infecting pathogen. Although the infected cells are recognized and thus prompt cytotoxic cell activation and clonal expansion, the resulting cytotoxic cell population fails to kill the infected antigen-presenting cells and to remove the source of stimulation. The ever-expanding population of cytotoxic lymphocytes produces large quantities of cytokines, such as IFN-γ, which sustain macrophage activation. There is a striking resemblance between biological changes induced by inflammatory cytokines, especially by exposure to TNF-α, and the clinical and laboratory findings that characterize HLH (17).

However, the fact that in most cases in humans, and in a few experimental setting in mice HLH is not associated with a high load of the triggering infectious agent, suggests that additional mechanisms may also play a role. Among them, cytotoxic lymphocytes may use a fratricide mechanism to kill each other. Such an hypothesis is supported by the observation that *in vitro* CTLs can be a target for sister CTLs, after they have captured membrane fragment from previously killed targets (3, 97, 98). The *in vivo* relevance of this mechanism is difficult to assess, but if it occurs *in vivo*, it should efficiently participate in the downregulation of the $CD8^+$ T-cell mediated immune response. In addition, subsets of regulatory T cells and NKT cells are armed with the same cytotoxic equipment that they can use to kill, at least *in vitro*, antigen-presenting cells or T cells in a CD1-restricted manner (99, 100). This process may also help to contain T-cell expansion.

Table 2. Comparison of human and murine HLH features

	Human	Murine
Body temperature	Increased*,†,‡, perimortem: decreased*,†,‡	Decreased*,†
Spleen size	Enlarged*,†,‡	Enlarged*,†,§
Hemoglobin	Decreased*,†,‡	Decreased
Neutrophil count	Decreased*,†,‡	Decreased*,†; increased§
Thrombocyte count	Decreased*,†,‡	Decreased*,†,§
Hemophagocytosis	Present*,†,‡	Present*,†
Ferritin levels	Increased*,†,‡	Increased*,†
Triglyceride levels	Increased*,†,‡	Increased*,†
Outcome without treatment	Death*,†,‡	Death*
	Rarely transient, relapsing†	Transient†

HLH, hemophagocytic lymphohistiocytic syndrome.
*Complete perforin deficiency.
†Complete Rab27a deficiency.
‡Complete Munc13-4 deficiency.
§Complete Unc13d deficiency.

A pivotal role for IFN-γ in HLH: toward an alternative therapeutic approach

Blocking IFN-γ did not only prevent HLH but was also recently shown to exert a therapeutic effect in experimental HLH (101) (*Fig.* 3). Therapeutic administration of an anti-IFN-γ antibody induced recovery from HLH in two different murine models of human HLH (perforin-deficient and Rab27a-deficient mice, both infected with LCMV), as shown by an increased survival in perforin-deficient mice and moderation of body temperature changes, correction of blood cytopenia, decreased cytokinemia, restoration of splenic architecture and reduced hemophagocytosis in the liver of both murine models (101) (*Table* 2, *Fig.* 3). CNS involvement in Rab27a-deficient mice was prevented by anti-IFN-γ therapy. After anti-IFN-γ therapy, T-cell infiltrates in the liver persisted, and LCMV could still be detected, with no detectable

