

Hemophagocytic Lymphohistiocytosis (HLH)

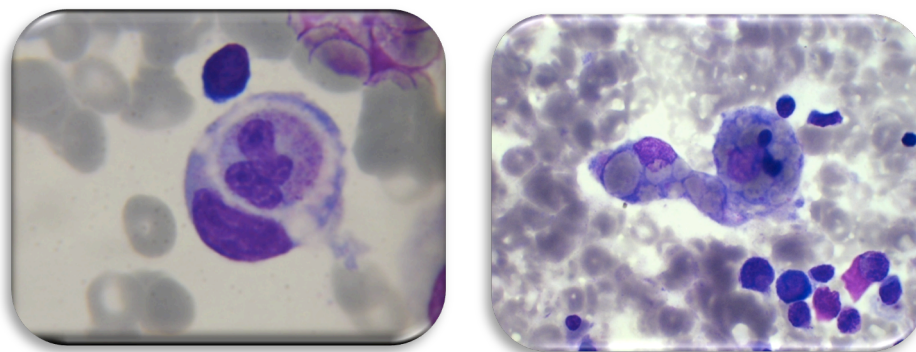


Figure 1 & 2 showing neutrophil phagocytosis & erythrophagocytosis respectively.

Introduction:

HLH (Hemophagocytic Lymphohistiocytosis) is not an uncommon disorder & every year we diagnose 25 cases of HLH at B J Wadia Hospital for children, Mumbai. Hemophagocytic Lymphohistiocytosis is an interim mechanism of disorder like DIC. It is an overreaction of the immune system & many diseases can trigger the same events.

Aetiology of HLH:

It can be familial or acquired & both share one common feature of a highly stimulated and ineffective immune response. Familial HLH is due to congenital defects in various proteins involved in granule exocytosis or due to defect in Perforin granzyme pathway. HLH is also commonly seen in primary immunodeficiency like XLP₁ (X Linked Lymphoproliferative Disorder) due to defect in SAP and XLP₂ due to defect in XIAP gene. Genes involved in Familial HLH are shown in Table (1) & (2).

Genetic aetiology of HLH: Table (1)

FHL	Gene defective	% of Familial HLH cases
FHL ₁	Chromosome 9	-
FHL ₂	Perforin	20 to 25%
FHL ₃	Munc 13/4	15 to 20%
FHL ₄	Syntaxin 11	10%
FHL ₅	Munc 18/2	20 to 25%
XLP ₁	SAP	-
XLP ₂	XIAP	-

Genetic aetiology of HLH Table (2)

Pigmentary Dilution	Gene	Comment
Chediak Higashi Syndrome (CHS)	Lyst	Accelerated phase of CHS
Griselli Syndrome (GS) Type 2	RAB 27 alpha	HLH does not occur in GS type I & 3
Hermansky Pudlak Syndrome type 2 (HP)	AP3B1	Only 2 cases in world literature

Acquired HLH is commonly seen with infections (IAHS – Infection associated Hemophagocytic Syndrome); Malignancies and Rheumatic diseases where it is also known as MAS (Macrophage Activating Syndrome). Thus a host of diverse diseases can activate HLH. The most common infections, which trigger HLH, are EBV, CMV, HSV, HHV, Koch, Salmonella, Malaria, Kalaazar in the Indian set up but virtually any infection can trigger HLH.

Pathophysiology & Understanding:

The Hallmark of HLH is defective NK Cell & Cytotoxic T cell activity.

NK cells & Cytotoxic T cells can be recognized morphologically as large lymphocytes with azurophilic granules in Wright preparation. These granules contain apoptosis inducing machinery the Perforin protein & Granzyme B.

To understand HLH we must understand the function of NK cell & Cytotoxic T cells. Both are designed to kill virally infected cells.

NK cells are our innate immune system cell that comprise 10 to 15% of Peripheral Blood Lymphocytes and are like Rambo's of the immune system with ready to kill receptors but have to be inhibited by receptors for MHC class I molecule, which override the kill signal. Thus MHC class I molecule protect us from NK cell toxicity.

Cytotoxic T cells are produced as an adaptive response to any infection. They recognize cytosolic protein antigen (e.g. Viral proteins) which are presented on the MHC class I molecule. Cytotoxic T cells are specific for that infection and are produced later after 4 to 5 days of infection. They are the atypical lymphocytes seen in Infectious mononucleosis & express Cd3+ & Cd8+ on their surface.

NK cell & Cytotoxic T cells eliminate a virally infected target cell via the Perforin Granzyme pathway. Once NK cell or Cytotoxic T cell binds the virally infected cell they form an immunologic synapse at the contact site. All the subsequent events occur at the immunologic synapse.

The azurophilic granule undergoes steps of vesicle maturation, polarization to the immunologic synapse, docking, and priming before it fuses with the surface membrane and releases its content within the immunological synapse.

Perforin is a protein like Complement C5-9, it perforates the target cell membrane forming a channel thus allowing Granzyme B to enter the target cell and induce apoptosis by activating the apoptotic mechanism.

Proteins important in the granule exocytosis process and the disease caused by their deficiency are shown in fig (3), (4), (5) & (6)

