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Review Article

A Scopic Review on the Diagnostic Dilemmas and Newer Treatment Modalities in Hemophagocytic Lymphohistiocytosis

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1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is an immune dysregulation syndrome which is caused due to defect of the Natural killer (NK) cells. Primary HLH is caused due to mutations of certain genes and secondary is due to infections (e.g.: EBV, Dengue), rheumatological issues and malignancy.

2. Pathophysiology

In a normal body NK cells and cytotoxic T cells (CTLs) identify the Antigen presenting cells (dendritic cells and histiocytes) and then release perforin and granzyme in a well-regulated fashion through a newly formed synapse called immunological synapse (IS) [1]. Perforins and granzymes are normally stored in granules in lysosomes of NK cells and CTLs [2]. Limited amounts of required Inflammatory mediators are also transported across the IS towards the APCs. Perforins form pores on the APCs and later granzymes help in apoptosis [2]. Together all

these processes orchestrate in a well regulated manner to first destroy the antigen and then set stage for apoptosis [1].

Following apoptosis, the dying cells secrete enzymes which stimulate each other in a cascade fashion to destroy the IS [3]. Any hindrance in this pathway leads to persistence of the antigen and gives a longer life to the IS, with further recruitment of CTLs, leading to more immune activation and release of cytokines, tumour necrosis factor (TNF) and interferons. This inflammatory cocktail stimulates macrophages and turns on the secretion of IL-6. The unchecked Macrophages hence activated, phagocytose the other blood cells causing cytopeneias [3-5]. The macrophages and histiocytes with the phagocytosed cells, infiltrate various organs, impeding their function like liver, spleen, and lymph nodes leading to hepatosplenomegaly and lymphadenopathy.

3. Etiology

3.1 Familial HLH and Primary HLH

The incidence of familial HLH was seen to be 1.2 in 1000000 children per year, according to a study conducted in 2009 [6]. An Indian meta - analysis review showed that the incidence of familial HLH in India is 1 in 50000 [7]. They are inherited in an autosomal recessive pattern. There are 5 main gene loci identified currently, which could cause familial HLH - a genetic loci on chromosome 9, PFR1, UNC13D, STX11, STXBP2 (Table 1). Seventy percent of all mutations noted are those of PRF1 and UNC13D causing FHL2 and FHL3 respectively [8]. Age of presentation is mostly during early infancy [9]. However, it is now understood that such children could stay normal till adolescence too. Other genetic diseases - Griscelli syndrome, Chediak Higashi, Hermansky-Pudlak and X linked lymphoproliferative disease (type 1 and 2) could also pre-dispose to HLH and when they occur, they are termed as primary HLH [10-12] (Table 2).

Gene	Protein	Function
Genetic loci- 9th chromosome	Unknown	Unknown
*PFR1	Perforin	Cytolysis and immune regulation
┼UNC13D	┼MUNC13-4	Prime cytolytic granules
‡STX11	Syntaxin	Transport cytolytic granules
& STXBP	Syntaxin binding protein	Fuse cytolytic granules to cell membrane, and release perforin

*-PFR –gene coding for perforin; ┼- UNC13D – codes for protein MUNC13 which plays a role in cytotoxic granule exocytosis; ‡- STX – A gene coding for syntaxin; &- STXBP- gene coding for syntaxin binding protein

Table 1: Genes causing Familial HLH.

Condition	Gene affected	Function affected	Associated feature
Griscelli	*RAB27A	Uncontrolled T cell and macrophage activation; Defective docking	Albinism
Chediak Higashi	┼LYST	Defective transport of cytolytic granules	Albinism
Hermansky Pudlak	‡AP3B1	Defective biogenesis of cytolytic granules	Albinism

X-linked lymphoproliferative disorder - 1	&SH2D1A,	Signaling lymphocytic activation molecule-associated protein (SAP) deficiency-inappropriate lymphocyte recruitment	Immunodeficiency Requires HSCT
X - linked lymphoproliferative disorder - 2	‖BIRC4	Inhibition of apoptosis	Immunodeficiency Uveitis (rare) Requires HSCT

*RAB27A- gene coding for RAB sub family of GTPases; ┼- LYST - gene coding for lysosomal trafficking regulator protein; ‡ - AP3B1 - A gene also called Adaptor related protein complex 3 subunit beta which codes for a protein that play a role in organelle biogenesis assosciated with melanosomes; &- SHD1A- gene coding for SH2 domain containing protein which signals lymphocyte activation; ‖- BIRC4- Gene coding for Baculoviral IAP repeat containing protein 4 which is involved in apoptosis

Table 2: Other conditions causing primary HLH.

