

Genetic Defects in Cytolysis in Macrophage Activation Syndrome

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Abstract Macrophage activation syndrome (MAS), typically presenting beyond the first year of life, is an often lethal cousin of familial hemophagocytic lymphohistiocytosis (fHLH). Defects in natural killer (NK) cell and CD8 T cell cytotoxicity result in a pro-inflammatory cytokine storm, cytopenia, coagulopathy, and multi-organ system dysfunction. MAS can occur in association with infections (herpes viruses), cancer (leukemia), immune deficient states (post-transplantation), and in autoimmune (systemic lupus erythematosus) and autoinflammatory conditions (systemic juvenile idiopathic arthritis). The distinction between fHLH, the result of homozygous defects in cytolytic pathway genes, and MAS is

becoming blurred with the identification of single or multiple mutations in the same cytolytic pathway genes in patients with later onset MAS. Here, we review the literature and present novel cytolytic pathway gene mutations identified in children with MAS. We study the inhibitory effect of one these novel mutations on NK cell function to suggest a direct link between fHLH and MAS.

Keywords Macrophage activation syndrome · Hemophagocytic lymphohistiocytosis · Cytolysis · Genetics · Mutation · Natural killer cell · CD8 T cell · Perforin · Interleukin-1 · Interferon-gamma

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Introduction

Macrophage activation syndrome (MAS) is quickly becoming a more recognized entity in both pediatric and adult hospital settings [1]. MAS is not a diagnosis of exclusion but can accompany infectious, oncologic, immunodeficiency, and rheumatic disorders [2•]. Among rheumatic diseases, MAS is most commonly seen in pediatrics in children with systemic juvenile idiopathic arthritis (sJIA), where it is believed by some to be inherent to the disease process in 40–50 % of cases, with approximately 10 % presenting clinically overtly [3, 4]. MAS is also seen in the related disorder, adult onset Still disease [5], but in adults it is more frequently seen in systemic lupus erythematosus (SLE) which is much more common [6•]. MAS also occurs in pediatric SLE [7•, 8] and in Kawasaki disease (KD) [9], along with a variety of other vasculitides and rheumatic disorders in children and adults [10•].

MAS is the terminology used by rheumatologists to refer to hemophagocytic lymphohistiocytosis (HLH) occurring in children and adults with rheumatic diseases. MAS, sometimes called reactive or secondary HLH, is likely similar to, if not the same disease process as, primary or familial HLH (fHLH),

which typically occurs in the first year of life as a result of homozygous defects in cytolytic pathway genes [1, 2•]. As such, the diagnosis of fHLH can be made if there is a molecular diagnosis of homozygous or compound heterozygous mutations in the known associated HLH genes (Table 1), but usually the diagnosis is made by meeting 5 of 8 established criteria [11]. The HLH criteria reflect the clinical manifestations and associated laboratory abnormalities and include the following: fever, splenomegaly, peripheral cytopenia (2 or more lineages), hypertriglyceridemia or hypofibrinogenemia, hemophagocytosis on biopsy or in cerebrospinal fluid, low or absent natural killer (NK) cell activity, hyperferritinemia, and elevated soluble CD25 (interleukin-2 receptor alpha chain) [11]. In part, because MAS may not be initially as severe as fHLH in presentation, and since measurement of NK cell activity and soluble CD25 is frequently not readily available, criteria have been proposed to diagnosis MAS for rheumatic disorders.

For rheumatic diseases, preliminary diagnostic criteria have been put forth for establishing the diagnosis of MAS in the setting of sJIA [12]. These criteria overlap some of the HLH criteria (cytopenias and hypofibrinogenemia) but also include an elevated liver enzyme and 3 sets of clinical criteria (central nervous system dysfunction, hemorrhages, and hepatomegaly). Recently, an international effort is in the process of generating new MAS criteria for children with sJIA using a rigorous statistical modeling approach, including testing the criteria on large sets of real patient data [13•]. Related criteria efforts have also been attempted for pediatric lupus [14]. Whether or not a single scoring system for all forms of MAS/reactive HLH will be sensitive and specific enough is yet to be determined [15•].

