Histiocyte Society blueprint for hemophagocytic lymphohistiocytosis research: deciphering underlying disease mechanisms to optimize diagnosis and therapy

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Abstract

Hemophagocytic lymphohistiocytosis (HLH) is a rare syndrome characterized by severe hyperinflammation, leading to end-organ damage and death in the absence of treatments directed at suppressing the overactive immune system. The study of mouse models and human biospecimens has provided insights into disease pathophysiology and enabled investigations of novel therapeutic strategies. Nevertheless, clinical outcomes remain suboptimal. Additional research is required to further our understanding of this highly heterogeneous disease in order to develop improved diagnostic and prognostic tools and identify and implement targeted and less toxic treatments. In this article, written on behalf of the Histiocyte Society, we highlight six key areas of ongoing research, including the remaining questions in each area. We also outline the challenges of global dissemination and implementation of this research and call for the establishment of local, regional, and national cooperative groups aimed at providing education for physicians and targeted diagnostic and clinical guidelines. Finally, we discuss the role of international societies, including the Histiocyte Society, in nucleating this work to ensure coordinated efforts aimed at improving outcomes for patients with HLH worldwide.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a severe hyperinflammatory syndrome characterized by an excessive but ineffective immune response leading to cellular hyperactivation and rampant cytokine secretion. Together, these factors mediate end-organ damage that is often fatal without therapy aimed at controlling the overactive immune response.[1] HLH can be subdivided into primary HLH (pHLH) and secondary HLH (sHLH). pHLH is best studied in the subset of patients with familial HLH (fHLH), characterized by germline bi-allelic loss-of-function variants affecting *PRF1*, *UNC13D*, *STX11*, and *STXBP2*, whose encoded proteins are essential for the formation and release of cytolytic granules from CD8 T and natural killer (NK) cells (Figure 1). As a result, patients with fHLH exhibit markedly reduced or absent CD8 T- and NK-cell-mediated killing.[1] In contrast, patients with sHLH generally lack bi-allelic mutations in pHLH genes but instead develop disease following exposure to strong immunological triggers, including infections, malignancies, and autoimmune diseases.[1] Treatment for HLH usually involves administration of etoposide and/or corticosteroids, with recent efforts also incorporating cytokine-targeting agents; however, clinical outcomes remain suboptimal.[2]

In this article, we highlight the major biological and clinical advances surrounding HLH in recent decades. We then provide an overview of six active areas of research, progress that has been made in each of these areas, and remaining challenges moving forward. We end by discussing the need to re-evaluate how we consider collaborative research and the conduct of HLH clinical trials on a global scale, as well as the role that cooperative groups play in supporting these efforts. In this manner, we put forth a "blueprint" to guide future HLH research.

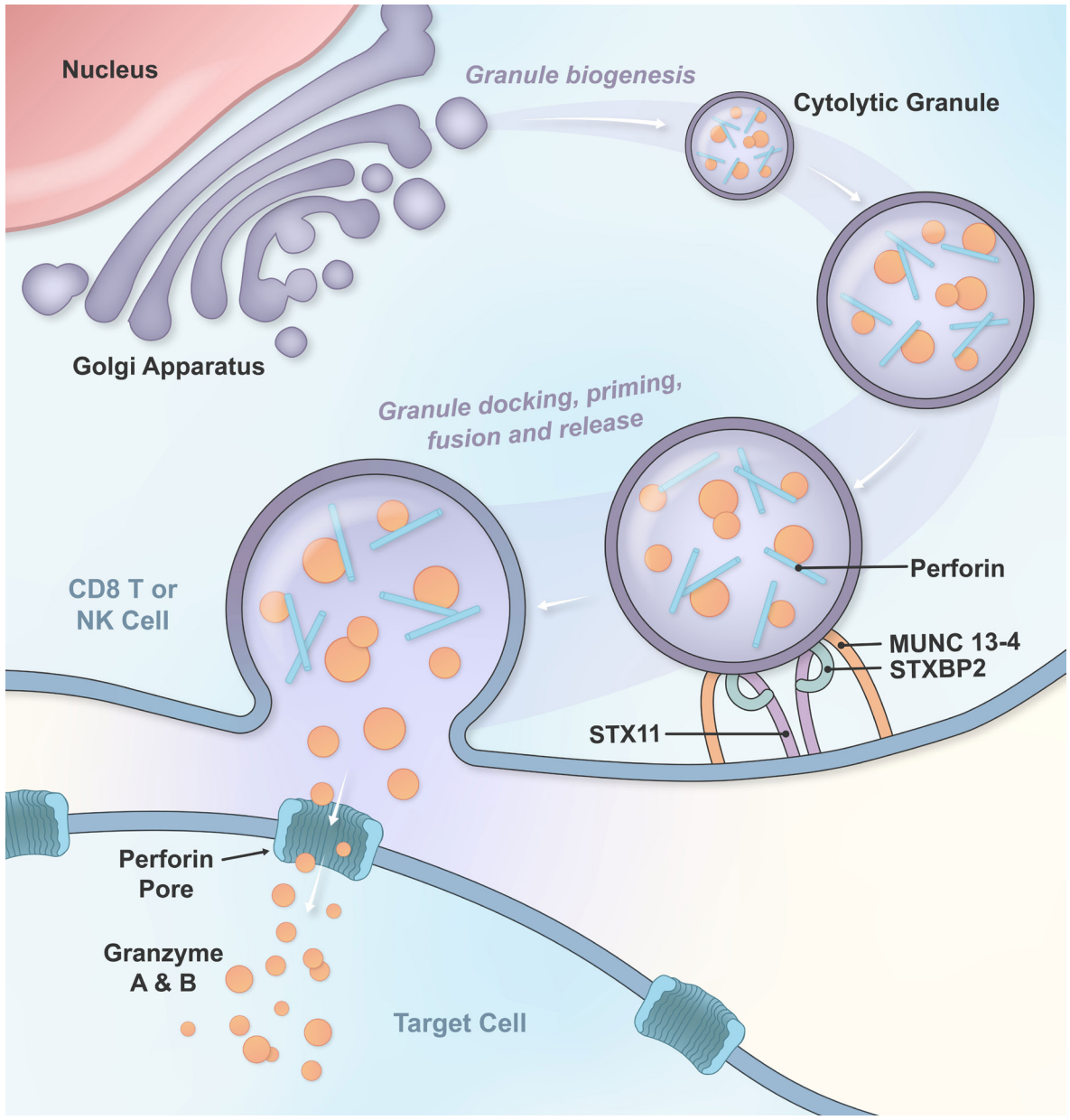

Figure 1. Cytolytic granule production and secretion. A series of intracellular proteins are required for the synthesis, trafficking, and release of cytolytic granules from CD8 T and natural killer cells at the site of target cell engagement. Defects in the genes encoding these proteins render this pathway non-functional, resulting in the impaired cytotoxicity characteristic of patients with primary hemophagocytic lymphohistiocytosis. NK: natural killer; MUNC: mammalian uncoordinated; STXBP: syntaxin-binding protein; STX: syntaxin.