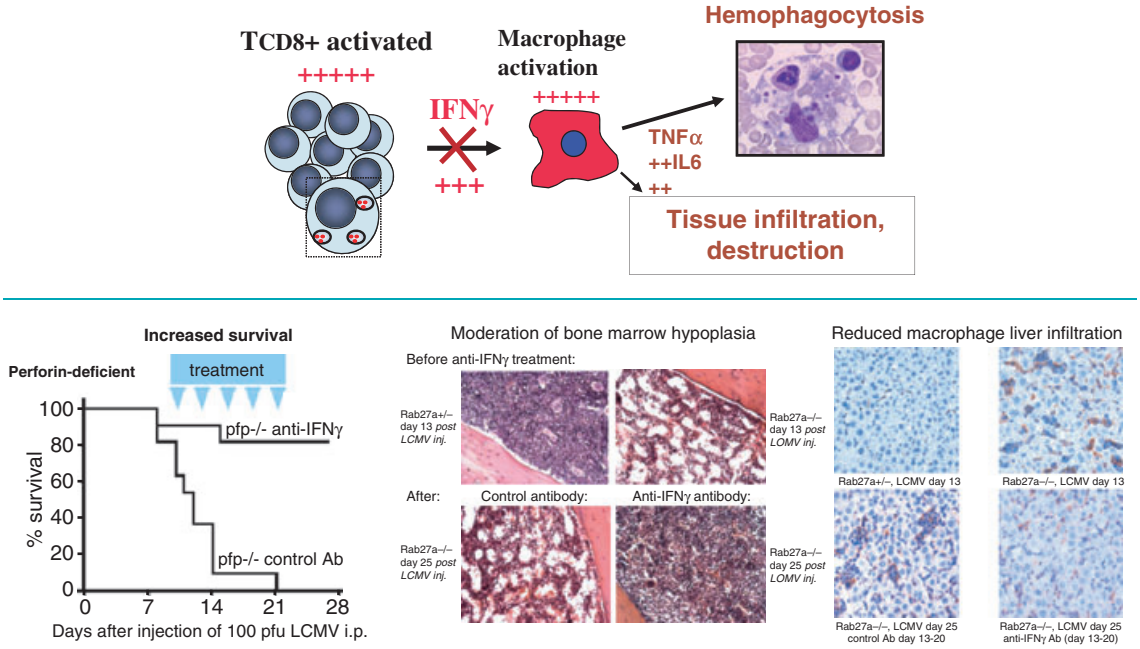

***Fig.* 3. Anti-interferon (IFN)-γ treatment defeats hemophagocytic lymphohistiocytic syndrome (HLH) in murine models of HLH.** Top: Treatment by anti-IFN-γ was done in perforin-deficient (day 8 after viral infection) and Rab27a-deficient mice (day 13 after viral infection) at the time the mice expressed the HLH full blown syndrome. Bottom: Wildtype and deficient mice treated by IFN-γ antibody or receiving control antibody were evaluated 2 weeks after beginning of treatment. Neutralization of IFN-γ increased survival of perforin-deficient mice and allowed recovery of bone marrow cellularity and reduced macrophage infiltration and activation (hemophagocytosis) in the liver. Reproduced from (101) with permission.

harm during the time course of these studies. It is likely that impaired killing of infected cells caused by the cytotoxicity defect enabled viral persistence. T-cell infiltrates were associated with the local detection of LCMV, suggesting the presence of ongoing, IFN-γ independent, partially inefficient, LCMV-driven T-cell responses. These results suggest that the pathophysiology of HLH can be divided into two steps: (i) virus-triggered $CD8^+$ T-cell activation and expansion resulting in high, sustained production of IFN-γ in the absence of virus clearance, and (ii) IFN-γ-mediated macrophage activation.

The second step only is inhibited by the anti-IFN-γ antibody. However, this is enough to alleviate most of the HLH symptoms in perforin- and Rab27a-deficient mice and to enable survival of LCMV-infected, perforin-deficient mice with HLH. These data suggest that neutralization of IFN-γ could be used in humans to alleviate the clinical manifestations of HLH. This treatment could be especially attractive for patients with inherited HLH, given that current drugs used to treat HLH, such as etoposide (102) or antithymoglobulins (103), can be toxic and are far more immunosuppressive than a transient IFN-γ blockade. Another potential advantage of an IFN-γ blockade in the management of inherited HLH might be its ability to improve engraftment in HSCT, as IFN-γ exerts a myelosuppressive effect. Neutralization of IFN-γ might also be an efficient and safe way to treat patients with acquired HLH, provided that the T-cell activation trigger is also amenable to therapy.