Until recently, the restrictive nature of the HLH criteria for diagnosing non-fHLH forms of MAS has hindered the recognition and timely diagnosis of MAS in the hospital setting. While fHLH is rare occurring in approximately 1 in 50,000 live births [2•], MAS/reactive HLH is much more common as it can arise in the setting of infectious, oncologic, rheumatic, and immune deficient states [16, 17]. In fact, MAS is still likely vastly under-recognized, particularly in the intensive care unit (ICU) setting, where it may be labeled multiple organ dysfunction syndrome (MODS), systemic inflammatory response syndrome (SIRS), or culture negative sepsis [18]. The recognition of MAS in the ICU is particularly important, as treatment for MAS is often radically different (immunosuppression or chemotherapy) than broad spectrum antibiotic coverage, and mortality for these ICU syndromes is high [19]. Similarly, children with KD who share many features of MAS, have been labeled “shock syndrome” [20]. Again, it is important to realize that MAS in the setting of KD may require specific treatment such as interleukin-1 (IL-1) blockade [21•]. Until a more global set of diagnostic criteria for all forms of MAS are validated, in the setting of a febrile, ill-appearing patient, a very sensitive, inexpensive, and simple screening tool remains an elevated serum ferritin level [22, 23].

As MAS is more frequently being recognized and diagnosed, the question arises as to why some individuals with certain infections, malignancies, immunodeficiencies, and rheumatic disorders are prone to develop MAS and others are not. This selectivity implies that not only is the particular condition (e.g., H1N1 influenza) [24, 25] a risk factor for MAS but the individual’s genetically susceptible host immune response may also play an important role in the development of MAS. Most of the understanding of the pathophysiology of MAS has come from the study of animals spontaneously or genetically engineered to be deficient in the same cytolytic pathway genes as found in fHLH (Table 1) [2•, 26••, 27••, 28]. Although there are most likely multiple pathophysiologic pathways capable of leading to the end common pathway of MAS that do not require defects in lymphocyte cytotoxicity [29••], there are emerging data that subsets of MAS patients have partial defects in the same genes which are homozygous deficient in fHLH infants [27••]. An exploration of genetic defects in cytolytic pathway proteins in patients with MAS/secondary HLH is the focus of this review [30].

Cytolysis and MAS Pathophysiology

A current hypothesis as to the pathophysiology of HLH proposes that the inability of NK or CD8 T cells to lyse infected antigen-presenting cells (APC) leads to ongoing infection as well as an amplifying crosstalk of pro-inflammatory cytokines elicited by the APC and the lymphocyte ineffective in cytotoxicity [2•, 26••, 27••, 31]. The resultant “cytokine storm” contributes to hemophagocytosis by activated macrophages/histiocytes, multi-organ dysfunction, pancytopenia, coagulopathy, and eventual host demise. This has perhaps been best explored experimentally in an animal model of perforin deficiency [32]. These mice deficient in perforin-dependent target cell cytotoxicity are perfectly healthy until exposed to a particular virus, LCMV, and then they develop a rapidly fatal condition similar to HLH. Elimination of CD8 T cells prior to infection rescues the mice from death, as does blockade of the pro-inflammatory cytokine, interferon-gamma (IFN- γ) [32]. Interestingly, as little as 20 % wild-type (WT) perforin-expressing CD8 T cell chimerism is also able to ameliorate the HLH picture in these animals [33•]. As APCs are also critically important to the process of HLH [34], elimination of antigen-presenting dendritic cells (DC) can also protect against the HLH picture in perforin-deficient mice [35••]. It has recently been proposed that the severity of the lymphocyte killing defect in different mouse strains positively correlates with the severity of the HLH phenotype [26••]; it is therefore intriguing to speculate that a subset of human MAS presenting later in life than fHLH (infancy) may result from more subtle genetic defects in lymphocyte cytotoxicity.