Advances in understanding disease biology through the study of animal models

Animal models have greatly expanded our understanding of the pathogenesis of HLH, including the diverse cellular and molecular drivers of disease. Numerous models have been developed to examine the biology of HLH and they recapitulate many of the cardinal disease manifestations observed in humans. Several excellent reviews on this topic have been published;[3,4] therefore, we will focus on two well-studied models and how these have elucidated key roles for innate and adaptive immune cells and cytokines as essential mediators of hyperinflammation and targets for therapeutic intervention.

The study of mice harboring natural or genetically engineered loss-of-function mutations in pHLH genes has demonstrated that perforin-dependent cytotoxicity is essential for downregulation of the normal immune response. Among existing models, perforin-deficient (*Prf1*$^{-/-}$) mice have been studied most extensively. Following infection with lymphocytic choriomeningitis virus (LCMV) or other pathogens, *Prf1*$^{-/-}$ mice develop fatal immunopathology due to robust CD8 T-cell activation and interferon-gamma (IFNγ) production.[5] These heightened T-cell functions are not due to a reduced CD8 T-cell activation threshold, but rather, they appear to result from the inability of *Prf1*$^{-/-}$ CD8 T cells to eliminate activated antigen-presenting cells.[6] As a consequence, the antigen-presenting cells continue to drive T-cell activation, proliferation, and production of IFNγ and tumor necrosis factor (TNF), leading to the downstream activation of macrophages that produce additional pro-inflammatory cytokines.[7] Altogether, these cytokines fuel a feed-forward loop of T-cell- and macrophage-mediated hyperinflammation (Figure 2).

To study sHLH, Behrens *et al*. developed a model in which wild-type mice receive doses of CpG DNA to engage toll-like receptor (TLR) 9.[8] This results in non-lethal inflammation

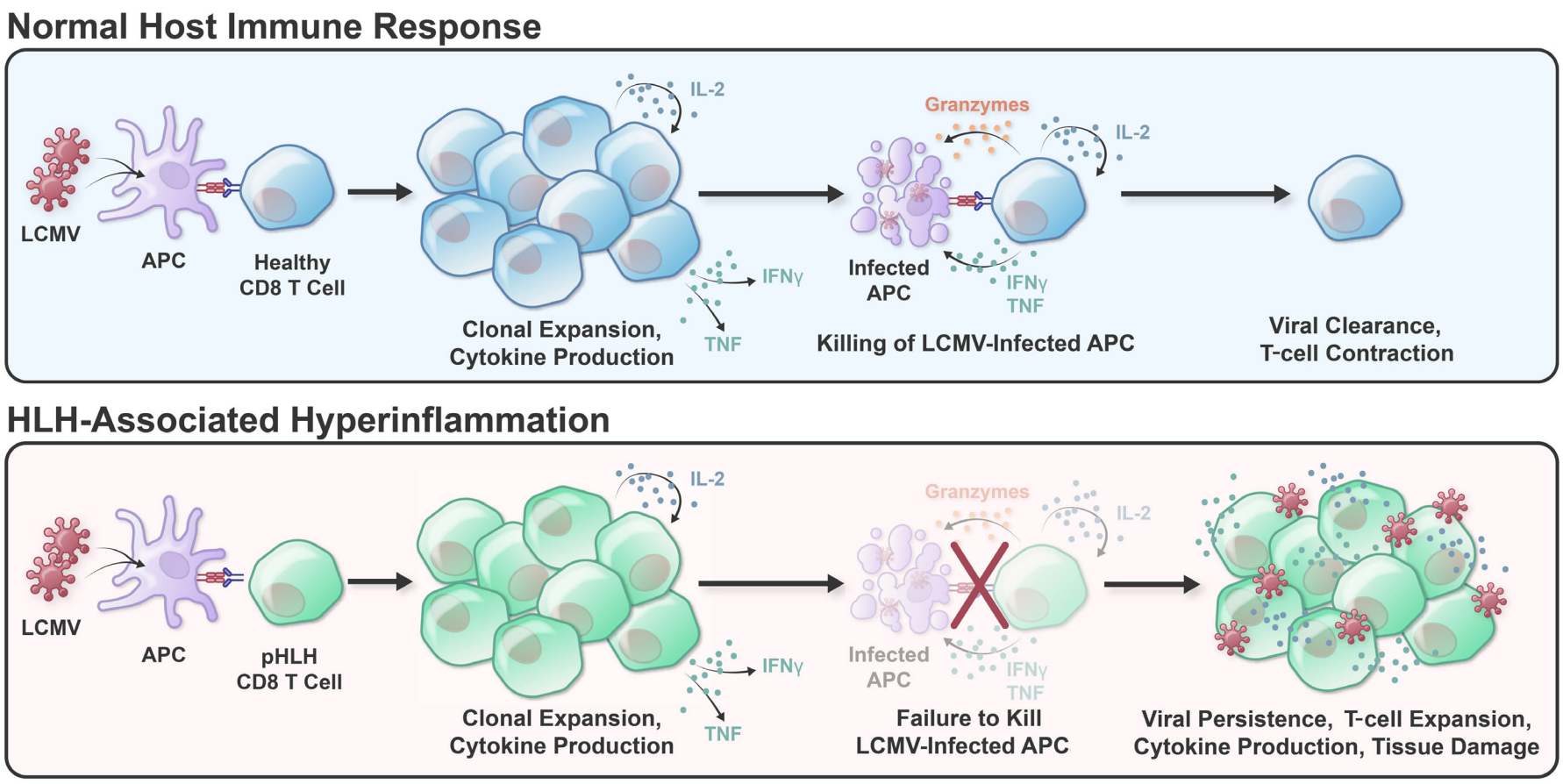

Figure 2. Pathophysiology of hemophagocytic lymphohistiocytosis. In a healthy immune response (top), CD8 T cells become activated in response to antigen presentation by antigen-presenting cells (APC), depicted here as APC activated by lymphocytic choriomeningitis virus (LCMV), resulting in clonal expansion and cytokine production, the latter of which results in activation of other innate and adaptive immune cells. The cytotoxic activity of CD8 T cells leads to elimination of LCMV-infected APC, resulting in a return to immune homeostasis. In contrast, in patients with primary hemophagocytic lymphohistiocytosis (bottom), CD8 T cells become appropriately activated in response to APC but are unable to effectively eliminate LCMV-infected APC owing to their impaired cytotoxic activity. As a result, there is persistent CD8 T-cell activation and cytokine secretion that drive a feed-forward loop perpetuating immune cell proliferation and cytokine secretion, with subsequent tissue damage. IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; HLH: hemophagocytic lymphohistiocytosis; pHLH: primary HLH.