HLH: unresolved questions

Triggering events of HLH

Infection is required to trigger an HLH-like condition in mice, and LCMV appears to be unique in this setting. This likely results from the ability of LCMV to stimulate a very strong immune T-cell and IFN-γ responses (93). In humans, EBV plays a similar role, being also a vigorous trigger of T-cell immune responses. Accordingly, EBV is a frequent trigger of HLH in humans. There is a tendency of an inverse correlation between the severity of the cytotoxic deficiency and the strength of the triggering agent required to induce HLH. A more pronounced cytotoxic deficiency results from perforin deficiency, compared to Rab27a or Munc13-4 deficiency. Consistently, a specific triggering agent is rarely identified in FHL2 patients who develop HLH very early in life, whereas EBV is the most frequently associated trigger of HLH in patients with GS and CHS, which occur generally later in life. Similarly, onset of HLH in perforin-deficient mice is induced with a lower viral dose than in Rab27a- or Munc13-4-deficient mice; these latter murine models in addition exhibit a better survival rate. Additionally, for a given gene defect, HLH occurs later in patients with missense mutations, which preserve some residual activity of the proteins than in patients with null mutations. Here again, EBV is more frequently found as an HLH trigger in patients with hypomorphic mutations. Thus, both murine models of and human patients with HLH support a similar correlation between the severity of the cytotoxic impairment and susceptibility to HLH occurrence. It is also notable that the consequences of granule-dependent cytotoxic defect are much more dramatic in humans than in mice, as some patients can develop HLH very early, during the neonatal period or even *in utero*, without clear-cut identification of a triggering event. Whether non-infectious environmental factors may also trigger these early HLH forms is an interesting possibility to consider. In contrast, murine models of HLH never spontaneously develop HLH, even when bred in non-protected animal facilities. All types of HLH murine models require a strong LCMV stimulus to trigger HLH.

Granule-dependent cytotoxicity is not the only mechanism by which lymphocytes can kill. Fas ligand expression can kill permissive Fas-expressing cells, and TNF-α can also exert cytotoxic functions. It is intriguing to observe that despite the fact that CTL and NK cells are armed with both granule-dependent and Fas-dependent cytotoxic pathways, they are obviously non-redundant in their functions. Genetic defects of the Fas ligand/Fas pathway lead to the autoimmune lymphoproliferative syndrome condition both in humans and mice, which differ from HLH and is related to autoimmunity (104, 105). No clear mechanisms have been provided to account for these striking observations.

Respective role of CTLs versus NK cells in HLH

The fact that the various genetic causes of FHL appears to differentially affect T- and NK cell cytotoxic activity, although the phenotype is indistinguishable among these various forms, is an intriguing observation. Null mutations in perforin, Rab27a, or Munc13-4 are consistently associated with a clear detectable impairment in both NK cells and CTL cytotoxic activity. In contrast, null mutations in STX11 or Munc18-2 impair exocytosis of cytotoxic granules from NK cells, a function partially restored upon IL-2 exposure. In addition, there are controversial results regarding the impairment of cytotoxic granule exocytosis of STX11 and Munc18-2 deficient CTLs. Indeed, defective cytotoxic degranulation of patient lymphocytes is not consistently observed, even in the absence of detectable protein expression. Several hypotheses

can be raised to explain this discrepancy: (i) NK cell functional deficiency could play the main role in the occurrence of the hemophagocytic lymphohistiocytosis phenotype. However, studies performed in the perforin-deficient mice, that were depleted either from the $CD8^+$ T-cell population or the NK cell population, strongly suggest that CTLs rather than the NK1.1 cell population play a critical role in the occurrence of HLH, at least in this condition (93). A similar conclusion was recently raised from the study comparing the ability of either WT, perforin$^{-/-}$ or RAG/perforin$^{-/-}$ donor bone marrow cells to re-establish normal immune regulation in perforin-deficient mice (106). In addition, the few patients with primary immune deficiencies, including with an absence of circulating NK cells, are more prone to viral infection with herpes virus than to development of an HLH. (ii) An alternative and likely hypothesis is that the *in vitro* cytotoxic assays used do not faithfully reflect the *in vivo* function of cytotoxic cells. The fact that some NK cell cytotoxic activity is partially restored by exposure to IL-2 *in vitro* supports this assumption. CTLs need to be cultured and activated before testing their cytotoxic activity *in vitro*; thus, these conditions could bypass the physiological step of the exocytic machinery, which is regulated by STX11 and Munc18-2. (iii) Finally, STX11 and Munc18-2 may have an additional role in other immune cells, as in antigen-presenting cells, which may operate in addition to the impairment of NK cell cytotoxic activity (57).