Table 1 HLH genes involved in the lymphocyte cytolytic pathway

Familial HLH	Gene	Location	Protein	Function	Reference
FHL1	Unknown	9q21.3-q22	Unknown	Unknown	[57]
FHL2	<i>PRF1</i>	10q21-22	Perforin	Pore-forming	[58]
FHL3	<i>UNC13D</i>	17q25	Munc13-4	Vesicle priming	[59]
FHL4	<i>STX11</i>	6q24	Syntaxin11	Vesicle docking	[60]
FHL5	<i>STXBP2</i>	19p13.2-3	Munc18-2	Vesicle membrane fusing	[50]
Immunodeficiency	Gene	Location	Protein	Function	Reference
Chédiak-Higashi	<i>LYST</i>	14q.1-q42.2	Lyst	Vesicle sorting	[61]
Hermansky-Pudlak-2	<i>AP3B1</i>	5q14.1	AP3B1	Vesicle trafficking	[62]
GrisCELLI-2	<i>RAB27A</i>	15q21	Rab27a	Vesicle fusing	[48]
XLP-1	<i>SH2D1A</i>	Xq25	SAP	SLAM signaling	[63]
XLP-2	<i>BIRC4</i>	Xq25	XIAP	Caspase inhibition	[64]

Cytolysis and Genetics of MAS in sJIA

In humans, non-familial MAS has been most rigorously studied in children with sJIA. It has been known for some time that children with HLH and their first degree relatives have defects in NK cell function [36], but not long after it was also recognized that children with sJIA and MAS had similar NK cell dysfunction [37]. This is in part why it has been proposed that MAS not be considered a complication but rather an inherent aspect of disease in a substantial proportion of children with sJIA [3]. In studying one of the known HLH-associated genes, Grom and colleagues identified a haplotype of polymorphisms across the *UNC13D* gene (Table 1) which encodes for Munc13-4, a protein critical for getting the cytolytic perforin-containing vesicles to the NK cell immunologic synapse. This particular haplotype was notably over-represented in sJIA patients with MAS compared to controls, implying a genetic link of an established lymphocyte cytolytic pathway gene and MAS [38]. Moreover, a known fHLH causative mutation in *UNC13D* was identified in a patient with sJIA, suggesting a particular risk for MAS development [39].

Another HLH gene critical to lymphocyte cytolytic activity, *PRF1*, encoding the perforin protein (Table 1), was found to be mutated resulting in an amino acid change (A91V) in 20 % of a European population of sJIA patients with MAS; it was also present in 10 % of sJIA patients without known MAS versus approximately 3 % of the general population [40]. Because this mutation is so common in the overall population, some have suggested it is strictly a polymorphism [41]; however, more recent functional analyses of this mutation have shown perforin A91V to impair NK cell lytic activity (Cron et al., in preparation) [42, 43–45]. Interestingly, at Children's of Alabama (CoA), we have identified this same perforin A91V mutation in one of our MAS patients of unknown etiology (Table 2, patient #4) and in 2 of our immunodeficiency patients (Table 2, patients #9 and #10), both of whom had decreased NK cell function. Most recently, we identified the common perforin

A91V mutation (Cron et al., in preparation) among the first 4 sequenced of a small cohort of fatal H1N1 influenza-infected adults with hemophagocytosis on autopsy [25]. Thus, single copy HLH gene mutations for both *PRF1* and *UNC13D* are associated with, and may be capable of contributing to, MAS development in children with sJIA and children and adults with other unrelated disorders or infections.

HLH Gene Mutations in a Pediatric MAS Cohort

From February 2008 to February 2012, 28 individuals with MAS, plus 2 children with immunodeficiency and decreased NK cell function, were identified at CoA. Ten of these children were tested for NK cell dysfunction, and 8 of them (Table 2, patients #1, 3, 5, 6, 9, 10, 11, 12) had decreased cytolytic activity (patients #4, 7, 8 were not studied). Seventeen of these patients were screened at the discretion of the treating physician at the time of hospitalization for MAS (or in clinic for the 2 immunodeficiency patients) for 5 of the HLH associated genes (*PRF1*, *UNC13D*, *STX11*, *STXBP2*, *RAB27A*) (Table 1). A rather surprisingly high percentage (13/17 or 76 %) of these children was found to have at least one mutation among these 5 selected genes (Table 2). Among the 11 kids with MAS, 9 met ≥ 5 or more HLH criteria, one met 4 criteria (Table 2, patient #13), and one had mild MAS as part of sJIA (Table 2, patient #8). Of the entire cohort of 28 MAS patients (not including 2 immunodeficiency patients with HLH gene mutations, but including 15 who were not sequenced for HLH genes), 39 % had at least one HLH gene mutation. It is possible that this percentage would be higher if the entire cohort (15 more of the 28) were sequenced, but it is likely that the sickest kids were sequenced as 4 of those with HLH gene mutations died from MAS, whereas 24 others, including all those not sequenced, all survived (Table 2). Thus, confounding by indication may have identified the bulk of those with HLH gene mutations. Nevertheless, comparable