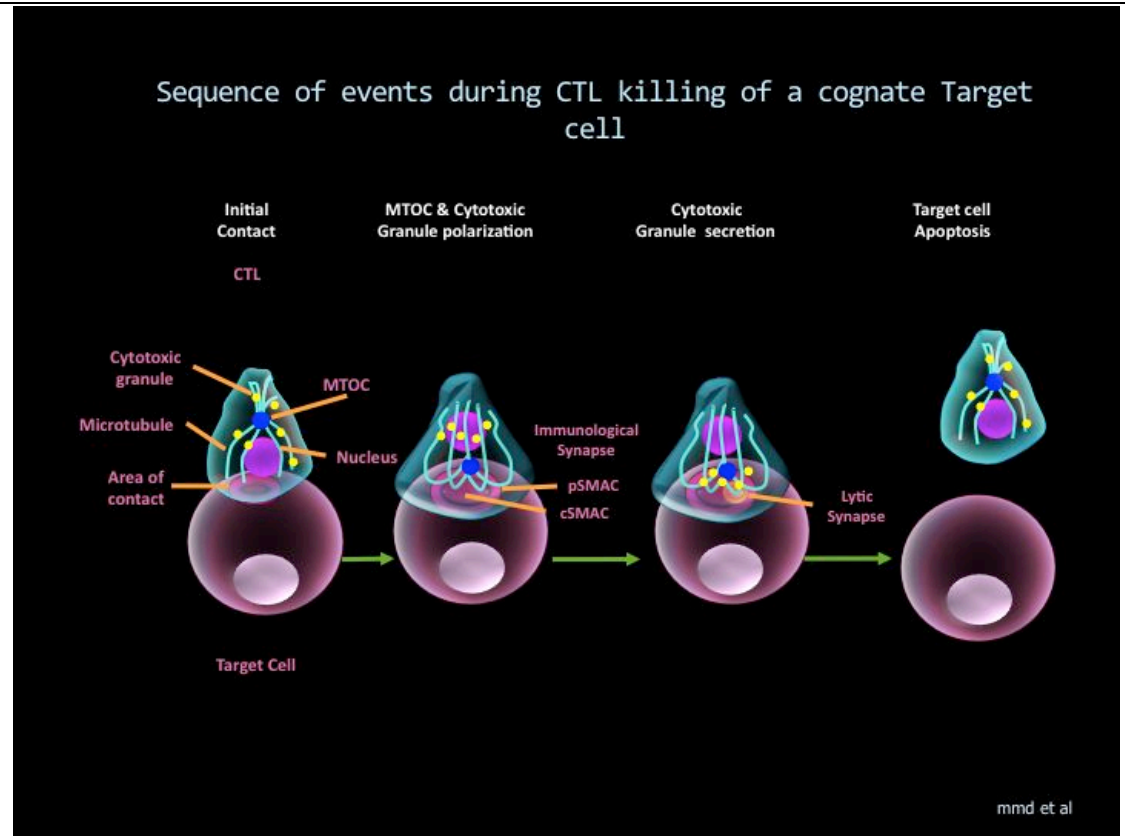


Figure (3) shows the sequence of events during CTL (Cytotoxic T Lymphocyte) killing of a cognate target cell. During initial contact an Immunologic Synapse (IS) is formed. MTOC (Micro Tubule Organizing Centre) reorients & polarizes the cytotoxic granule to the IS. At the periphery of Immunologic synapse is the pSMAC (Peripheral Supra Molecular Activation Complex), which is rich in Integrins and it marks the outer boundary of the IS. At the center, is the cSMAC (central SMAC); cSMAC is rich in T cell receptors, and CD8 molecule. The polarized cytotoxic granules fuse with the membrane and form a lytic synapse where released Perforin will perforate the target cell membrane and allow granzyme to enter to induce apoptosis. Only few cytotoxic granules are secreted. The Cytotoxic T cell can bind another target and begin the same process again.

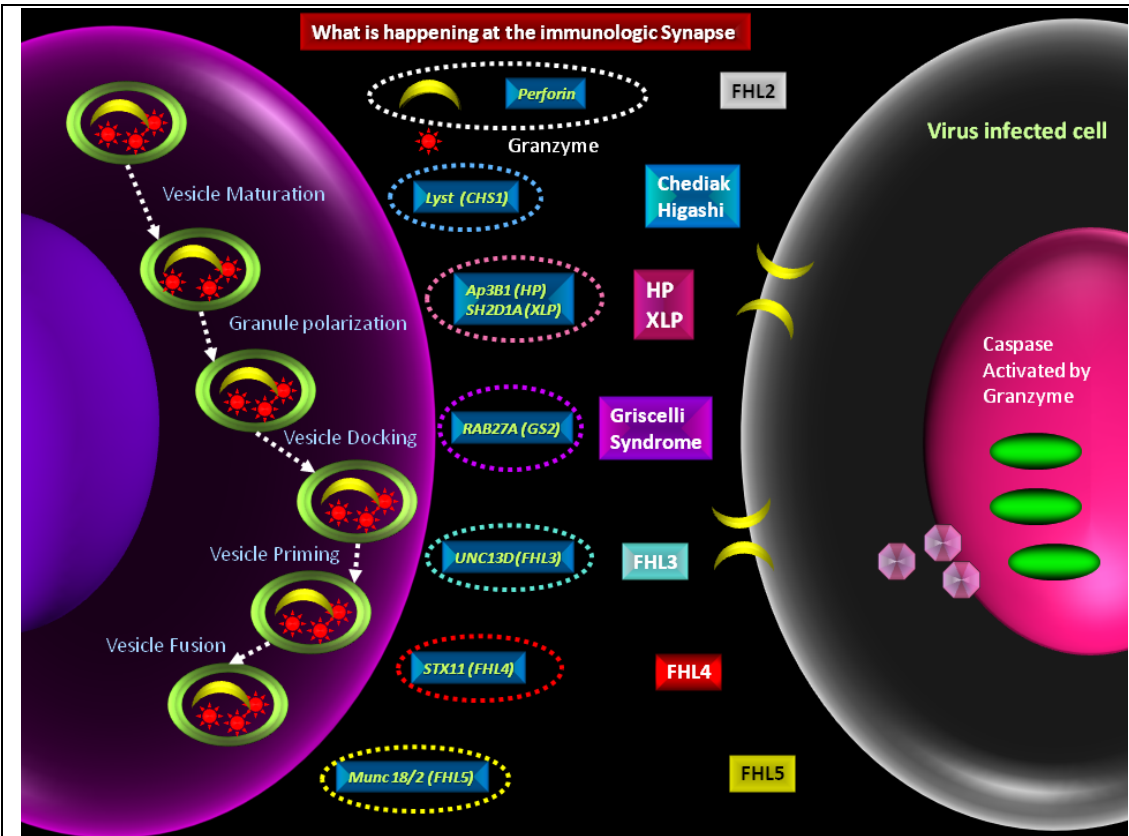
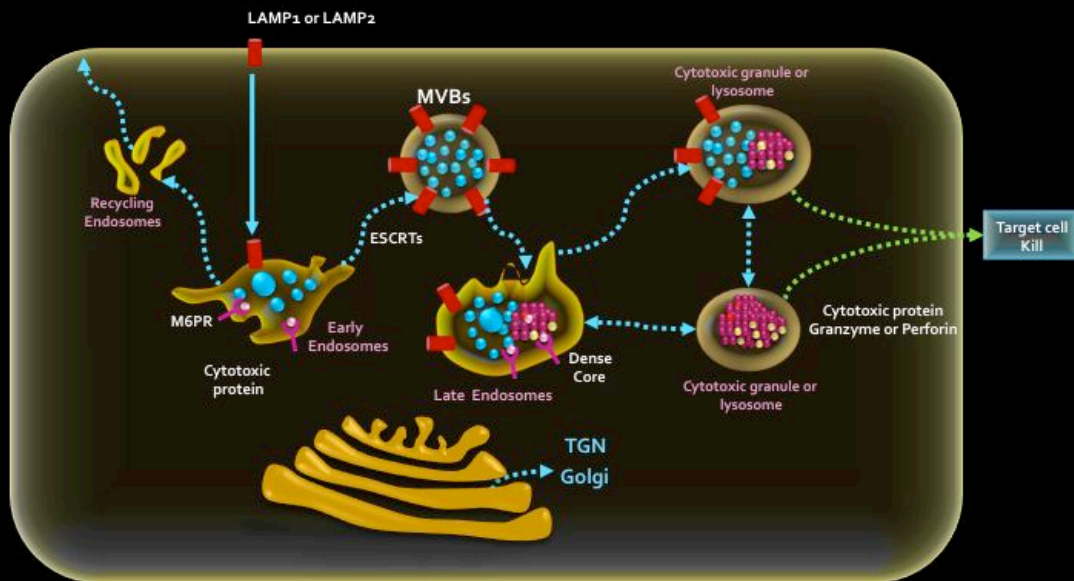