resembling macrophage activation syndrome, a subtype of sHLH. As is the case with LCMV-infected *Prf1*$^{-/-}$ mice, disease is dependent upon IFNγ but, surprisingly, does not require CD8 T cells. These data reveal that TLR9-induced activation of innate immune cells is sufficient to initiate inflammation. Co-administration of an interleukin (IL)-10 receptor blocking antibody or transgenic expression of IL-18 exacerbates inflammation,[9] revealing important negative and positive regulatory roles, respectively, for these cytokines in driving TLR9-associated hyperinflammation.

By highlighting the cell types and cytokines central to disease, animal models have also provided opportunities to test novel therapies, particularly those aimed at blocking cytokine activity. To this end, antibody-mediated neutralization of IFNγ can reverse anemia and prolong survival in LCMV-induced pHLH[5] and TLR9-induced sHLH.[10] Similarly, the Janus kinase (JAK) inhibitor ruxolitinib, which blocks signaling downstream of IFNγ and several other HLH-associated cytokines, significantly limits inflammation and ameliorates survival in both HLH models.[11] Notably, ruxolitinib inhibits signaling induced by IL-2, a molecule that upregulates expression of pro-survival proteins in activated CD8 T cells. In so doing, ruxolitinib inhibits the expression of these proteins, enhances the sensitivity of activated T cells to dexamethasone-induced cell death, and works cooperatively with dexamethasone to curtail inflammation in LCMV-infected *Prf1*$^{-/-}$ mice.[12] Collectively, these and other mouse models have been instrumental in evaluating the efficacy of numerous cytokine-directed therapies as well as gene correction approaches in HLH.[3,4]

Advances in the clinical management of patients with hemophagocytic lymphohistiocytosis

The first international prospective clinical trial for pediatric pHLH was the Histiocyte Society HLH-94 protocol, which evaluated the use of dexamethasone and etoposide as an 8-week induction therapy. Patients could then receive optional continuation therapy with dexamethasone, etoposide, and cyclosporine as a bridge to hematopoietic cell transplantation (HCT). This trial resulted in a substantial improvement in the estimated 5-year overall survival from less than 10% in the 1980s to 54%.[13] A subsequent trial, HLH-2004, incorporated cyclosporine during the 8-week induction.[14] This modification did not significantly improve

survival and possibly increased toxicity.[2] Thus, HLH-94 remains the standard of care for pHLH. If cyclosporine is used to control inflammation, care should be given not to incorporate this medication before week 3 of induction. Furthermore, lower trough levels (120-150 μg/L) should be maintained. Together, these maneuvers may lessen the risk of HLH reactivation and cyclosporine-associated toxicity.[15] Most recently, a large international retrospective survey reported an impressive 3-year overall survival of 77% for patients with pHLH receiving etoposide-based front-line regimens.[16] Although not a standardized clinical trial, this report further emphasized the efficacy of etoposide as a gold-standard treatment for pHLH. Etoposide-based regimens have also been reported as effective in controlling inflammation for patients with sHLH including those with Epstein-Barr virus (EBV)-associated HLH[17,18] as well as HLH occurring in the context of rheumatological disorders.[19]

The enrollment criteria used in HLH-2004 have been widely adopted as diagnostic criteria for HLH (Table 1).[14] Recenty, Henter *et al*. conducted a study to further validate these criteria for diagnosing HLH.[20] The criteria demonstrated high sensitivity and specificity for distinguishing pHLH from other inflammatory diseases, although most cases of sHLH were specifically excluded. They also concluded that NK functional studies could be excluded from the HLH-2004 criteria. Accordingly, these criteria remain the gold standard for diagnosing pediatric HLH. Another widely used diagnostic tool, used primarily in adult patients, is the HScore (Table 1), which was developed and validated in a population of adults with sHLH due to infection and/or malignancy.[21] In a recent multicenter validation study, both the HLH-2004 criteria (with fulfillment of 4 of 8 cri-

Table 1. Diagnostic schema for hemophagocytic lymphohistiocytosis.

HLH-2004 Criteria	HScore
The diagnosis of HLH requires at least one of the two following criteria: • A molecular diagnosis consistent with pHLH • At least 5 of the following: ○ Fever ≥38.5˚C ○ Splenomegaly ≥2 cm below the costal margin ○ Cytopenias affecting at least two lineages: ❐ Hemoglobin <90 g/L ❐ Platelets <100x10^9/L ❐ Neutrophils <1.0x10^9/L ○ Hypertriglyceridemia and/or hypofibrinogenemia ❐ Fasting triglycerides ≥3.0 mmol/L ❐ Fibrinogen ≤1.5 g/L ○ Hemophagocytosis ○ Ferritin ≥500 μg/L ○ sIL-2Rα ≥2400 U/mL ○ Low or no natural killer cell activity*	• Known underlying immunosuppression ○ No: 0 points ○ Yes: 18 points • Temperature (˚C) ○ <38.4: 0 points ○ 38.4-39.4: 33 points ○ >39.4: 49 points • Organomegaly ○ None: 0 points ○ Hepatomegaly or splenomegaly: 23 points ○ Hepatomegaly and splenomegaly: 38 points • Number of cytopenias ○ One lineage: 0 points ○ Two lineages: 24 points ○ Three lineages: 34 points • Ferritin (ng/mL) ○ <2,000: 0 points ○ 2,000-6,000: 35 points ○ >6,000: 50 points • Triglyceride (mmol/L) ○ <1.5: 0 points ○ 1.5-4: 44 points ○ >4: 64 points • Fibrinogen (g/L) ○ >2.5: 0 points ○ ≤2.5: 30 points • Aspartate aminotransferase (IU/L) ○ <30: 0 points ○ ≥30: 19 points • Hemophagocytosis ○ No: 0 points ○ Yes: 35 points