3.2 Secondary HLH

This is caused due to conditions which initiates inflammation mediated by NK cells or cytotoxic T lymphocytes (CTLs). NK cells constitute the early defenders against intracellular pathogens such as viruses, and CTLs are recruited later. Hence viruses are the most common infectious agents leading to HLH [8]. In a meta-analysis by Srinivas Rajagopala and Navneet singh it was found that viral infections were the most common cause of Secondary HLH. EBV and dengue were the most common viruses to cause HLH in children. In the current period, it is worth mentioning that many of the deaths occurring due to COVID 19 are also due to severe HLH like cytokine storm [8, 13]. COVID 19 causes blunting of NK cell number and functions [8]. Leishmaniasis, Ricketsia, Malaria, Histoplasmosis, Enteric fever, and Tuberculosis were also responsible agents in adults. The other causes were connective tissue disorders such as stills disease, juvenile idiopathic arthritis and SLE [7]. Malignancies especially those affecting T cells - like anaplastic T cell lymphoma and acute lymphoblastic lymphoma were seen to cause HLH in a study by Lehmberg et al. in 2015 [14]. Few B cell lineage neoplasias like hodgkins lymphoma were also seen. Secondary HLH could also occur following bone marrow transplant.

4. Diagnosis

Prolonged high grade fever is present in 90-100% of the children, and hence HLH is one of the differential diagnosis of Pyrexia of unknown origin [15]. Splenomegaly is also seen in more than 80% of children according to all the studies reviewed [9, 15, 16] and lymphadenopathy is seen in approximately 50% of cases [17]. The child at presentation appears very sick with features mimicking sepsis and septic shock (tachycardia, tachypnea, hyperpyrexia and occasionally hypotension). Pancytopenia or bicytopenia is always present because of significant phagocytosis, and the differential count always show lymphocyte predominance. Neutropenia is occasionally noted. CRPH which is a Marker of inflammation is seen to be very high. Activated histiocytes bind with factor 10, hence activating the common pathway of coagulation and consuming fibrinogen leading to hypofibrinogenemia [18]. ESR is hence low, since erythrocyte sedimentation is dependent on fibrinogen. Ferritin is elevated because ferroportin mediated iron efflux increases due to increase in growth differentiation

factor 15 during the hyperinflammation in HLH. Inflammation also causes upregulation of hemoxygenase - which breaks heme to iron. Other markers of inflammation which increase are IL-6, IL-18, IFN-gamma, TNF-alpha. Hypertriglyceridemia occurs due to inhibition of lipo-protien lipase by TNF alpha [19]. Diagnostic criteria for HLH was formulated in 2004 and confirms the diagnosis if any of the gene abnormalities can be detected (molecular diagnosis) or any 5 of the 8 criteria laid out as shown in Table 3 [20]. These diagnostic criteria were formulated according to prevalence of these features in 369 patients in HLH study of 2004. More than 70% of children in this study showed all these features [20].

Number	Criteria	Cut off value
1)	Fever	>38.5 degree centigrade
2)	Splenomegaly	-
3)	Cytopenias (atleast 2 cell lines):	-
a)	Hemoglobin	< 9 g/dl
b)	Neutrophil count	<1000/microliter
c)	Platelet count	<100000/microliter
4)	Hypertriglyceridemia or Hypofibrinogenemia	-
a)	Hypertriglyceridemia	>265 mg/dl
b)	Hypofibrinogenemia	<150 mg/dl
5)	Hemophagocytosis in bone marrow or lymph node or spleen and no evidence of malignancy	-
6)	* Hyperferritinemia	>500 ng/mL
7)	* Low or absent Natural Killer cell activity	-
8)	*Soluble CD 25 (IL- 2 receptor)	> 2400 U/ml

*- New criteria added in HLH 2004 guidelines

Table 3: Diagnostic criteria for HLH (HLH-2004)-Any 5 of the following 8 criteria to be fulfilled.