Defective cytotoxic function and immune surveillance

Evidence of a link between the functional defect of granule-dependent cytotoxicity and cancer susceptibility has been brought by the analysis of the mouse model of perforin deficiency. Early experiments have pointed out that perforin knockout mice are more susceptible to tumorigenesis than wildtype mice and that old perforin knockout mice have an increased susceptibility to the development of spontaneous lymphomas (107). Whatever the genetic background, approximately half of a cohort of aging perforin-deficient mice develops spontaneous mature B-cell malignancy, between 1 and 1.5 years after birth. More recent studies have reinforced the role of perforin in immune surveillance of lymphomas (108). By analyzing perforin loss associated with either loss of Mlh1 tumor suppressor allele or oncogene-expressing transgenic mice, perforin was shown to act as a suppressor of B-cell malignancies (108). A role for perforin in surveillance of B-cell lymphomagenesis is now well established in mice, even though the precise reason why perforin loss specifically favors B-cell lymphoma development remains unresolved.

In humans, the role of perforin, and more broadly of granule-dependent lymphocyte cytotoxicity, in influencing the susceptibility to cancer remains difficult to assess. FHL patients succumb to overwhelming HLH at a young age and die, unless they undergo HSCT. These past years, several correlative studies have attempted to demonstrate such a link. A few reports have shown that some patients with lymphoma carry perforin mutations, especially the Ala91Val mutant, potentially supporting a role for perforin in immune surveillance (42, 109–111). However, given the small size of the cohort of patients screened in these studies, together with the high frequency of the specific Ala91Val mutant in the normal population, which in addition highly differ within the various populations, these results should be interpreted with caution. Today, the most compelling evidence for a link between perforin and immune surveillance in humans relies on the recent observation that temperature-sensitive missense mutations in perforin are frequently inherited in patients with delayed onset of FHL and increased susceptibility to hematological malignancies. These mutations affect protein folding and thus protein function when expressed at 37 °C, but a functional defect can be rescued at a permissive temperature (30 °C). Thus, in contrast to truly null mutations with no possibility of rescue associated with early HLH occurrence, the amount of residual activity of the protein remaining at permissive temperature fully correlates with the age of HLH onset and with the occurrence of lymphoma or leukemia (44). Most likely, partially active perforin enables patients to survive long enough without developing HLH to unmask their predisposition to cancer.

By extrapolating these data, one can suggest that for the other genetically determined defects of the cytotoxic activity of lymphocytes, hypomorphic mutations may similarly be associated with increased susceptibility to lymphomagenesis. This hypothesis is now worth considering.

Another important question is to determine the minimal level of cytotoxic activity required to maintain immune homeostasis and avoid predisposition to cancer. Clearly, a single normal perforin allele expressed in the parents of children with inherited HLH is sufficient to maintain health. These obligatory carriers were never reported to exhibit increased predisposition to malignancies, although exhaustive studies have not yet been performed in this population.

Concluding remarks

The studies of human inborn errors affecting lymphocyte cytotoxic activity have been extremely rewarding by leading

to the identification of critical effectors of the exocytic machinery of cytotoxic cells. The multiplicity of genetic forms now identified allows for dissection of the cascade of events which drive the maturation, docking, priming, and fusion of the cytotoxic granules of lymphocytes when these cells recognize target cells. Few inherited HLH conditions remain to be molecularly identified, which again may allow identification of additional effectors/regulators of the cytotoxic function of lymphocytes. These discoveries have also provided insight into the unexpected regulatory function of the cytotoxic lymphocytes during an immune response. It remains; however, to better assess the consequence on immune surveillance of moderate reduction of the granule-dependent cytotoxic function of lymphocytes resulting from hypomorphic mutations of HLH-associated genes.