According to the HLH-2004 criteria (left), patients can be diagnosed with hemophagocytic lymphohistiocytosis (HLH) if they have a molecular diagnosis consistent with HLH or if they meet at least five of eight clinical, laboratory, and histopathologic criteria.[14] The HScore consists of nine variables with points assigned to various values of those variables. The point total in turn corresponds to a probability of having HLH.[21] *Of note, a recent retrospective study recommended exclusion of natural killer-cell functional studies from the HLH-2004 criteria. pHLH: primary HLH; sIL-2Rα: soluble interleukin-2 receptor alpha.

teria) and the HScore (with a cut off of 169) performed well in distinguishing sHLH from other hyperinflammatory disorders in critically ill patients.[22]

Recent therapeutic advances have focused on cytokine-directed therapies and response-adapted approaches, allowing for individualized treatment tailored to disease biology, severity, and therapeutic response. For example, the humanized anti-IFNγ monoclonal antibody emapalumab, when combined with dexamethasone, demonstrated efficacy in children with relapsed or refractory HLH with an estimated 12-month survival of 73.4%.[23] While data on the use of emapalumab in adults with HLH were lacking at the time, in 2018 emapalumab received approval from the Food and Drug Administration (but not the European Medicines Agency) for children and adults with relapsed or refractory pHLH or intolerance of conventional therapies. Subsequent data on its use in adults remain sparse. Ruxolitinib has also shown promise in multiple case reports and clinical trials.[24,25] Finally, anakinra, an IL-1 receptor antagonist, has demonstrated efficacy in sHLH, particularly in patients who fail conventional regimens.[26] These advancements signify a paradigm shift in the treatment of HLH to include more rational and potentially less toxic therapies.

Finally, the past decades have seen tremendous advances surrounding HCT for patients with pHLH, which remains the only known curative therapy. Fortunately, many patients with pHLH can successfully proceed to HCT. To this end, pre-HCT survival was reported to be 89% at 2 months in the HLH-2004 study with 80% of patients undergoing HCT.[2] Furthermore, in the recent international retrospective study, 91% proceeded to HCT.[16] Studies evaluating reduced intensity conditioning regimens have demonstrated decreased rates of transplant-related toxicity and mortality, although outcomes are mixed regarding graft failure.[27-30] An alternative approach involves the use of reduced toxicity conditioning, which aims to lessen the adverse effects of traditional myeloablative conditioning while also reducing the risk of mixed chimerism and graft failure that can be seen using approaches based on reducing intensity conditioning.[27,29,31] Additional research is needed to identify the optimal conditioning regimen for patients with pHLH.

Future directions for research

Genetics of adult hemophagocytic lymphohistiocytosis

Much of our current understanding of genetic predisposition to HLH has come from the study of children with pHLH, in whom bi-allelic loss-of-function mutations in a pHLH gene result in disease manifestations.[1] More recently, germline genetic alterations resulting in pathological inflammasome activation or T-cell dysregulation have also been implicated in the development of pediatric HLH.[32] Despite these advances, the role of genetics in adults with HLH remains largely unknown. There are rare reports of pHLH presenting in adolescence or adulthood, however, the phenotypic spectrum of pHLH in adults remains to be defined.[33,34] Multiple retrospective studies have evaluated for pHLH gene variants in adult HLH cohorts.[35-38] Although classic autosomal recessive cases appear to be rare, heterozygous germline variants, primarily missense and splice-site variants, in HLH-associated genes have been identified with frequencies as high as 40%.[36] Results of variant filtering approaches vary, but these heterozygous variants appear to associate with disease severity and outcome, raising suspicion of a potential role in disease pathogenesis. However, conflicting studies also exist,[38] and similar variants are found in control cohorts at rates as high as 9%.[35] Due to their retrospective nature, these cohorts are prone to confounding factors and bias, and functional evaluation of genetic variants has not been consistently performed. Parsing the true impact of these genetic findings is challenging, especially for hypomorphic variants, such as *PRF1* p.A91V, which is present at a relatively high frequency in the general population but whose impact on protein function remains unclear.[33] Nevertheless, partial genetic defects in pHLH genes may be important contributors to the development of sHLH across multiple inflammatory contexts.[4]

In addition to germline changes, recent studies have implicated a potential role for somatic variants as contributors to HLH risk. Somatic clones involving classic clonal hematopoiesis (CH) genes have been found at greater frequencies in adults with HLH. This is not entirely unexpected as inflammatory disease can drive the production of these clones. However, CH by itself can contribute to immune dysregulation, and mouse models suggest that the presence of CH can influence HLH pathogenesis.[39] Additionally, somatic variants in *FAS* have been found at higher rates in patients with malignancy-associated HLH (mHLH)[40] and somatic variants in *NLRC4* have been associated with autoinflammatory diseases.[41] Further evaluation is needed to understand the contribution of somatic variants to HLH risk in adults.

Remaining questions to be addressed

Overall, there is a substantial need to further elucidate the genetic basis of adult HLH. To do so, it will be essential to recruit broad, prospective, longitudinal cohorts of adults with HLH and conduct comprehensive genomic testing. Analysis of such well-annotated cohorts will enable evaluation of the role of genetic predisposition in driving adult HLH and the role of genetics in influencing overall outcomes.