Ferritin levels for diagnosis of HLH is a very debated topic. Though 500ng/ml is considered significant, various authors have opined about increasing the threshold for confirmation of the diagnosis. Ferritin levels of 10000 ng/ml was considered significant by Allen et al. and 6000 ng/ml by Belfike et al. [21, 22]. CD107a was seen to be upregulated in all activated NK cells and hence was considered to be a marker of NK cell activity. As mentioned earlier, infiltration of macrophages and histiocytes in various organs cause multi organ failure. Liver involvement is seen in most of the children with HLH. Hence HLH is considered to be a differential diagnosis of Acute Liver Failure (ALF) during the first year of life. In a study on 251 children with HLH in Korea, 86% of the children were seen to have hepatomegaly, with 63% having transaminitis and 35% showing icterus. Around 27% of them had prolonged APTT. Hypofibrinogenemia was seen in 62% of the children [17]. Histology of affected liver shows lymphohistiocytic infiltration in the portal system, along with bile duct injury. However lobular histology is preserved. Kupffer cell hyperplasia is also noted along with sinusoidal congestion with histiocytes, along with hemosiderosis [23]. Symptoms and signs of lung

infiltration are very difficult to be differentiated from other infections [10]. CNS infiltration is seen in mostly the familial variant, and its symptoms are varied ranging from neck rigidity due to meningeal involvement to irritability due to encephalitis. Three stages were described by Henter and Nennesmo in 1997, where stage 1 showed only meningeal infiltration in the mildest form and stage 3 showed diffuse infiltration of parenchyma, meninges, along with multifocal tissue necrosis [24]. Features of raised ICP - bulged fontanelle, vomiting, blurring of vision are also seen. Convulsions and palsies of Cranial nerves 6 and 7 are occasionally noted.

It must be noted that the diagnosis of HLH is mostly made with progression of symptoms, not responding to the usual therapies, and hence the commoner possibilities must always be considered first. SIRS, sepsis induced MODS, and MAS secondary to JIA share the same clinical and laboratory features as that of HLH. This dilemma leads to over diagnosis of HLH [25]. Arico et al hence made an algorithm with three tests - perforin expression (by flow cytometry), lymphocyte 2B4 receptor function (antibody dependent cellular cytotoxicity assay) and NK cell activity [26]. In centers which do not have such facilities- supportive therapies for sepsis and MODS should be initiated and chemotherapy for HLH should be reserved for children who do not respond to them [25]. However, delay in treatment of HLH is also a determinant of mortality.

5. Treatment

The most important goal in treatment is to arrest the stormy hyperinflamatory response within the body and to promote apoptosis of the antigen presenting cell to prevent further inflammatory cells' recruitment. Etoposide is a chemotherapeutic drug which does both the above functions. Once this is achieved- the familial, persistent or relapse disease is treated with hemopoeitic stem cell transplant (HSCT) [20].

To achieve appropriate immune suppression, the HLH society had come up with a consensus guidelines in 1994 and later revised it in 2004, as shown in Table 4 [20]. There were marginal improvement in the survival with HLH 2004 as compared to HLH 94 guidelines - 62% with the former and 54% with the latter [20]. In v/o of significant complications of cyclosporine (renal toxicity and posterior reversible encephalopathy syndrome) many experts do not start early cyclosporine therapy, though some clinicians opine that it helps in maintaining remission for longer time [27]. Early initiation of steroids after ruling out malignancy must be considered even before all investigation reports are obtained [27]. Treatment should be tailored according to the disease severity and liver and renal functions. Children who show a very rapid response should be weaned off faster. Dexamethasone which is started at $10mg/m^2$ could also be tapered down as per severity [27]. At the same time any flare in the disease warrants further intensification of both steroid dose and etoposide frequency.