Analyses of murine models of HLH support a primary role of IFN-γ secreted by lymphocytes in the excessive macrophage activation that drives disease pathogenesis. The recent demonstration of a therapeutic role of IFN-γ neutralization on disease manifestations in murine models of HLH may be in the future transposed to the clinic. Important advances have been made during the last 20 years in the treatment of these inherited conditions, based on the usage of cytolytic and immunosuppressive drugs combined with allogenic HSCT. A considerable challenge remains in the development of efficient and less toxic drugs to be used in order to achieve remission of HLH.

References

1. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol 2002; **20**:323–370.
2. Bottino C, Moretta L, Moretta A. NK cell activating receptors and tumor recognition in humans. Curr Top Microbiol Immunol 2006;**298**:175–182.
3. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity 2001;**15**:751–761.
4. Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol 2004;**5**:524–530.
5. Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. Nature 2006;**443**:462–465.
6. Jenkins MR, Tsun A, Stinchcombe JC, Griffiths GM. The strength of T cell receptor signal controls the polarization of cytotoxic machinery to the immunological synapse. Immunity 2009;**31**:621–631.
7. Beal AM, et al. Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. Immunity 2009;**31**:632–642.
8. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;**2**:735–747.
9. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 2003;**3**:361–370.
10. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol 2002;**2**:401–409.
11. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol 2006;**6**:940–952.
12. Cullen SP, Martin SJ. Mechanisms of granule-dependent killing. Cell Death Differ 2008;**15**:251–262.
13. Martinvalet D, Dykxhoorn DM, Ferrini R, Lieberman J. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. Cell 2008; **133**:681–692.
14. De SaintBasile G, Fischer A. The role of cytotoxicity in lymphocyte homeostasis. Curr Opin Immunol 2001;**13**:549–554.
15. Menasche G, Feldmann J, Fischer A, de Saint Basile G. Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis. Immunol Rev 2005;**203**:165–179.
16. Henter JI, Elinder G, Ost A. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. Semin Oncol 1991;**18**:29–33.
17. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. Blood 1991;**78**:2918–2922.
18. Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-gamma-producing lymphocytes and IL-6- and TNF-alpha-producing macrophages. Blood 2005;**105**:1648–1651.
19. Henter JI, Arico M, Elinder G, Imashuku S, Janka G. Familial hemophagocytic lymphohistiocytosis: primary hemophagocytic lymphohistiocytosis. Hematol Oncol Clin North Am 1998;**12**:417–433.
20. Farquhar J, Claireaux A. Familial haemophagocytic reticulosis. Arch Dis Child 1952;**27**:519–525.
21. Haddad E, Sulis ML, Jabado N, Blanche S, Fischer A, Tardieu M. Frequency and severity of central nervous system lesions in hemophagocytic lymphohistiocytosis. Blood 1997;**89**:794–800.
22. Osugi Y, et al. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. Blood 1997;**89**:4100–4103.
23. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. Acta Paediatr Scand 1991;**80**:428–435.
24. Lipton JM, Westra S, Haverty CE, Roberts D, Harris NL. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 28-2004. Newborn twins with thrombocytopenia, coagulation defects, and hepatosplenomegaly. N Engl J Med 2004;**351**:1120–1130.
25. Schneider EM, Lorenz I, Muller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. Blood 2002;**100**:2891–2898.
26. Ohadi M, et al. Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3-22 by homozygosity mapping. Am J Hum Genet 1999;**64**:165–171.
27. Dufourcq-Lagelouse R, et al. Linkage of familial hemophagocytic lymphohistiocytosis to 10q21-22 and evidence for heterogeneity. Am J Hum Genet 1999;**64**:172–179.