Mechanisms of immune dysregulation

The pathogenesis of pHLH is well-described, with cytotoxicity defects driving persistent T-cell activation and production of IFNγ and several other proinflammatory cytokines.[1]

Related proteomic profiles have been identified in pediatric patients with sHLH, including those with inflammation triggered by EBV, and in patients with sHLH occurring in the context of systemic juvenile idiopathic arthritis and adult-onset Still's disease.[1,42,43] Although studies are largely lacking for adult HLH, proteomic evaluations of adults with mHLH have identified a similar IFNγ signature.[44] IL-18 also plays a key role in the pathogenesis of HLH, especially in patients with gain-of-function variants in *NLRC4* or with *XIAP* deficiency.[45,46]

Immunophenotyping studies in pHLH have identified a distinct hyper-activated $CD38^{hi}/HLADR^{+}$ effector T-cell population.[47,48] These pathological T cells are usually oligoclonal but may not be pathogen-specific.[49,50] Analagous activated T cells are not found in sepsis, and this may have clinical utility in differentiating HLH from sepsis.[47] Studies of children with systemic juvenile idiopathic arthritis have revealed that the $CD38^{hi}/HLADR^{+}$ T-cell phenotype is specific to sHLH episodes and not flares of the systemic arthritis.[51] Although these hyperactivated T cells have now been described in other contexts, their level is markedly lower than in HLH.[52,53] Additionally, the levels of $CD38^{hi}/HLADR^{+}$ T cells correlate with CXCL9 and soluble IL-2 receptor alpha (sIL-2Rα) levels, known markers of disease activity in pHLH. While hyperactivated cytotoxic T cells are clearly linked to the pathogenesis of pHLH, these observations further support their role in HLH more broadly.[48]

Although data are limited in adults, phenotyping studies have also identified $CD38^{hi}/HLADR^{+}$ T cells in adult sHLH. These T cells demonstrate increased cycling and, in severe HLH, there are distinct hyperactivated T cells that downregulate CD3.[54] Interestingly, in patients with human immunodeficiency virus-associated inflammatory syndromes, where many patients meet HLH criteria, comparable pathways are activated, with expansion of hyperactivated T cells producing IFNγ and pathological inflammasome activation with increased IL-18.[55,56] These findings further emphasize the potentially convergent pathophysiological mechanisms of pHLH and other HLH-like inflammatory syndromes and warrant broad evaluation for markers that drive severe and persistent disease.

Remaining questions to be addressed

Future studies elucidating HLH pathophysiology are vital and should capitalize on mouse models and well-annotated clinical samples to comprehensively interrogate the mechanisms driving persistent hyperinflammation. In sHLH, substantial work is required to define the immunological spectrum of disease across different etiologies and to determine the extent of overlapping mechanisms with pHLH. Expanding our understanding of HLH pathophysiology will allow for identification of key prognostic factors to differentiate mild from more severe forms of disease and to identify novel signaling pathways that can be targeted therapeutically.

Malignancy-associated hemophagocytic lymphohistiocytosis

Malignancy, together with infection, are the two most common triggers for HLH in adults.[57,58] HLH can occur as the initial presentation of a new malignancy or can arise in the setting of chemotherapy.[59,60] Among patients with mHLH occurring at diagnosis, hematologic malignancies are by far the most common trigger, with T- and NK-cell lymphomas and mature B-cell lymphomas being responsible for the majority of cases.[59] HLH arising during chemotherapy has been reported in patients with a variety of cancer diagnoses, including myeloid malignancies.[61-63] mHLH carries a particularly poor prognosis, with the median overall survival being significantly shorter than that in patients with sHLH arising from other etiologies, primarily due to the aggressive nature of the underlying malignancies and associated challenges related to delivering full-dose chemotherapy to patients with cytopenias and/or organ dysfunction.[64-66] In adults, the 5-year overall survival rate is approximately 15%,[67] while survival in children is significantly higher.[61]

While mHLH shares many clinical features with pHLH, the extent of pathophysiological overlap remains incompletely understood. Several mechanisms have been proposed.[68] In patients with malignancy as the trigger for HLH, it has been hypothesized that tumor cells mediate persistent antigen presentation and cytokine secretion, driving hyperinflammation. It has also been proposed that host factors may predispose to the development of the underlying malignancy and to HLH. For example, heterozygous germline variants in pHLH genes have been identified in many patients with hematologic malignancies both with and without concomitant HLH.[69-72] These findings support the data from mouse models demonstrating that wild-type mice eliminate tumors more effectively than $Prf1^{-/-}$ mice,[73] suggesting that intact cytotoxicity is required for optimal immune surveillance against the development of malignancy and for controlling hyperinflammation. As above, acquired somatic variants in the form of CH may similarly contribute to the risk of both hematologic malignancies and mHLH.[39] Finally, it has been proposed that infectious triggers may play a role in the development of mHLH. For example, many mHLH-associated lymphomas are driven by EBV,[74] and it remains unclear the extent to which EBV itself *versus* the associated malignancy contributes to hyperinflammation. The role of infections in mHLH may also be particularly relevant in patients who develop HLH while receiving cancer-directed therapy, a time during which they are immunosuppressed secondary to both the malignancy and chemotherapy.[68] Many patients with HLH arising during therapy have a concomitant infection,[62,71] again making it challenging to attribute hyperinflammation to the infection *versus* the underlying malignancy, but most likely both conditions contribute according to the threshold model described by Brisse *et al.*[4]

There is also little consensus regarding the optimal diag-

nostic approach for patients with mHLH. While the HLH-2004 diagnostic criteria are commonly applied, they were not developed for use in patients with malignancy and their utility in this population has not been rigorously studied. Furthermore, many of the signs and symptoms associated with HLH are common features of hematologic malignancies, making it challenging to identify patients with pathological hyperinflammation.[60] Application of the HLH-2004 criteria does not readily facilitate identification of the underlying disease etiology, which can contribute to delaying the diagnosis of the malignancy in patients meeting HLH diagnostic criteria and can in turn delay treatment initiation.[71] These factors highlight the importance of developing better tools for diagnosing and predicting outcomes in mHLH. One recently reported tool is the optimized hyperinflammatory index (OHI), a model that considers combined elevation of ferritin and sIL-2Rα, which was predictive of mortality in this population, in which those with negative OHI scores (including those who fulfilled criteria for HLH) fared significantly better than those with positive scores.[75] Another study identified 18 variables described in the literature that are closely associated with HLH and determined that patients with at least five of the 18 variables have a high likelihood of having mHLH.[76] Finally, Yang *et al*. proposed a model to enhance the utility of ^{18}F-fluorodeoxyglucose positron emission tomography in identifying malignancies in patients with HLH, in whom inflammation can result in a high false-positive rate.[77] These studies represent important advances in diagnostic tools for patients with mHLH, but additional prospective studies are needed for the development of rigorous diagnostic tools.