Anti thymocyte globulin (ATG), which causes T cell lymphopenia, has also been used along with steroids instead of etoposide and has shown to have good efficacy with lesser degree of cytopenia. A study done by Mahlaoui et al from showed this regimen to have complete remission in 73% and partial remission in 24% of the 38 children enrolled [28]. Intravenous immunoglobulins could be used in the treatment of HLH as a primary agent or an adjunct. Studies showing the efficacy of this drug have been shown in Table 5. It is to be noted that IVIG is most effective in secondary HLH and must be given as early as possible [29-34]. EBV induced HLH has shown good response to addition of Rituximab (anti CD 20 antibody). Anakinra

(IL1 recptor antagonist) was found to be beneficial as an early add on in Systemic onset juvenile idiopathic arthritis induced HLH [35, 36]. HLH like presentation following bone marrow transplant can be treated by adalimumab - TNF alpha inhibitor [37]. Various Biologicals used to treat HLH are shown in Table 6.

Initial intensive therapy: (2 weeks)	**HLH 1994 protocol**		**HLH 2004 protocol**	
	Dose	Number of weeks (frequency)	Dose	Number of weeks (frequency)
Drug				
Dexamethasone	10 mg/m2	2 weeks (daily)	10 mg/m2	2 weeks (daily)
Etoposide	150 mg/m^2	2 weeks (twice weekly)	150 mg/m^2	2 weeks (twice weekly)
Cyclosporine	-	-	*┼3 mg/kg	*┼2 doses daily
Initial Continuation therapy (2-8 weeks)				
Drug				
Dexamethasone	5 mg/m^2	2 weeks (daily) 3rd and 4th weeks	5 mg/m^2	2 weeks (daily) 3rd and 4th weeks
	2.5 mg/m^2	2 weeks (daily) 5th and 6th weeks	2.5 mg/m^2	2 weeks (daily) 5th and 6th weeks
	1.25 mg/m^2	2 weeks (daily) 7th week	1.25 mg/m^2	2 weeks (daily) 7th week
	Taper - stop	1 week 8th week	Taper - stop	1 week 8th week
Etoposide	150 mg/m^2	6 weeks(once a week) 3rd, 4th, 5th, 6th,7th,8th weeks	150 mg/m^2	6 weeks(once a week) 3rd, 4th, 5th, 6th,7th,8th weeks
Cyclosporine	–	-	*┼3 mg/kg	*┼2 doses daily
CNS involvement				
Intrathecal drug: (3, 4, 5, 6 weeks)				
Methotrexate	<1yr–6 mg 1-2yr-8 mg 2-3yr-10mg >3 yr-12mg	4 weeks (once a week)	<1yr–6 mg 1-2yr-8 mg 2-3yr-10mg >3 yr-12mg	4 weeks (once a week)
┼prednisolone	-		<1yr-4 mg 1-2yrs-6mg 2-3yrs-8mg >3yrs-10mg	4 weeks (once a week)
Continued therapy				
Dexamethasone	10 mg/m^2 for 3 days	Till remission/HSCT (once in 2 weeks)	10 mg/m^2 for 3 days	Till remission/HSCT (once in 2 weeks)

Etoposide	150 mg/m^2	Till remission/HSCT (once in 2 weeks)	150 mg/m^2	Till remission/HSCT (once in 2 weeks)
Cyclosporine	*3mg/kg	*2 doses daily	*3mg/kg	*2 doses daily

*- Cyclosporine levels to be maintained at 200 microgram/L; ┼- Addition in the HLH 2004 protocol

Table 4: Treatment protocol as per 1994 and 2004 HLH guidelines.

Study		**Number of IVIG patients**	**Primary HLH**	**Secondary HLH**	**Dose of IV IG**	**Compelete remission**
1	**Larroche C et al. [29]**	17	8	9	1.6g/kg- 2 doses	17 (100%)
2	**Rajajee S et al. [31]**	22	0	22	1g/kg – 2 doses	19 (86.3%)
3	**Nandkumar D et al. [30]**	17	0	17	1g/kg – 2 doses	17 (100%)
4	**B Ramachandran et al. [32]**	33	19	14	1g/kg – 2 doses	21 (63.6%)
5	**Yingkang Jin et al. [33]**	06	0	6	1g/kg – 2 doses	4 (66%)
6	**Bertrand Dunogué et al. [34]**	46	0	46	2g/kg- 1 dose	21 (45.6%)

Table 5: Treatment of HLH with IV IG.