28. Feldmann J, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell 2003;**115**:461–473.
29. zur Stadt U, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum Mol Genet 2005;**14**:827–834.
30. Côte M, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. J Clin Invest 2009;**119**:3765–3773.
31. zur Stadt U, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. Am J Hum Genet 2009;**85**:482–492.
32. Stepp S, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science 1999;**286**:1957–1959.
33. Lichtenheld MG, et al. Structure and function of human perforin. Nature 1988; **335**:448–451.
34. Uellner R, et al. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. EMBO J 1997;**16**:7287–7296.
35. Voskoboinik I, Mar J, Camakaris J. Mutational analysis of the Menkes copper P-type ATPase (ATP7A). Biochem Biophys Res Commun 2003;**301**:488–494.
36. Rosado CJ, et al. A common fold mediates vertebrate defense and bacterial attack. Science 2007;**317**:1548–1551.
37. Feldmann J, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. Br J Haematol 2002;**117**:965–972.
38. Voskoboinik I, et al. The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. J Exp Med 2004;**200**:811–816.
39. Feldmann J, et al. Severe and progressive encephalitis as a presenting manifestation of a novel missense perforin mutation and impaired cytolytic activity. Blood 2005;**105**:2658–2663.
40. Zur Stadt U, Beutel K, Weber B, Kabisch H, Schneppenheim R, Janka G. A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype. Blood 2004;**104**:1909; author reply 1910.
41. Trambas C, et al. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. Blood 2005;**106**:932–937.
42. Santoro A, Cannella S, Trizzino A, Lo Nigro L, Corsello G, Arico M. A single amino acid change A91V in perforin: a novel, frequent predisposing factor to childhood acute lymphoblastic leukemia? Haematologica 2005;**90**:697–698.
43. Voskoboinik I, et al. Perforin activity and immune homeostasis: the common A91V polymorphism in perforin results in both presynaptic and postsynaptic defects in function. Blood 2007;**110**:1184–1190.
44. Chia J, Yeo KP, Whisstock JC, Dunstone MA, Trapani JA, Voskoboinik I. Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. Proc Natl Acad Sci USA 2009;**106**:9809–9814.
45. Marcenaro S, et al. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. Blood 2006;**108**:2316–2323.
46. Santoro A, et al. Novel Munc13-4 mutations in children and young adult patients with haemophagocytic lymphohistiocytosis. J Med Genet 2006;**43**:953–960.
47. Zur Stadt U, et al. Mutation spectrum in children with primary hemophagocytic lymphohistiocytosis: molecular and functional analyses of PRF1, UNC13D, STX11, and RAB27A. Hum Mutat 2006;**27**:62–68.
48. Ueda I, Ishii E, Morimoto A, Ohga S, Sako M, Imashuku S. Correlation between phenotypic heterogeneity and gene mutational characteristics in familial hemophagocytic lymphohistiocytosis (FHL). Pediatr Blood Cancer 2006;**46**:482–488.