Remaining questions to be addressed

Our limited understanding of disease pathophysiology and ongoing diagnostic challenges have impeded the establishment of clear treatment recommendations for mHLH, as well as other forms of HLH in adults. To date, there are limited investigations focusing on adults with HLH – a factor that significantly hampers progress in this area. Accordingly, there is a compelling need for more comprehensive investigations in this domain. For example, it currently remains unknown how best to combine HLH-directed therapy with cancer-directed therapy. Some guidelines suggest prioritization of HLH-directed therapy in patients with severe hyperinflammation,[78] as it has been shown that some patients die of aggressive HLH while receiving cancer-directed therapy alone.[68] However, some studies suggest that outcomes are superior in patients who receive therapy targeting hyperinflammation and the malignancy simultaneously.[79] When HLH-directed therapy is given, current treatment strategies are based largely on pediatric protocols,[14] and there have not been any completed large-scale, prospective clinical trials in adults. This is particularly problematic for mHLH, which disproportionately impacts adults. It also remains unclear how to optimally integrate anti-cytokine therapies into the management of mHLH and when to utilize HCT, independently of malignancy-associated indications.[80] Ongoing research into disease pathophysiology will facilitate accurate identification of patients with mHLH and guide the development of novel treatment strategies for evaluation in prospective clinical trials.

Immune effector cell hemophagocytic lymphohistiocytosis-like syndrome

Immune effector cell hemophagocytic lymphohistiocytosis-like syndrome (IEC-HS) is a life-threatening hyperinflammatory syndrome associated with chimeric antigen receptor (CAR) T-cell therapy. It shares pathophysiological overlap with cytokine release syndrome (CRS) and HLH, but seems distinct in its mechanisms, clinical course, and therapeutic implications.[81] The syndrome often follows CRS abatement, and although IEC-HS shares key cytokine signatures with pHLH and sHLH,[81-83] its pathogenesis remains incompletely understood. Whereas CRS is the initial cytokine burst upon T-cell activation, IEC-HS represents a "second wave" of immune persistence and amplification in which recruitment of additional immune effectors fuels a self-perpetuating cytokine storm.[84,85]

There is growing recognition that IEC-HS can emerge from severe CRS, in which the inflammatory response is refractory to IL-6 blockade. This raises the question of whether IEC-HS represents a distinct entity or an extreme phenotype of CRS, with some studies implicating the heightened role of IL-10 and IL-18 in distinguishing IEC-HS from severe CRS.[86,87] This distinction is clinically relevant since management of IEC-HS differs therapeutically from that of severe CRS in its refractoriness to targeted anti-cytokine therapies, requiring treatment mirroring that of HLH with lymphocytotoxic agents or with therapies targeting multiple cytokines, such as ruxolitinib and emapalumab.[88,89] These strategies aim to suppress the broader inflammatory network driving IEC-HS, which cannot be adequately controlled by blockade of single cytokines.

Additionally, persistent immune activation in IEC-HS raises concerns regarding the functional integrity of CAR T cells. Prolonged cytokine signaling may lead to T-cell exhaustion, impairing their persistence and cytolytic function against malignant cells. Some studies suggest that sustained CAR T-cell expansion, a characteristic of IEC-HS, may not always correlate with therapeutic efficacy but instead contribute to toxicity.[86]

To mitigate life-threatening IEC-HS related to CAR T-cell therapies, numerous strategies have been developed to allow for selective attenuation or elimination of hyperactive CAR T-cell responses. Among these, suicide gene systems have been investigated as a means to rapidly curtail adverse events using an externally administered activating agent. One example is the inducible caspase 9 system, which incorporates a modified caspase-9 protein in T cells that is activated upon administration of a

small molecule chemical inducer of dimerization, triggering apoptosis. A first-in-human clinical trial evaluating this approach demonstrated a 90% reduction in transgenic T cells within 30 minutes, effectively mitigating toxicity.[90] An alternative strategy involves engineering CAR T cells to express targetable surface markers, such as truncated epidermal growth factor receptor or CD20. These markers can be selectively targeted using monoclonal antibodies such as cetuximab or rituximab, respectively, providing an additional layer of control to mitigate hyperinflammatory states.[91,92] More advanced approaches involve CAR T cells with microenvironment-responsive regulatory elements. While CAR T cells inherently lack control over cytokine profiles generated during activation, SynNotch receptors offer a solution by enabling customized, context-dependent cytokine expression. SynNotch receptors contain the regulatory domain from the Notch receptor coupled with synthetic extracellular recognition domains and intracellular transcriptional domains. When engaged by a cognate antigen, the Notch receptor undergoes cleavage, releasing the intracellular transcriptional domain to enter the nucleus and induce target gene expression. In this way, they can be programmed to deliver therapeutic payloads locally rather than provoking systemic inflammation.[93] Other CAR use biological targeting systems that require multiple antigens for activation. For example, AND-gate CAR are activated only when two or more antigens are simultaneously detected, minimizing off-target effects and reducing the likelihood of systemic hyperinflammation.[94]

Remaining questions to be addressed

Overall, there is a need to understand the mechanisms driving IEC-HS better and how to target them effectively. Beyond cellular engineering, pharmacological and dosing strategies may mitigate the risk of severe hyperinflammation. Step-up dosing regimens, in which IEC therapies are administered in smaller, fractionated doses with close monitoring for adverse effects,[95] is already employed for all Food and Drug Administration-approved bispecific antibodies across hematologic and solid malignancies.[96] This enables gradual immune activation with the goal of reducing toxicity while maintaining efficacy, which preliminarily appears to be the case for the first approved CAR T-cell employing this strategy.[97] Collectively, these strategies represent a growing arsenal of tools aimed at enhancing the safety and precision of CAR T-cell therapies.

Anti-cytokine therapies

While traditional HLH therapy relies on lymphocyte depletion and macrophage suppression, the recognition that specific cytokines mediate disease manifestations has led to the development of strategies aimed at neutralizing these inflammatory mediators (Figure 3). Optimal integration of these therapies remains a subject of debate, particularly in determining their role in frontline *versus* relapsed or refractory settings, defining their efficacy across different HLH etiologies, and refining cytokine profiling approaches to guide therapy selection.

Early integration of anti-cytokine therapies has the potential to improve outcomes while reducing the toxicity of broad immunosuppressive agents. The rationale for IFNγ blockade in HLH is based on the role of IFNγ as a macrophage-activating signal, sustaining the hyperinflammatory state by amplifying phagocytosis, cytokine production, and hemophagocytosis. In pHLH, in which cytotoxic lymphocyte defects impair antigen clearance, uncontrolled IFNγ production becomes a self-reinforcing loop that drives disease progression.[5] The ability of emapalumab to suppress this pathway while preserving broader immune function distinguishes it from conventional cytotoxic therapies, raising the question of whether earlier initiation of IFNγ blockade could supplant etoposide in certain patients, particularly those with contraindications to myelosuppressive chemotherapy. Real-world data are emerging to support its use across many HLH triggers,[98-100] although prospective trials as a front-line agent for children with pHLH and comparative trials against HLH-94-based regimens remain an unmet need.