Figure (4) depicts the events occurring at the immunologic synapse and highlights molecules important in process of granule exocytosis.

Steps of granule exocytosis along with proteins required at each step with their defects are given below:

1. Maturation of cytotoxic granule (LYST protein - Disease: Chediak Higashi Syndrome)
 2. Granule polarization (Ap3B1 – Disease: Hermansky Pudlak); (SH2D1A; SAP – Disease: X linked Lymphoproliferative Syndrome)
 3. Vesicle docking (RAB27 α - Griscelli Syndrome)
 4. Vesicle priming (UNC13D - FHL3) (Familial HLH)
 5. Vesicle Fusion (STX11 - FHL4).
 6. Vesicle docking priming & fusion (Munc 18/2 – FHL5)
- Defect in Perforin results in FHL2

Biogenesis and exocytosis of Cytotoxic granules



Adapted from; Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules
Geneviève de Saint Basile, Gaël Ménasché & Alain Fischer *Nature Reviews Immunology* 10, 568-579
(August 2010)

27/07/11; mmd et al

Figure (5) model depicting the biogenesis and exocytosis of cytotoxic granules. Granzymes and other soluble proteins, including lysosomal hydrolases, are sorted from the *trans*-Golgi network by the mannose-6-phosphate receptor (M6PR) and transported to early endosomes before they are sorted to late endosomes. M6PR is then recycled to the *trans*-Golgi network. Lysosome-associated membrane protein 1 (LAMP1) or LAMP2 travel directly to the plasma membrane where they are internalized and sorted by adaptor protein complexes (not shown) to the endosomal pathway. The recruitment of the endosomal-sorting complex required for transport (ESCRT) machinery and the formation of luminal vesicles occur in early endosomes and also in late endosomes. Cytotoxic granules are hybrid organelles that contain both lysosome and late endosome components. They have various proportions of dense core domains, where perforin and granzymes are stored, and multivesicular bodies, which contain acid hydrolases