49. Hong W. Cytotoxic T lymphocyte exocytosis: bring on the SNAREs! Trends Cell Biol 2005;**15**:644–650.
50. Brose N, Hofmann K, Hata Y, Sudhof TC. Mammalian homologues of *Caenorhabditis elegans* unc-13 gene define novel family of C2-domain proteins. J Biol Chem 1995;**270**:25273–25280.
51. Brose N, Rosenmund C, Rettig J. Regulation of transmitter release by Unc-13 and its homologues. Curr Opin Neurobiol 2000;**10**:303–311.
52. Menager MM, et al. Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. Nat Immunol 2007;**8**:257–267.
53. Wood SM, et al. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. Blood 2009;**114**:4117–4127.
54. Prekeris R, Klumperman J, Scheller RH. Syntaxin 11 is an atypical SNARE abundant in the immune system. Eur J Cell Biol 2000;**79**:771–780.
55. Jahn R, Scheller RH. SNAREs – engines for membrane fusion. Nat Rev Mol Cell Biol 2006;**7**:631–643.
56. Bryceson YT, et al. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. Blood 2007;**110**:1906–1915.
57. Zhang S, et al. Syntaxin-11 is expressed in primary human monocytes/macrophages and acts as a negative regulator of macrophage engulfment of apoptotic cells and IgG-opsonized target cells. Br J Haematol 2008;**142**:469–479.
58. Arneson LN, Brickshawana A, Segovis CM, Schoon RA, Dick CJ, Leibson PJ. Cutting edge: syntaxin 11 regulates lymphocyte-mediated secretion and cytotoxicity. J Immunol 2007;**179**:3397–3401.
59. Toonen RF, Verhage M. Vesicle trafficking: pleasure and pain from SM genes. Trends Cell Biol 2003;**13**:177–186.
60. Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins. Science 2009;**323**:474–477.
61. Verhage M, et al. Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 2000;**287**:864–869.
62. Toonen RF, Verhage M. Munc18-1 in secretion: lonely Munc joins SNARE team and takes control. Trends Neurosci 2007;**30**:564–572.
63. Ménasché G, et al. Mutations in RAB27A cause Griscelli syndrome associated with hemophagocytic syndrome. Nat Genet 2000;**25**:173–176.
64. Stinchcombe JC, et al. Rab27a is required for regulated secretion in cytotoxic t lymphocytes. J Cell Biol 2001;**152**:825–834.
65. Menasche G, et al. A newly identified isoform of Slp2a associates with Rab27a in cytotoxic T cells and participates to cytotoxic granule secretion. Blood 2008;**112**:5052–5062.
66. Holt O, et al. Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse. Traffic 2008;**9**:446–457.
67. Wu XS, et al. Identification of an organelle receptor for myosin-Va. Nat Cell Biol 2002;**4**:271–278.
68. Stinchcombe J, Bossi G, Griffiths GM. Linking albinism and immunity: the secrets of secretory lysosomes. Science 2004;**305**:55–59.
69. Tardieu M, et al. Progressive neurologic dysfunctions 20 years after allogeneic bone marrow transplantation for Chediak-Higashi syndrome. Blood 2005;**106**:40–42.
70. Nagle DL, et al. Identification and mutation analysis of the complete gene for Chediak-