Table 2 HLH mutations in pediatric MAS and immunodeficiency patients

Pt	Age	Sex	Diagnosis	Trig-ger	Gene	Mutation	Amino acid change	Prior Association	Outcome
1	15	M	Leukemia	?	<i>UNC13D</i>	2896 C>T	R966W	Pt. 6 only	Died
2	1.5	M	Uveitis	?	<i>UNC13D</i>	847 A>G	I283V	4 % AA	Alive
3	18	F	Uveitis	?	<i>RAB27A</i>	259 G>C	A87P	Griscelli-2	Alive
4	9	F	?	?	<i>PRF1</i>	272 C>T	A91V	3 % USA	Alive
5	16	F	Gastroparesis	CMV	<i>STXBP2</i>	1298 C>T	A433V	None	Died
6	12	M	Systemic JIA	HSV1	<i>UNC13D</i>	2896 C>T	R966W	Pt. 1 only	Alive
7	7	M	Polyarteritis	?	<i>STXBP2</i>	1782 G>A	3' UTR	6 others	Alive
8	1.5	F	Systemic JIA	?	<i>UNC13D</i>	2368 C>T	Splice site	None	Alive
9	3	M	? Immune deficiency	?	<i>UNC13D</i>	869 C>T	S290L	One prior	Alive
					<i>PRF1</i>	272 C>T	A91V	3 % USA	
10	1	F	Chronic HepB	HepB	<i>PRF1</i>	272 C>T	A91V	3 % USA	Alive
11	17	M	?	EBV	<i>STXBP2</i>	822 G>A	A274A	None	Died
12	8	M	?	HHV6	<i>STXBP2</i>	1782 G>A	3' UTR	None	Died
					<i>STXBP2</i>	1782 G>A	3' UTR	None	
					<i>STXBP2</i>	1026 C>G	Non-coding	None	
					<i>STXBP2</i>	1357 G>A	Non-coding	None	
13	14	F	Sjogren	?	<i>STXBP2</i>	1590 G>A	Splice site	3 others	Alive
					<i>STXBP2</i>	-37 delete	5' UTR	linked	

numbers have recently been observed in Cincinnati (Grom et al., submitted) implying that many children with MAS may carry a risk allele with a mutation of at least one of the classic HLH genes (Table 1). Similar reports of HLH gene mutations in 14 % of 175 adults with MAS/secondary HLH have also been reported, with almost half having the perforin A91V mutation [46••]. Thus, the distinction between primary/fHLH and secondary or reactive HLH/MAS is becoming genetically blurred.

In the cohort reported here (Table 2), as might be predicted, 3 of the 13 patients (23 %) possessed the relatively common (3 % of the North American general population) perforin A91V mutation (Table 2, patients # 4, 9, 10). However, the HLH genes most commonly mutated in this cohort were *UNC13D* and *STXBP2* with 5 patients each (Table 2). Several of these mutations had not been previously described in the medical literature, including one Munc13-4 mutation (R966W) that was identified in two of the children, one with leukemia-associated MAS and the other with sJIA-associated MAS (Table 2, patients #1 and #6, respectively). Another mutation, Munc13-4 I283V (Table 2, patient #2), has previously been considered a benign polymorphism as it is present in 4 % of African-Americans, but it was recently reported as a cause of late onset fHLH in another cohort [47•]. The patient with the Munc13-4 I283V amino acid change in our cohort developed uveitis and spondyloarthritis after the MAS was clinically controlled. Intriguingly, a previously well 18-year-old female in our cohort (Table 2, patient #3) with a Rab27a A87P mutation also developed uveitis and spondyloarthritis after MAS resolution. This particular *RAB27A* mutation is a known cause of Griscelli-2 fHLH when present on both