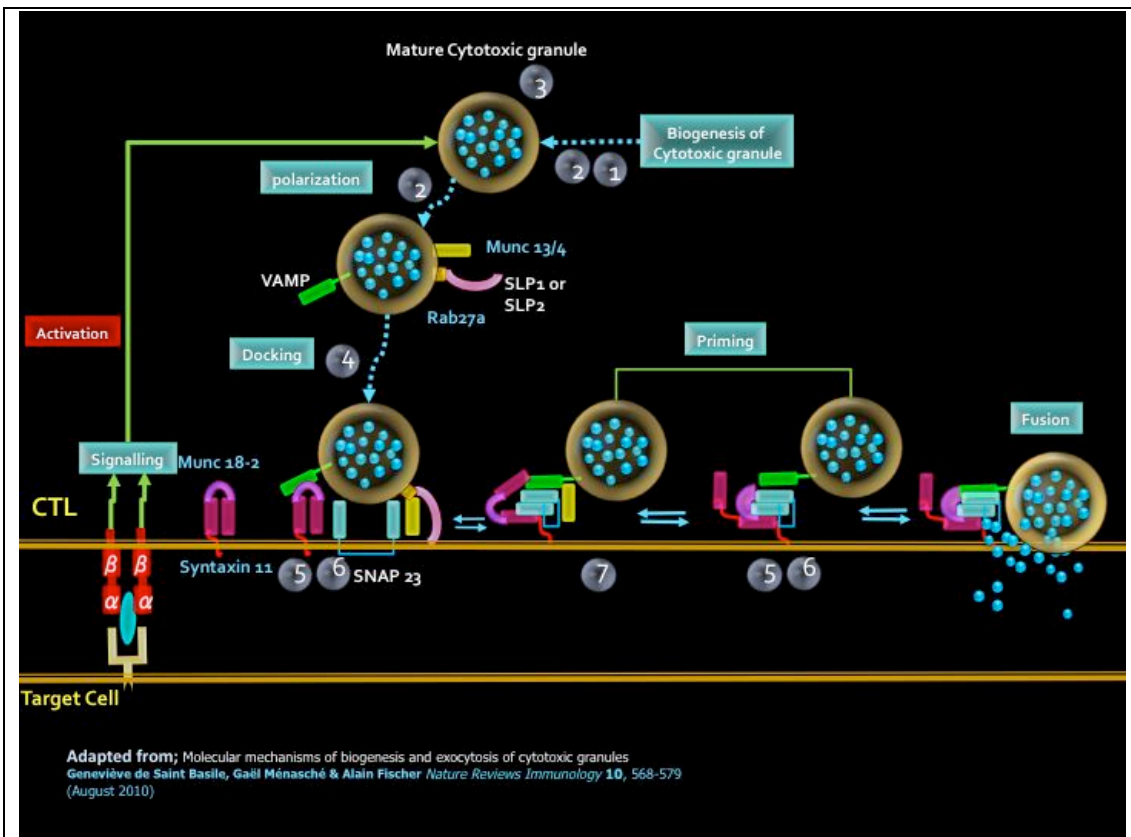


Figure (6) model depicting the biogenesis and exocytosis of cytotoxic granules. Mature cytotoxic granules dock at the plasma membrane through the interaction of RAB27a with synaptotagmin-like protein 1 (SLP1) or SLP2. In addition, cytotoxic granules interact with a docking complex of MUNC18-2 and syntaxin 11 in the closed conformation. Docked vesicles are then primed by MUNC13-4, which probably triggers the switch of syntaxin 11 from a closed to an open conformation. A SNARE (soluble *N*-ethylmaleimide-sensitive factor accessory protein receptor) complex then forms between a vesicle membrane SNARE, possibly vesicle-associated membrane protein 7 (VAMP7) and/or VAMP8, on one side and the target membrane SNAREs syntaxin 11 and SNAP23 (synaptosomal-associated protein of 23 kDa), on the other side. Completion of the fusion reaction occurs when MUNC18-2 clasps across the zippering four-helix SNARE complex bundle. Formation of the SNARE complex precedes membrane fusion. Numbers indicate the different steps that are impaired owing to loss of function of the proteins causing inherited haemophagocytic lymphohistiocytosis: defects in lysosomal trafficking regulator (LYST) (1), adaptor protein 3 (AP3) (2), perforin (3), RAB27a (4), syntaxin 11 (5), MUNC18-2 (6) and MUNC13-4 (7).

It is thus easy to comprehend why such diverse defects result in a common pathophysiologic process (HLH).

XLP (X linked Lymphoproliferative disorder) is of two types XLP1 due to defect in SAP protein & XLP2 due to defect in XIAP (X-linked Inhibitor of Apoptosis) gene. 60% of XLP1 patients develop EBV induced HLH while 90% of XLP2 develop HLH. Currently it XLP2 is being reclassified as HLH causing disease rather than causing lymphoproliferation.

The pathophysiology of HLH is shown in fig (7) & (8).

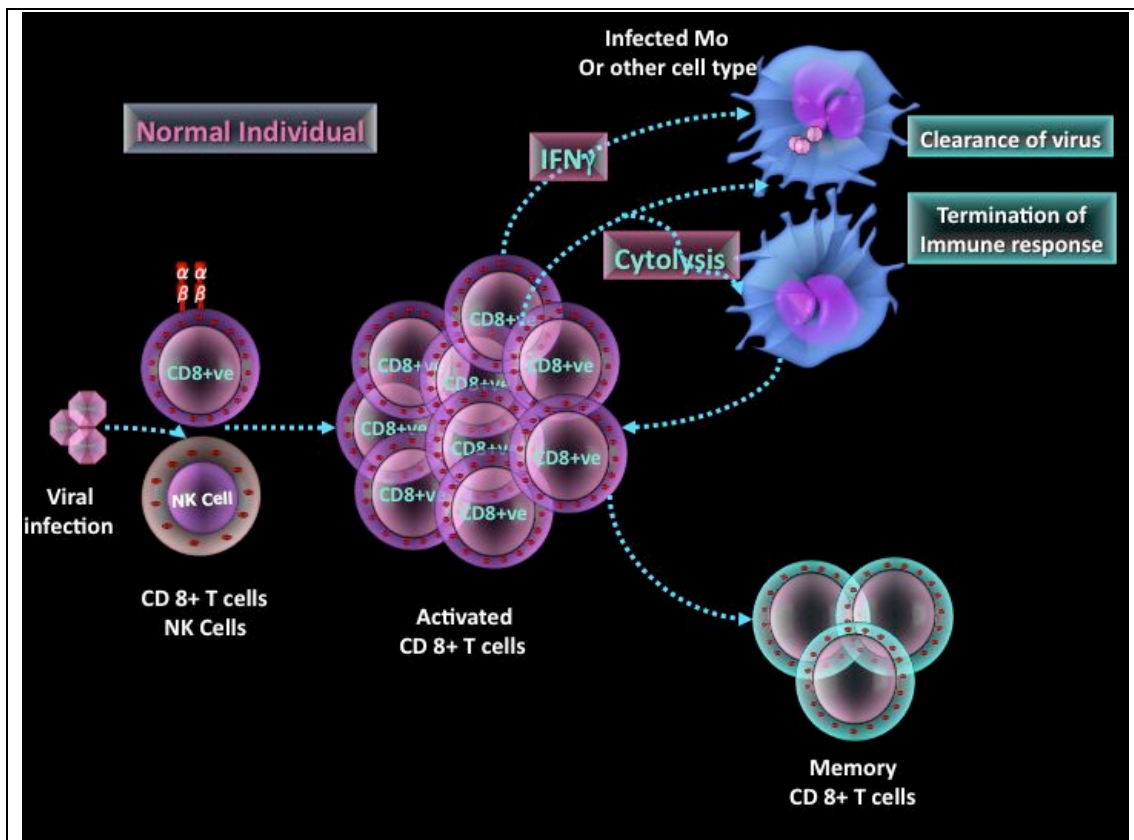


Figure (8) shows NK cell & CTL (Cytotoxic T Lymphocyte) response to virally infected cells. In normal patients there is clonal expansion, secretion of Interferon Gamma and killing of virally infected cell resulting in control of viral infection. Once infection is controlled the CTL are culled and immune homeostasis achieved. Some of these cells become memory cells.

Since NK cell & Cytotoxic T cells are unable to kill target cells following events occur. There is over stimulation of the immune system, excess antigen presentation, Excess T cell proliferation with infiltration of various organs like CNS, Liver, Spleen, & Lymph nodes. This results in formation of excess Cytokines like TNF alpha, INFG, IL1, Gm-CSF. The Antigen Presenting Cell needs to be culled by NK cells to achieve immune homeostasis. This does not happen and persistent antigen presentation occurs with further activation of immune system. The cytokine IFNG activate the Macrophages that result in Hemophagocytosis, thus giving the name to this syndrome.

In normal immune response, once the infection is eliminated, the effector immune cells needs to be culled to achieve immune homeostasis. In HLH this does not occur resulting in massive expansion of effector T cells, organ infiltration resulting in massive tissue necrosis and multiorgan failure.

The cells participating in HLH are not only NK cells & Cytotoxic CD8+ve T cells but also other cells with cytotoxic activity like CD4+ cytotoxic T cells & iNKT cells.

There is some correlation between hypercytokinemia and the diverse clinical manifestations in HLH.

- IL1 is responsible for fever.
- TNF alpha is responsible for Hepatic injury, coagulopathy.
- TNF alpha inhibits lipoprotein lipase resulting in hypertriglyceridemia
- IFNG causes Macrophage activation, Hemophagocytosis; Cytopenias & muscle wasting.
- Soluble IL2R alpha (sCD25) & Beta 2 Microglobulin levels in serum suggest excess T cell activation.
- Elevated Serum Ferritin is due to Macrophage activation.