Higashi syndrome. Nat Genet 1996; **14**:307–311.
71. Barbosa MDFS, et al. Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature 1996;**382**:262–265.
72. Jogl G, et al. Crystal structure of the BEACH domain reveals an unusual fold and extensive association with a novel PH domain. EMBO J 2002;**21**:4785–4795.
73. Gebauer D, Li J, Jogl G, Shen Y, Myszka DG, Tong L. Crystal structure of the PH-BEACH domains of human LRBA/BGL. Biochemistry 2004;**43**:14873–14880.
74. Martens S, McMahon HT. Mechanisms of membrane fusion: disparate players and common principles. Nat Rev Mol Cell Biol 2008;**9**:543–556.
75. Tchernev VT, et al. The Chediak-Higashi protein interacts with SNARE complex and signal transduction proteins. Mol Med 2002;**8**:56–64.
76. Williams RL, Urbe S. The emerging shape of the ESCRT machinery. Nat Rev Mol Cell Biol 2007;**8**:355–368.
77. Burgess A, Mornon JP, de Saint-Basile G, Callebaut I. A concanavalin A-like lectin domain in the CHS1/LYST protein, shared by members of the BEACH family. Bioinformatics 2009;**25**:1219–1222.
78. Gunay-Aygun M, Huizing M, Gahl WA. Molecular defects that affect platelet dense granules. Semin Thromb Hemost 2004; **30**:537–547.
79. Wei ML. Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. Pigment Cell Res 2006;**19**:19–42.
80. Nazarian R, Huizing M, Helip-Wooley A, Starcevic M, Gahl WA, Dell'Angelica EC. An immunoblotting assay to facilitate the molecular diagnosis of Hermansky-Pudlak syndrome. Mol Genet Metab 2008;**93**:134–144.
81. Clark RH, et al. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. Nat Immunol 2003;**4**:1111–1120.
82. Enders A, et al. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. Blood 2006;**108**:81–87.
83. Jung J, et al. Identification of a homozygous deletion in the AP3B1 gene causing Hermansky-Pudlak syndrome, type 2. Blood 2006;**108**:362–369.
84. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS. Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. Mol Cell 1999;**3**:11–21.
85. Dell'Angelica EC, Ohno H, Ooi CE, Rabinovich E, Roche KW, Bonifacino JS. AP-3: an adaptor-like protein complex with ubiquitous expression. EMBO J 1997;**16**:917–928.
86. Dell'Angelica EC, Ooi CE, Bonifacino JS. Beta3A-adaptin, a subunit of the adaptor-like complex AP-3. J Biol Chem 1997; **272**:15078–15084.
87. Benson KF, et al. Mutations associated with neutropenia in dogs and humans disrupt intracellular transport of neutrophil elastase. Nat Genet 2003;**35**:90–96.
88. Massullo P, et al. Aberrant subcellular targeting of the G185R neutrophil elastase mutant associated with severe congenital neutropenia induces premature apoptosis of differentiating promyelocytes. Blood 2005;**105**:3397–3404.
89. Honing S, Sandoval IV, von Figura K. A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J 1998;**17**:1304–1314.
90. Latour S, Veillette A. The SAP family of adaptors in immune regulation. Semin Immunol 2004;**16**:409–419.
91. Rigaud S, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature 2006;**444**:110–114.
92. Badovinac VP, Hamilton SE, Harty JT. Viral infection results in massive CD8 + T cell expansion and mortality in vaccinated perforin-deficient mice. Immunity 2003; **18**:463–474.
93. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8 + T cells and interferon gamma are essential for the disorder. Blood 2004;**104**:735–743.
94. Crozat K, et al. Jinx, an MCMV susceptibility phenotype caused by disruption of Unc13d: a mouse model of type 3 familial hemophagocytic lymphohistiocytosis. J Exp Med 2007;**204**:853–863.
95. Pachlopnik Schmid J, et al. A Griscelli syndrome type 2 murine model of hemophagocytic lymphohistiocytosis (HLH). Eur J Immunol 2008;**38**:3219–3225.
96. Mazodier K, et al. Severe imbalance of IL-18/IL-18BP in patients with secondary hemophagocytic syndrome. Blood 2005;**106**:3483–3489.
97. Huang JF, et al. TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. Science 1999;**286**:952–954.
98. Hudrisier D, Riond J, Mazarguil H, Gairin JE, Joly E. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. J Immunol 2001;**166**:3645–3649.
99. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 2004;**21**:589–601.
100. Ho LP, Urban BC, Jones L, Ogg GS, McMichael AJ. CD4(-)CD8alphaalpha subset of CD1d-restricted NKT cells controls T cell expansion. J Immunol 2004;**172**:7350–7358.
101. Pachlopnik Schmid J, et al. Neutralization of IFNg defeats hemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. EMBO Mol Med 2009;**1**:112–124.
102. Henter JI, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007;**48**:124–131.
103. Mahlaoui N, et al. Immunotherapy of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins: a single-center retrospective report of 38 patients. Pediatrics 2007;**120**:e622–e628.
104. Rieux-Laucat F, Fischer A, Deist FL. Cell-death signaling and human disease. Curr Opin Immunol 2003;**15**:325–331.
105. Bidere N, Su HC, Lenardo MJ. Genetic disorders of programmed cell death in the immune system. Annu Rev Immunol 2006;**24**:321–352.
106. Terrell C, Zoller E, Jordan M. Perforin-Dependent Cytotoxicity Regulates the Immune Response in Trans: How Much Donor Chimerism is Enough? Bilbao, Spain: Bilbao: Histiocyte Society, 2009 September 15–17.
107. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med 2000;**192**:755–760.
108. Bolitho P, et al. Perforin-mediated suppression of B-cell lymphoma. Proc Natl Acad Sci USA 2009;**106**:2723–2728.
109. Cannella S, et al. Germline mutations of the perforin gene are a frequent occurrence in childhood anaplastic large cell lymphoma. Cancer 2007;**109**:2566–2571.
110. Mehta PA, et al. Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Leukemia 2006;**20**:1539–1541.
111. Clementi R, et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. Blood 2005;**105**:4424–4428.