Both pHLH and sHLH are associated with cytokine profiles typified by elevations in cytokines such as IFNγ, IL-6, and other JAK-dependent cytokines. Given this inflammatory profile, JAK inhibition with ruxolitinib has emerged as a promising strategy in HLH. Preclinical models of pHLH demonstrate that ruxolitinib-mediated JAK inhibition reduces the inflammatory transcriptional signature of T cells and macrophages[101,102] and sensitizes CD8 T cells to steroid-induced apoptosis.[12] In clinical applications, ruxolitinib has been used in the treatment of multiple forms of HLH with positive results.[24,25,101] Given the reliance of cytotoxic T cells on JAK-STAT signaling, one concern is whether JAK inhibitors might exert immunomodulatory effects at the expense of antitumor activity, a concern requiring further investigation in mHLH and IEC-HS.

Infection-associated HLH presents another setting in which cytokine-directed therapy may provide benefit while avoiding the risks of profound immunosuppression. Viral HLH, particularly EBV-associated disease, can sometimes lead to malignant transformation into aggressive lymphoproliferative disorders. Treatment with rituximab to eliminate B cells, the reservoir for EBV, has proven benefical in certain cases.[103] While IFNγ blockade theoretically holds promise in this setting, concerns remain regarding whether suppressing IFNγ might impair viral clearance, increasing the risk of persistent or recrudescent infection, though these concerns have been somewhat allayed by a demonstrated improvement in HLH symptoms and viral load following ruxolitinib treatment.[25] This has led some investigators to consider IL-1 blockade as another adjunctive therapy, particularly given that IL-1 plays a major role in inflammasome activation.[104,105] Anakinra has been used in sHLH

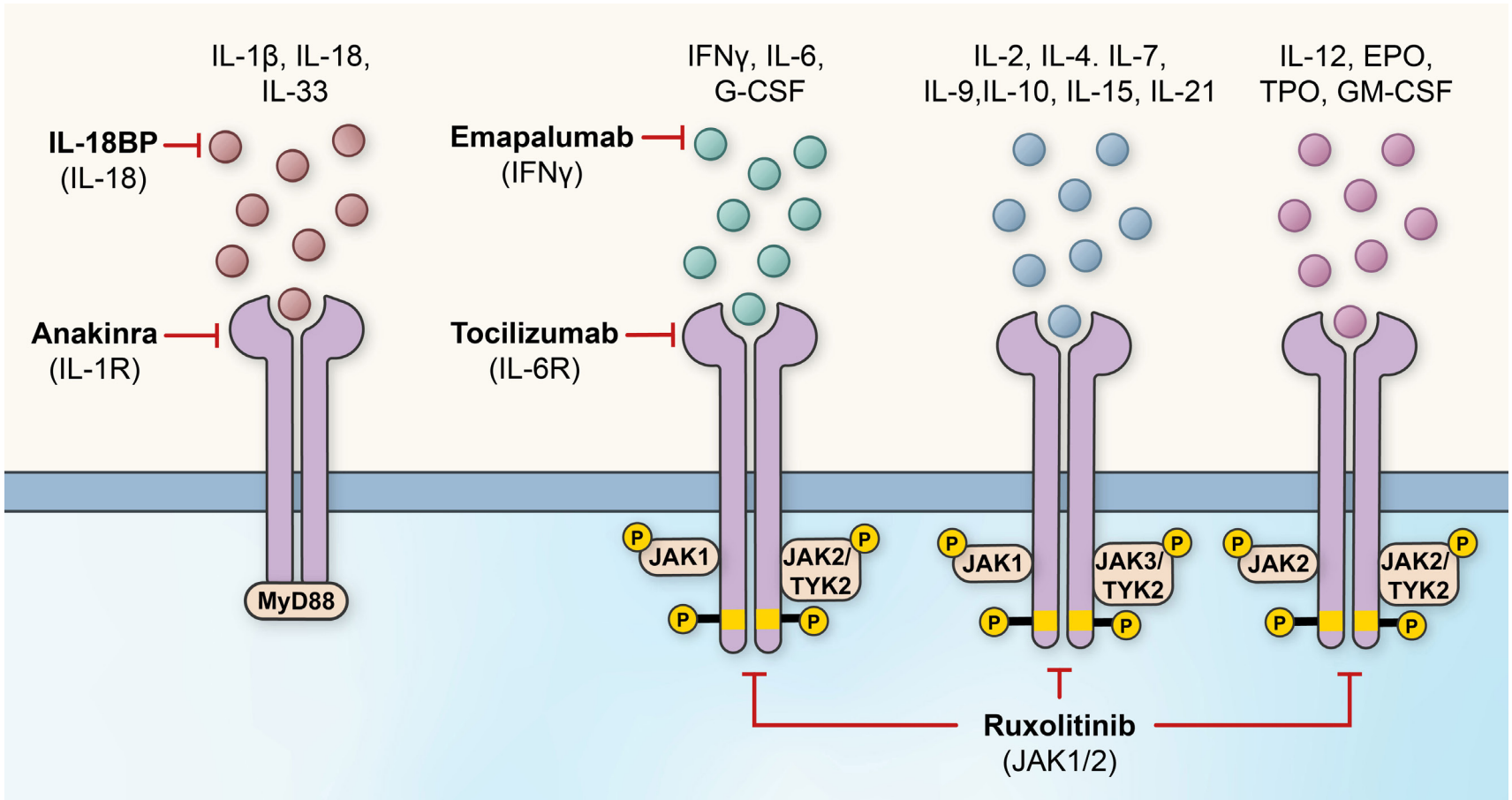

Figure 3. Cytokine receptor signaling and anti-cytokine therapies. Multiple inflammatory cytokines activate common downstream signal transduction pathways mediated by MyD88 or the Janus kinases. Targeted inhibitors have been developed that inhibit the activity of individual cytokines, their cell surface receptors, or downstream signal transduction. IL-18BP: interleukin 18 binding protein; IL-1R: interleukin 1 receptor; MyD88: myeloid differentiation primary response 88; IL: interleukin; IFN: interferon; IL-6R: interleukin 6 receptor; G-CSF: granulocyte colony-stimulating factor; EPO: erythropoietin; TPO: thrombopoietin; GM-CSF: granulocyte-monocyte colony-stimulating factor; JAK: Janus kinase; TYK: tyrosine kinase.

and macrophage activation syndrome with favorable safety profiles, particularly in patients at high risk of infection. These retrospective and small prospective studies suggest efficacy in suppressing hyperinflammation across various HLH etiologies,[106-108] though its role relative to IFNγ inhibition, JAK-STAT blockade, or even cytotoxic chemotherapy remains uncertain.