Biologicals used in HLH	**Mechanism of action**	**Specially used in**
Rituximab	CD 20 antibody	EBV associated HLH
Anakinra	IL-1 receptor antagonist	sJIA associated HLH
Alemtuzumab	CD 52 antibody	Serotherapy in HSCT for HLH
Tocilizumab	IL-6 receptor antagonist	Trials ongoing
Ruxolitinib	JAK 1,2, receptor antagonist	Trials ongoing
Emapalumab	IFN gamma antibody	Trials ongoing
Adalimumbab	TNF alpha inhibitor	Post-transplant associated HLH

Table 6: Biologicals used in treatment of HLH.

Response to therapy could be monitored using ferritin levels or soluble IL2 receptor concentration. A drop of 15% of the former within first 48 hours and any decrease in the latter is considered to be a positive response [27]. Decrease in triglyceride level also suggest improvement [19]. Refractory cases (nil response after 8 weeks standard therapy) could be treated with Alemtuzumab (anti CD 52 antibody) along with steroids, etoposide and cyclosporine as shown in Marsh et al. 1 mg/kg of alemtuzumab was given as an

add on to 14 children and 25% of them showed partial remission [38]. Doxorubicin (liposomal), Etoposide and Methylprednisolone (DEP) is another regimen used as a salvage therapy especially in adult refractory cases [39].

6. Hematopoietic stem cell transplant (HSCT)

HSCT for this disease was first carried out in 1995, and advances from then in this field have led to better outcomes. The stem cells are usually harvested from peripheral blood (78%) or bone marrow (22%) of the donor [40]. Umblical cord stem cells have also been used for transplant and have shown good results with better survival in infants with primary HLH [41]. However, this cannot be done in bigger children and adults, since umblical cord may not have sufficient stem cells. Finding a fully matched donor for HSCT could be difficult, and hence partially matched family member's stem cells are used in few centers - haploidentical HSCT. The chances of the donor also having the abnormal gene causing HLH is high in such cases and if so the recipient would go into relapse [38].

Following HSCT, it takes 10-20 days for engraftment to occur. If all hematopoietic cells post-transplant are of donor origin, then the recipient is called a complete chimera and shows complete chimerism. 20-30% stable chimerism is required for a successful transplant, and to keep the child in sustained remission [38]. Increasing level of recipient cells could lead to graft rejection, and the opposite may result in the complication of graft vs host disease (GVHD). During mixed chimerism, whether an increased recipient origin cells indicates graft failure or a graft, recipient peaceful coexistence is difficult to assert [42]. Hence, clinical monitoring of remission, and early identification of GVHD is prime following the procedure.

Myelo-ablative conditioning regimens are nowadays being used to kill the recipient marrow cells, so that there is enough room for the donor cells in the recipient marrow. The advent of this therapy, also known as conditioning, preceding transplant has improved the outcomes in HLH quite significantly. Initially Busulphan and cyclophosphamide were used. However adverse effects such as veno-occlusive disorders ensued leading to adverse outcomes [38]. Better outcomes were noticed with reduced intensity conditioning regimens like melphalan and fludarabine, or treosulfan and thiotepa, though at the expense of higher rates of mixed chimerism. The results were further enhanced with addition of serotherapy with alemtuzumab which is a monoclonal antibody of CD52 (present on all mature lymphocytes, but absent in the stem cells) [43]. Alemtuzumab could be given at the proximal, distal or intermediate phase of the regimen. Data from various studies favor both proximal and intermediate phase administration. Mixed chimerism is seen in such cases, but with adequate tolerance, because the levels of alemtuzumab is seen to be high enough till around 60 days [44]. Also Alemtuzumab mediated depletion of T cells and APCs bring down the inflammation prior to HSCT - improving the chances of survival. However it is to be noted that low donor chimerism lesser than 20% warrants post HSCT cell therapy, which could be either donor lymphocyte infusion (DLI) or a second HSCT. Post-transplant, another cytokine storm can occur which looks like the original HLH itself. This condition is seen to have good response to adalimumbab [37]. Table 7 shows various studies on HSCT, with low and high intensity conditioning and their outcomes [45-48].