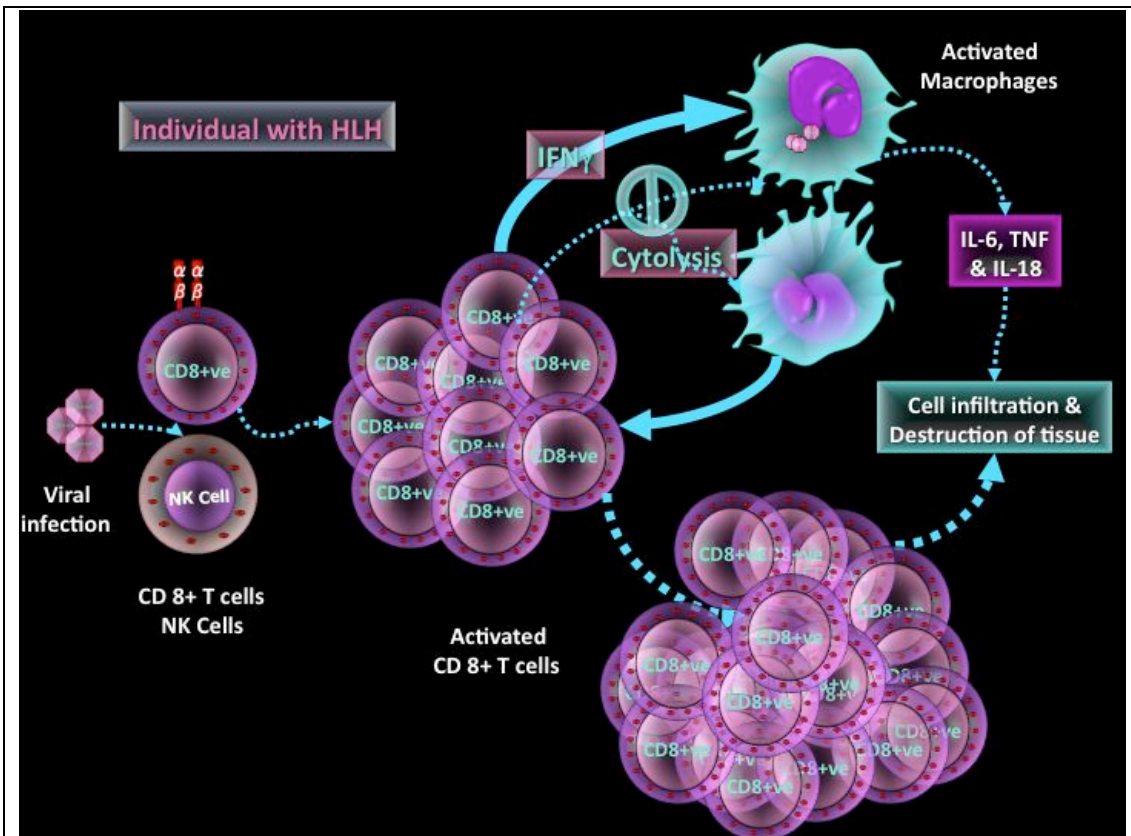


Figure (9) shows that in patients of HLH due to genetic defect; there is inability to kill virally infected cell as well as Antigen presenting cells resulting in massive clonal expansion of CTLs, secretion of their cytokines like Interferon Gamma (IFNG), TNF (Tumor Necrosis Factor) alpha, IL6, IL18 & GM-CSF (Granulocyte Macrophage Colony Stimulating Factor). IFNG activate Macrophages, which then phagocytose blood cells resulting in haemophagocytosis. Since the cytokines are produced in a massive amount by Macrophages, T cells & NK cells, hyper stimulation continues and the patient has a cytokine storm with signs & symptoms of HLH. The normal contraction of the immune system also does not take place resulting in persistent cytokine secretion, infiltration of various organs by T cells, massive tissue necrosis & organ failure.

Does this understanding of pathophysiology & role of cytokine in HLH have any therapeutic implications? Probably yes; clinical trials with Anti TNF alpha or Anti IFNG antibodies are already being done with success in animal model.

What is the clinical presentation of HLH?

HLH should be suspected in a patient when they have following signs & Symptoms.

1. Fever
2. Rapidly progressive cytopenias
3. LFT abnormalities
4. Organomegaly like Hepatosplenomegaly, Lymphadenopathy
5. Coagulopathy
6. BM or Organ biopsy with hemophagocytosis
7. Hyperferritinemia
8. Hypertriglyceridemia
9. Hypofibrinogenemia
10. Hyponatremia.

We very often suspect HLH in case of fever, organomegaly, icterus and rapidly evolving cytopenias; especially in an intensive care setting. In such cases besides looking for LFT abnormalities we specifically ask for Coagulation profile with 'D' Dimer; Serum Ferritin, Serum Triglycerides & Serum Electrolytes.

A BM is very often diagnostic in HLH.

Some times the hemophagocytes may be few but if the clinical suspicion is strong it may be worth repeating a BM examination.

Rarely Hemophagocytosis may be seen in Lymph nodes or on liver biopsy

In 2009 Filipovich et al has revised the HLH diagnostic criteria, which are practical and very much applicable in our set up. These criteria are shown in Table (3):

HLH Diagnostic Criteria 2009: Table (3)

1. Molecular Diagnosis of HLH or XLP
2. At least $\frac{3}{4}$
Fever
Splenomegaly
Hepatitis
Cytopenias
3. At least $\frac{1}{4}$
Hemophagocytosis,
Hyperferritinemia
Increased Soluble IL2Ralpha
Absent or very decreased NK cell function.
4. Supportive of HLH
Hypertriglyceridemia,
Hypofibrinogenemia
Hyponatraemia

Familial HLH present generally early in life with 70 to 80% less than 1 yr. of age. However presentation later on in life is also seen. In a recent study it was shown that

37% of adult with HLH (age>18 yrs.) had mutations in Perforin (Prf) gene. Thus today, we can better understand the spectrum of disease.

Prf A91V mutation is common polymorphism seen in 4 to 11% (Heterozygous) & 1 to 4% (Homozygous) of western population. It is now believed that it significantly decreases Perforin expression due to protein misfolding & is an HLH inducing mutation. How common is this polymorphism In India we do not have any data.

In our experience we see HLH at all age groups and we do have significant no of HLH in adults.