chromosomes [48]. We have recently found that over-expression of this *RAB27A* mutation in the NK-92 NK cell line [49] delays cytotoxic granule polarization to the immunologic synapse and dramatically lowers NK cell cytotoxicity (Zhang et al., in preparation). Thus, single copy variants of HLH genes (Table 1) likely contribute to late onset HLH/MAS via partial dominant negative or hypomorphic effects [46••] resulting in decreased lymphocyte cytolytic activity.

A Novel *STXBP2* Mutation Decreases NK Cell Lytic Function

Intriguingly, 5 of the 13 patients in this cohort (Table 2) had mutations in *STXBP2*, the cause of fHLH5 (Table 1) [50]. A similar relative abundance of *STXBP2* and *UNC13D* mutations was also recently noted in a group of 14 sJIA patients with MAS (Grom et al., submitted). In the current cohort (Table 2), many of the *STXBP2* mutations were novel or had rarely been reported in association with HLH. Several of these mutations were present in non-coding regions, including 5' and 3' untranslated regions and RNA splice sites. It is possible that these non-coding mutations may alter Munc18-2 levels and thus diminish lymphocyte cytolytic function, as recent reports have demonstrated that mutations in intronic and transcriptional regulatory regions of HLH genes can contribute to HLH and immunodeficiency [51••, 52•]. Furthermore, a sizeable cohort of fHLH5 patients was reported with splice-site mutations in *STXBP2* resulting in later onset HLH [53•]. Thus, it is believable that the *STXBP2* splice-site mutations in the cohort reported here likely contributed to MAS, in particular for two of

the patients with multiple *STXBP2* mutations, one associated with the death of the child (Table 2, patients #12 and #13).

One of the novel *STXBP2* mutations identified led to an amino acid change, Munc18-2 A433V, and was associated with decreased NK cell activity and the death of a child with MAS/secondary HLH (Table 2, patient #5). To test the plausibility that this single copy mutation contributed to MAS development, we first cloned the wild-type (WT) *STXBP2* mutation into a lentiviral expression vector [54] that co-expressed a green fluorescent protein (GFP) for tracking transduced cells. Using site directed mutagenesis [54], we further generated a lentiviral construct expressing the novel Munc18-2 A433V patient mutation. The *STXBP2* WT, mutant, or an empty lentiviral expression vector were independently transduced into the human NK cell line, NK-92 [49], and cells were sorted for GFP expression. These cells were co-cultured with fluorochrome-labeled K562 target cells [55] at increasing effector (NK-92) to target (K562) cell ratios, and cell lysis was determined using 7AAD staining by flow cytometry [56]. We repeatedly found that over-expression of WT Munc18-2 augmented NK cell lysis of K562 target cells compared to empty vector control, but over-expression of the Munc18-2 A433V mutant consistently and statistically significantly reduced NK cell lytic function (Fig. 1). These results confirmed the concept that a single copy HLH mutation may contribute to decreased NK cell function and serve as a risk for development of later age onset HLH or MAS.

Conclusions

MAS belongs on the spectrum of fHLH and secondary or reactive HLH sharing many clinical, laboratory, and pathologic features. MAS is a consequence of a pro-inflammatory cytokine storm that results from a dysregulated immune response which can occur associated with a variety of infectious, oncologic, rheumatic, and immune deficient conditions. The frequently fatal condition of MAS/secondary HLH is still widely under-recognized and therefore undertreated in children and adults, particularly in the setting of the hospital ICU in patients with MODS. It is important to understand that MAS is an end common pathway from many disease states and not a diagnosis of exclusion. Until improved and timely diagnostic aids are developed, it is likely worth screening for MAS by serum ferritin levels in febrile acutely ill individuals.