Study		Number of patients	Conditioning agents (number of patients)	Serotherapy agent (number of patients)	Outcome %survival
1	Carl et al. [45]	36	*Melphelan + Fludarabine (36)	Alemtuzumab (36)	82.4% (after 1 yr)
2	Katherine et al. [46]	60	*Fludarabine + Treosulphan (44) *Fludarabine + Melphelan (15)	Alemtuzumab (40) Ⱨ Ⱨ ATG (20)	75% (after 5 years)
3	Nikiforow et al. [47]	20	Ⱨ Busulphan + Etoposide + Melphelan	-	70% (after 5 years)
4	Hong Hao Ma. [48]	15	Ⱨ Busulphan + Fludarabine + Cyclophosphamide	Ⱨ Ⱨ ATG	71% (after 3 years)

*- Low intensity conditioning; Ⱨ- High intensity conditioning; Ⱨ Ⱨ- Anti Thymocyte Globulin

Table 7: Studies of HSCT with various conditioning agents and survival.

7. Outcomes

Children with this condition do not survive more than 2 months without treatment [9, 11] Younger age, CNS involvement, severe elevation in transaminase, severe cholestasis, and coagulation abnormalities are considered to be indicators of poor outcomes according to a study by Koh et al. [16]. Outcomes are also negative if the treatment is delayed, and hence at least steroids should be commenced immediately after ruling out all other possibilities such as malignancy. In some children repeated, multiple blood and plasma exchange have shown positive response, and have been used to buy some time till diagnosis is confirmed [49].

Hematopoietic stem cell transplant is the final curative treatment available, and in familial cases, this must be performed as soon as possible during remission. Hence HLA typing and searching an appropriate donor is commenced as soon as the disease is confirmed in such cases. Having active disease during transplant, using haploidentical stem cells and a poor match in HLA typing were seen to have high rates of failure following HSCT [38].

8. Recent updates and research

Pre B cell colony enhancing factor (PBEF) - a marker of severe inflammation, has been shown to be a very good marker for diagnosis and disease activity. This has shown a better specificity because with remission it touches the baseline levels, as compared to IL2 receptor levels, which stays mildly elevated for some time [50]. Hybrid immunotherapy is a new treatment modality which is being tried in few centers where steroid, etoposide and ATG are being used. Other regimens with the primary agent being alemtuzumab and tocilizumab (IL-6 receptor inhibitor) are being studied in France and Philadelphia respectively. However, there are no published results from these studies. Cytokines cause the inflammatory storm through Janus kinase (JAK1,2) and signal transducer and activator of transcription (STAT) associated receptors. Ruxolitinib causes inhibition of JAK1,2 receptors with amelioration of inflammation in murine models [51]. This agent as primary agent is being tried in Michigan. Anti IFN-gamma antibodies - Emapalumab is also being tried. A study on 13 children treated with Emapalumab showed improvement of parameters in 9 children and 7 of them went for HSCT [52]. Hematopoietic stem cell gene

therapy with selected cells of high level perforin expression led to better survival in animal model studies. Perforin gene transfer using lenti virus as vector into progenitor cells of perforin deficient mice was performed and significant reduction in cytokine secretion in mice, with more than 30% engraftment, was noted [53]. In another study Micro RNA -126 (enhancer of perforin expression) was placed in the perforin DNA loci of the stem cells, and was transplanted into perforin deficient mice. These mice showed a dose dependent increase in survival and better clearance of pathogen [54]. Such gene therapy if successful in humans could improve the outcome remarkably. Extracorporeal adsorption technique has shown success in adults. It was used in 2 adults with HLH secondary to herpes infection, and was found to be effective in bringing the cytokines' level down. An adsorption cartridge made up of polystyrene divinylbenzene copolymer beads is used, which binds to hydrophobic compounds with a molecular weight of 10-55 kDa [55].

9. Conclusion

HLH is a fatal storm of inflammation in the body, which mimics other severe illnesses such as SIRS and sepsis. Differentiating between them clinically is a challenge. Treatment of both sepsis and HLH are also very different with former being treated with broad spectrum antibiotics and latter with immunosuppressants. The recent diagnostic guidelines have led to an over diagnosis of HLH leading to flare up of infections and missing malignancies at an earlier stage due to the immunosuppressants used for the same. At the same time delaying the treatment of HLH also causes mortality. This dilemma warrants immediate but prudent treatment with exercise of cautious monitoring.

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