It is important to highlight over here that in India especially at B J Wadia Hospital for children we also see Peripheral Blood HLH that was 1st reported by Dr Zinet Currimbhoy and we do consistently see cases of PB HLH. We recently saw 2 newborns and 1 older boy with PB HLH. This has not been reported from abroad.

Perforin mutations are also seen in patients of Chronic Active EBV (CAEBV) disease & in 5% of patients with NHL. We have seen an interesting case of Hodgkin's disease being referred from TMC who developed HLH on treatment and his Perforin levels were Zero. Thus his first presentation was with malignancy and then developed HLH on treatment. Why he did not develop HLH with zero Perforin level? We do not have answer to these questions, but it opens our eyes to the spectrum of disease and the fact that other factors besides Perforin mutations are important for manifestation of HLH.

In 2 patients HLH presented as chronic meningoencephalitis suggesting that a localized organ involvement may be the only presentation of HLH.

In a patient of ALPS (Autoimmune Lymphoproliferative Syndrome) both mutation in FAS gene and Perforin gene was detected.

HLH is a fulminant disease but some patient can have a mild chronic relapsing course. We had a 9 yr. old boy who had persistent pancytopenia with persistent HLH for more than 2 yrs.

Very often HLH may be triggered by infection and it may be impossible to do distinguish primary HLH from secondary infection associated HLH. Hence it is very important to establish molecular diagnosis.

Once HLH is diagnosed a search for triggering infection has to be done especially for EBV, CMV, Koch, Enteric, Malaria, Kalaazar depending upon clinical presentation.

Thus we understand that the spectrum of HLH can be as diverse as fulminant HLH to chronic HLH. It may have multiorgan affection or single system presentation. In setting of CAEBV or malignancy associated HLH and MAS also possibility of Prf mutations have to be considered.

MAS (Macrophage Activation Syndrome) occur in the setting of RA, SLE & other collagen vascular defects & more than 100 cases are published in literature. MAS tend to occur in the early active phase of the disease. Leucopenia, thrombocytopenia & fibrinogen levels are not markedly decreased, as there is preexisting inflammation that results in neutrophilic leukocytosis, thrombocytosis & elevated plasma fibrinogen levels. Serum ferritin levels are markedly elevated and

we have seen levels up to 200,000 ng/ml in MAS secondary to RA. Besides clinical features typical of HLH there is often pronounced coagulopathy & cardiac involvement that is responsible for mortality with MAS. MTX, NSAID's & Gold salt may be the triggering event. The mortality is high at 10 to 15%. A few patients of MAS have been found to have Perforin & SAP mutation.

Lymphoma associated HLH very often is triggered by EBV, which infect the NK & NKT cells unlike the B cells in Infectious mononucleosis. Commonly associated with peripheral T cell lymphoma.

How do we work up a patient of HLH?

Laboratory evaluation of HLH is directed with following considerations

1. Establish diagnosis of HLH

CBC platelet
ESR
PS (look for peripheral Blood HLH)
LFT
Creatinine
LDH
Serum Electrolytes
Serum Ferritin
Serum Triglycerides
Coagulation Profile (PT, PTT, Plasma Fibrinogen & 'D' Dimer.)
CSF for pleocytosis & elevated proteins (50% of cases)

2. Supportive evidence for HLH

PB or Bone Marrow Aspiration
Liver Biopsy
Lymph node Biopsy

3. Sophisticated Lab investigations

sCD25 > 2400 IU/L
NK cell activity (May be normal in 30% of cases)
Serum Beta2 Microglobulin

4. Aetiological work up

Anti EBV VCA IgM, PCR
Anti CMV Abs, Antigen, PCR
Appropriate Microbiological Cultures
Hair Mount studies for pigmentary dilution disorders.

5. Work up for familial HLH

Perforin by flow cytometry

GRA (Granule Release Assay)
Gene sequencing to identify mutations

Following fig (10), (11) & (12) highlight our approach to patients with HLH.