In both children and adults, there is mounting evidence that genes associated with fHLH (Table 1) are found to be mutated in late onset HLH and MAS. Even single copy mutations have been shown to decrease NK cell cytolytic activity and thus likely directly contribute to the pathophysiology of MAS/secondary HLH. From the data presented in this present pediatric MAS cohort (Table 2), both the large number and varied HLH genes and individual mutations identified suggest

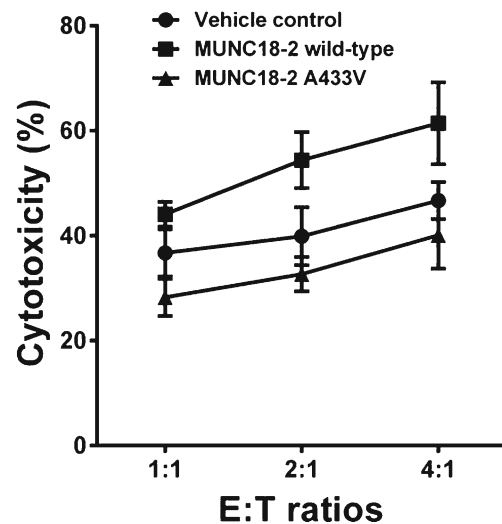


Fig. 1 Decreased cytolysis in NK cells overexpressing a novel MUNC18-2 mutation. NK-92 human NK cells (49) were transduced with a green fluorescent protein-expressing (GFP) lentiviral vector (z-368-ΔNP) co-expressing wild-type MUNC18-2 (*squares*), mutant MUNC18-2 (A433V) (*triangles*), or GFP alone (*circles*) under the control of the immediate-early cytomegalovirus transcriptional promoter. GFP+ flow cytometry (FCM) sorted NK-92 cells were cultured with eFluor450-labeled K562 target cells at graded effector to target (E:T) cell ratios, and cell death was measured by 7AAD staining at 4 h post-incubation using FCM. Summary data ($n=3$) are presented as means±SEM percent cell lysis (cytotoxicity) along the Y-axis and increasing E:T ratio on the X-axis. All 3 conditions were statistically significantly different from one another (2-way ANOVA, $p=0.0005$)

causality rather than a simple association with MAS and decreased NK cell function. Moreover, the demonstration that over-expression of one of the novel *STXBP2* mutations found in this MAS cohort experimentally decreased NK cell cytolytic function (Fig. 1) further strengthens this argument.

It is somewhat sobering to consider the fact that some of the identified MAS patient mutations are relatively frequent in the general population (up to 3–4 %) and likely contribute to MAS in a large variety of settings (intracellular infections, rheumatic conditions, leukemia, immune deficiency states). The association of the perforin A91V mutation with H1N1 influenza is a prime example of this disconcerting concept. A large percentage of the general public is exposed to various infections, H1N1 influenza, for example. Many in the population will contract the infection, but only a subset of individuals will require hospitalization or die from the infection. In these cases, it is not only the infection that is a trigger, but it is the individual's genetically determined immune response to the infection that can be lethal as a result of developing MAS. It is therefore not all that surprising that fHLH genes are found mutated in a substantial percentage of individuals identified with MAS and secondary HLH. In addition to the genetic risk, there may also be a second “hit” contributed by the infection, or, in the case of rheumatic diseases, a hyper-inflammatory state that results in MAS. Increased awareness of MAS, and knowledge of the genetic susceptibility for the development of

MAS, are both likely to help combat the fatal nature of this more common than previously considered hyper-inflammatory disorder.

Compliance with Ethics Guidelines

Conflict of Interest Randy Q. Cron has received <\$5,000 each as consulting fees from Genentech, Novartis, and Swedish Orphan Biovitrum. Alexei A. Grom has received <\$5,000 each as consulting fees from Novartis and Roche. Mingce Zhang, Edward M. Behrens, T. Prescott Atkinson, and Bitu Shakoory declare that they have no conflict of interest. The work used to generate the data in Fig. 1 was supported by a grant from the Kaul Pediatric Research Institute to Randy Q. Cron.

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- Of importance
- Of major importance

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