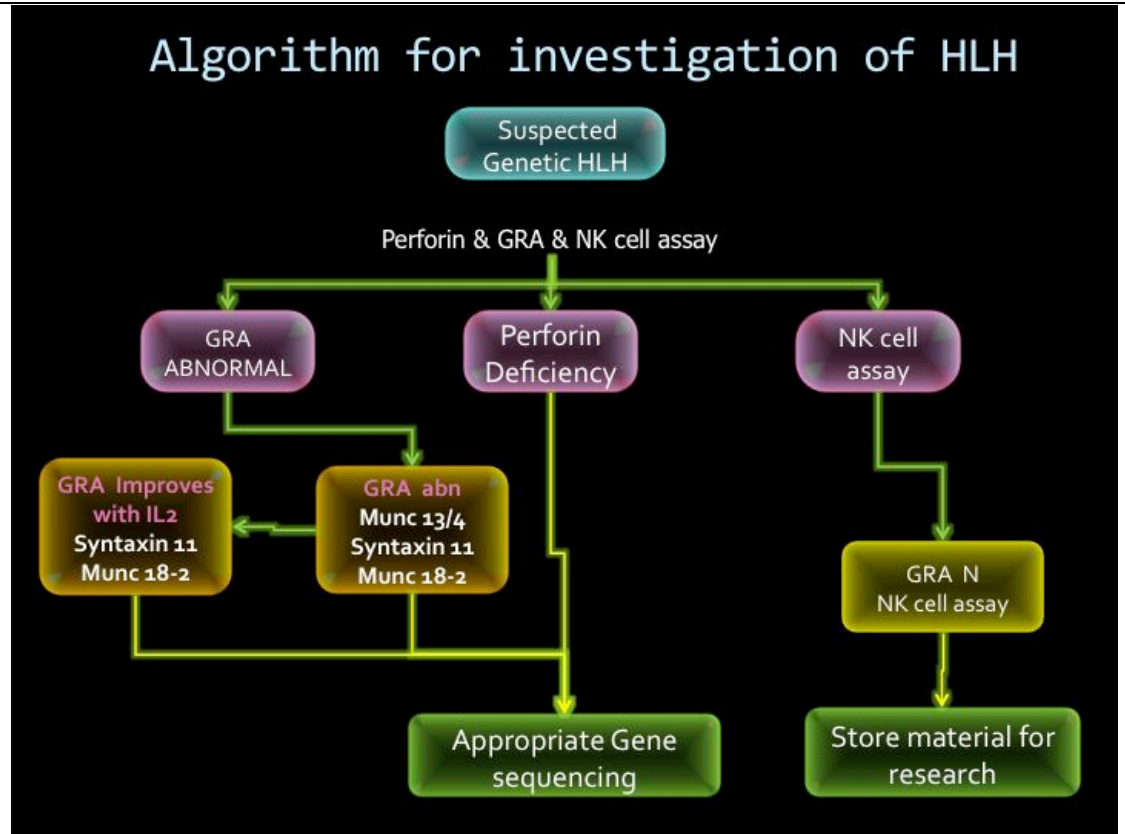


Figure (10) shows our approach to work up of patient with genetic HLH

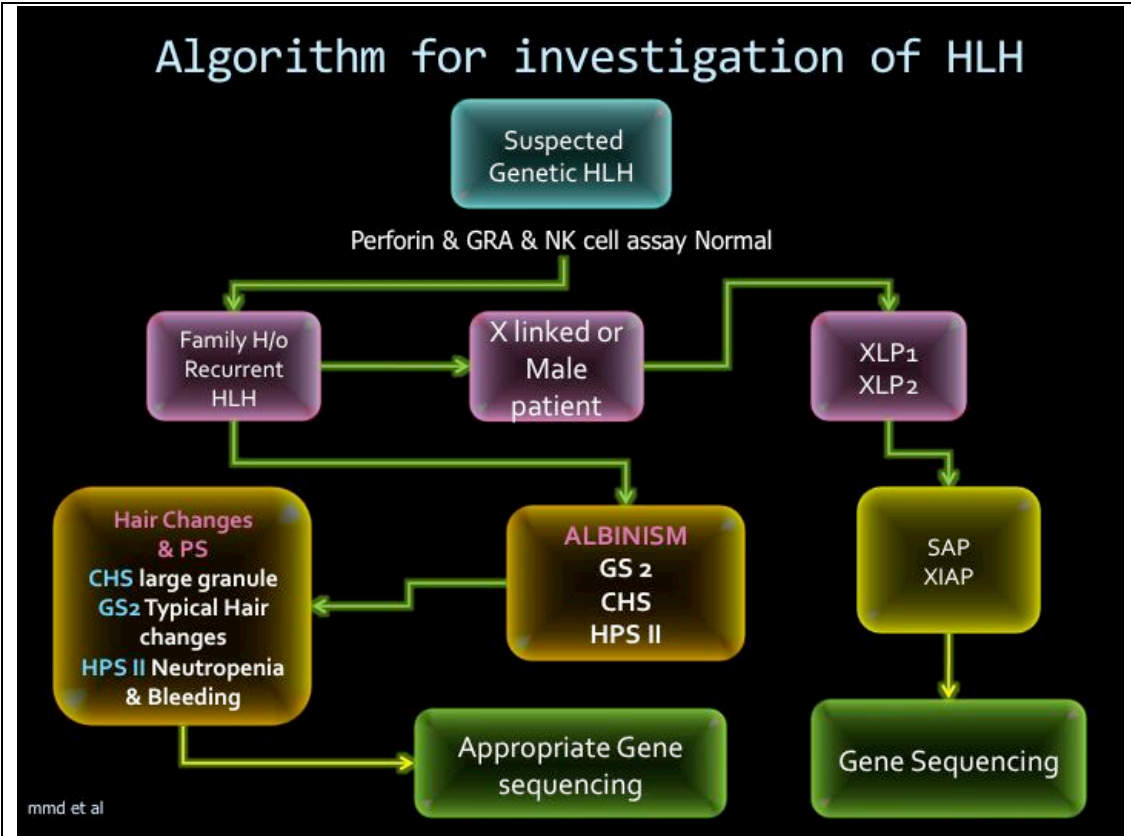


Figure (11) shows our approach to work up of patient with genetic HLH

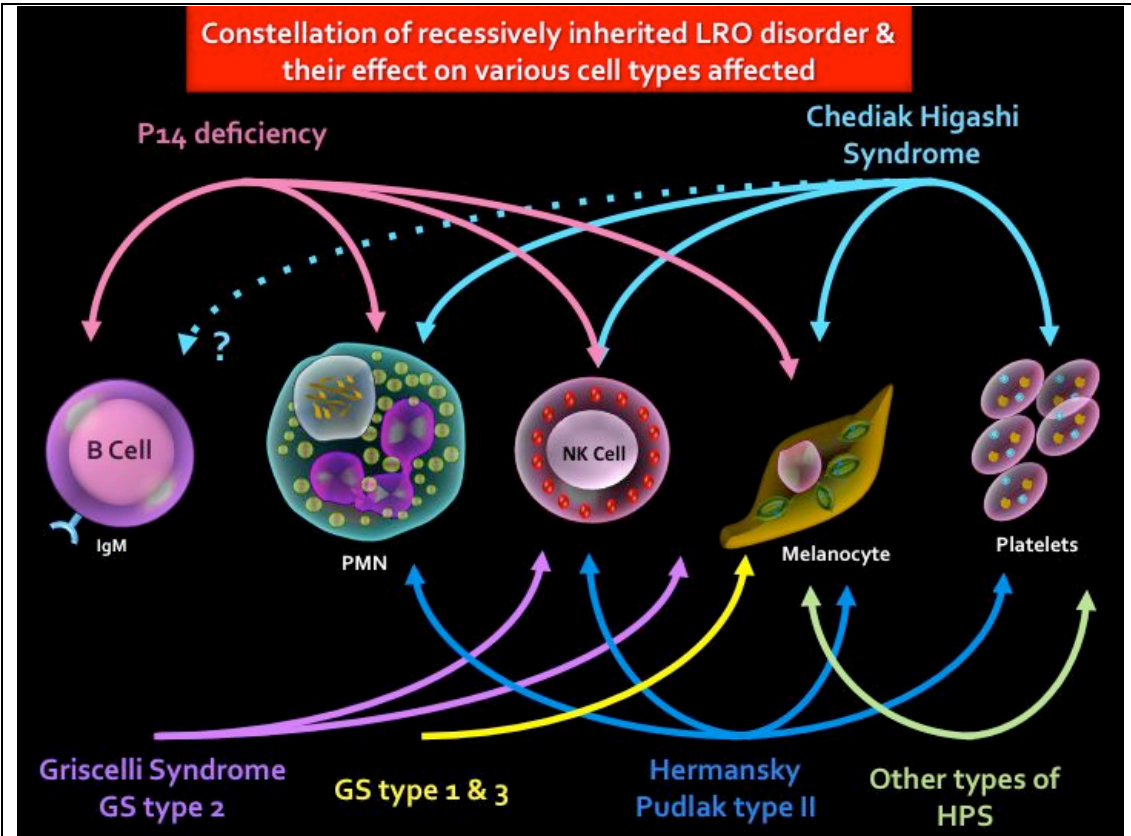


Figure (12) highlights the cells affected in various pigmentary dilution syndromes thus providing a molecular explanation for different organ system affection in pigmentary dilution syndromes. Since NK cells are affected in CHS, Hermansky

podlak syndrome & Griscelli Syndrome type 2 HLH can occur in these diseases. Endosomal protein 14 also affects NK cells but no cases of HLH are yet described in world literature.

Management of HLH:

Principle of treatment:

The immediate aim of treatment is to suppress the severe hyper inflammation. The secondary aim is to eliminate pathogen activated APCs (Antigen Presenting Cells) so as to remove the stimulus for ineffective activation of T cells.

Please note in secondary HLH pathogen directed therapy is not sufficient to control the hyper inflammation. Leishmaniasis treated with Liposomal Amphotericin B is the only exception.

The treatment recommended is HLH 2000 protocol that is devised by Histiocytic society. It essentially consists of 3 drugs.

Dexamethasone:

- Lympholytic
- Inhibit expression of cytokines
- Suppresses maturation of APCs
- Better CNS penetration hence preferred over prednisolone

Cyclosporine A:

- Prevents T cell activation & proliferation

Etoposide (VP-16):

- Activity against Monocytes & Macrophages
- Inhibits EBNA synthesis I EBV infected cells

IV Gammaglobulins:

- Provide cytokine & pathogen specific antibodies
- Immunomodulation

Etoposide is a genotoxic drug and there are concerns regarding development of late effects like MDS (Myelodysplastic Syndrome); but it can be life saving especially in EBV associated HLH. Cytopenias is common in HLH and there is this constant dilemma & tussle (between the intensivist & Immunologist) as to give Etoposide or not as Etoposide is a myelosuppressive drug. Do not withhold Etoposide due to cytopenias. At least the first two doses must be given before modifying the schedule due to presence of a very low ANC (Absolute Neutrophil Count) < 500. Unless we control the hyper inflammation there is no chance of cytopenias improving.

The French group does not use Etoposide and they use ATG (Anti Thymocyte Globulin) to control HLH.

Up to 4 doses of Intra Thecal MTX (Methotrexate) are recommended I HLH 2004 protocol if the 1st 2 weeks of therapy fail to improve neurological symptoms & if there is progressive CSF pleocytosis. Effective systemic therapy can result in CNS control of the disease.

Once inflammation is controlled a search for a potential Bone Marrow donor is done and if a match is available the child should be transplanted to achieve a cure. In HLH 1994 protocol median survival was 64% with OS (Over all Survival) of 55%.

In secondary HLH treatment is given for 8 wks. HLH reevaluation is done & if normal treatment is stopped and child is observed periodically for recurrence.

Once hyper inflammation is controlled there can be reactivation of HLH or development of CNS events. Intra Thecal MTX would benefit for CNS activation.

The HLH therapy is reinforced in case of systemic reactivation.

Indications for BMT:

- FHL (Familial Hemophagocytic Lymphohistiocytosis)
- Relapsing secondary HLH.

Results of BMT are:

- Matched Related Donor: 71 % ± 16%
- Matched Unrelated Donor 71 %
- HLA mismatched Unrelated Donor: 54% ± 27%
- Haploidentical Related Donor: 50 % ±24%

Disease directed therapy:

Anti Leishmania therapy
Anti Lymphoma therapy.

If there is poor or no response to 4 weeks of HLH 2004 protocol treatment, HLH is probably refractory & continuing HLH 2004 protocol will be of no further benefit. Salvage treatment options that can be tried in such a situation are shown in table (). There is no established salvage regime. ATG is rarely effective if Etoposide based regimen has been ineffective. Search for BM donor should be done & one should attempt to transplant these patients.

ATG
Fludarabine
Alemtuzumab
Daclizumab
IVGG
Chemotherapeutic agents
Anti TNF alpha receptor blocking agents
Anti Interferon Gamma Antibodies

In patients with EBV induced HLH or XLP with fulminant EBV induced HLH we can use Injection Rituximab (monoclonal Ab to CD 20) at doses of 375 mg / m² weekly for 4 weeks, to knock out the B cells harboring EBV. This strategy works very well to control EBV induced HLH & in fulminant infectious mononucleosis.

In desperate situations Anti TNF alpha-receptor blocking agents have been tried.

Experimental works with Anti Interferon Gamma Antibodies have shown excellent results in mouse model of HLH. Human trials are about to begin. We are awaiting those results.

Prognosis: The prognosis of familial HLH is guarded in India. We have seen more than 11 cases of Perforin deficiency and have lost all of them. Most of the mortality is due to hepatic failure, multiorgan failure, Coagulopathy, infection and CNS events. If a successful BMT is done than the chances of cure are more than 50%. The first patient of HLH was transplanted at Necker hospital Paris 28 years back and is alive and fine. We need to establish a successful transplant programme in India to save our babies with HLH

Conclusion:

HLH is not an uncommon disease. a practicing pediatrician is likely to see these patients in a tertiary care set up. HLH should be considered in the differential diagnosis when we have a constellation of symptoms like fever, organomegaly, rapidly evolving cytopenias, LFT disturbances, and coagulopathy. One should evaluate further with estimation of serum ferritin, Serum Triglycerides, Coagulation profile & serum electrolytes. A Bone Marrow should be done to confirm one's suspicion. An aggressive approach is warranted even in secondary HLH, as the hyper inflammation is not possible to control by infection directed therapy. Therapy should be given for at least 8 weeks in secondary HLH. In India peculiarly peripheral blood HLH is not uncommon. Awareness is the clue. As more pediatricians get sensitized we should be able to diagnose more cases of HLH and offer them prompt treatment. HLH in adults is also not uncommon and we need to create awareness about the same in adult physicians too. There is an urgent need to set up BMT facilities to cure some of these patients.

The Division of Immunology at B J Wadia Hospital for children along with NIIH runs PID (Primary Immunodeficiency) services at B J Wadia Hospital. NIIH has established Perforin & granule release assay which can be done in suspected cases of HLH.

We hope we are able to establish transplant facilities to cure some of our patients.

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