The ability to use cytokine profiling to guide therapeutic decision-making represents a major opportunity in HLH treatment, as cytokine signatures differ by etiology and may predict response to targeted interventions. IFNγ and its downstream chemokine CXCL9 are particularly elevated in pHLH, making them potential biomarkers for emapalumab responsiveness.[109] Notably, IL-18 is markedly increased in macrophage activation syndrome, suggesting IL-18 inhibition as a potential therapeutic avenue in sHLH associated with rheumatological disease.[110] Prospective validation of these biomarkers remains a critical need, as the ability to stratify patients based on cytokine signatures could allow for precision-targeted interventions rather than empiric treatment escalation.

Remaining questions to be addressed

While biomarkers of response to anti-cytokine therapies are beginning to emerge, defining resistance mechanisms remains a greater challenge. Many patients treated with IFNγ blockade fail to achieve adequate disease control, suggesting either redundancy in inflammatory pathways or compensatory cytokine upregulation. Persistent elevation of sIL-2Rα despite IFNγ inhibition may indicate ongoing T-cell activation driving disease pathology, raising the question of whether dual IFNγ and JAK-STAT inhibition may be required. Similarly, patients receiving IL-1 blockade may show persistent elevations in IL-18, suggesting an alternative inflammatory axis driving disease in macrophage activation syndrome. Future directions will likely focus on combination regimens that target multiple inflammatory mediators while minimizing toxicity. Additionally, efforts to develop real-time cytokine monitoring strategies could allow dynamic therapeutic adjustments based on evolving inflammatory profiles. As the field advances, the challenge will be to integrate cytokine-directed therapies into HLH management in a way that maximizes efficacy while minimizing the long-term risks of immune suppression.

Gene therapy

Currently, HCT is the only available curative therapy for patients with pHLH.[111] However, there has been considerable interest in the development of gene therapy strategies, which have been widely investigated for other inborn errors of immunity. Gene therapy strategies can be subdivided into approaches that replace the entire defective

gene and those that edit the deleterious mutation in the native gene.[112] Gene replacement involves the use of γ-retroviral or lentiviral vectors carrying the gene of interest. These vectors permanently and semi-randomly integrate into the host cell genome and are carried forward to host cell progeny, enabling expression of the therapeutic gene sequence. In contrast, gene editing involves programmable nucleases that catalyze double-strand DNA breaks at specific target sequences. This recruits DNA damage repair machinery, which, when provided with a homologous DNA template carrying the desired gene sequence, repairs the break via high fidelity homology-directed repair, replacing the native sequence with the sequence encoded by the provided template.[112] These nuclease technologies include zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems,[112] the last of which have already shown considerable clinical promise in patients with hemoglobinopathies.[113]

Many proof-of-concept studies have effectively modified CD8 T cells or hematopoietic stem cells (HSC) from animal models or from patients with pHLH, demonstrating the potential efficacy of gene therapy in this disease. Ghosh *et al*. used a retroviral vector system to express wild-type *Prf1* in CD8 T cells from *Prf1*$^{-/-}$ knockout mice. When compared to untransduced CD8 T cells, these transduced cells provided complete protection from HLH.[114] Li *et al*. subsequently used CRISPR/Cas9 to edit CD8 T cells from *Prf1*$^{-/-}$ mice, fully ameliorating hyperinflammation *in vivo*. The authors went on to apply this approach to memory T cells from two patients with pHLH and demonstrated complete restoration of *in vitro* cytotoxic function.[115] It has also been shown that introduction of wild-type *Prf1* into HSC from *Prf1*$^{-/-}$ mice leads to perforin protein expression in mature T and NK cells with recovery of cytotoxicity and attenuation of hyperinflammation *in vivo*.[116] Similar studies have been performed in the context of patients with loss-of-function variants in *UNC13D* and the related HLH predisposition syndrome X-linked lymphoproliferative disease, mediated by defects in the *SH2D1A* or *XIAP* genes. In these studies, the use of viral vectors or nuclease technology to restore gene expression in CD8 T cells or HSC resulted in rescue of cellular function and protection against hyperinflammation.[117-121]

Gene therapy in pHLH carries the potential to overcome many of the limitations of HCT. Foremost, gene therapy does not require identification of a donor, which can be limiting in patients from certain ethnic groups.[122] The use of autologous cells eliminates the need for post-transplant immunosuppression and the risk of development of graft-*versus*-host disease, which contributes to suboptimal post-HCT survival.[111] Furthermore, it has been shown that correction of the cytotoxicity defect in only a minority of cells can overcome the pHLH phenotype. Specifically, in the *Prf1*$^{-/-}$ model, restoration of perforin expression in as few as 10% of CD8 T cells or HSC is sufficient to restore functional immune regulation.[123] Comparably, in a retrospective cohort of patients who underwent HCT for pHLH, post-HCT donor chimerism as low as 20% protected against disease reactivation.[124] These studies suggest that strategies aimed at correcting even a small percentage of immune cells may have significant clinical benefit. These preclinical data demonstrating the efficacy of adoptive T-cell transfer in murine models of pHLH[123,125] also suggest that such a strategy may be efficacious in humans for mitigating hyperinflammation as a bridge to HCT or transfer of edited HSC, as uncontrolled hyperinflammation remains a significant barrier to successful HCT in patients with pHLH.[2]

Remaining questions to be addressed

Despite the progress in this area, multiple questions and limitations remain, with safety being a primary concern. While viral vectors have shown clinical efficacy,[112] they carry the potential to induce oncogenesis. For example, there have been reports of patients receiving viral gene therapy and subsequently developing hematologic malignancies secondary to insertion of the viral vector into a proto-oncogene.[126] Since these reports, there have been efforts to develop safer viral vectors to decrease the risk of mutagenesis events,[127,128] although the long-term safety of these strategies requires further investigation. While nuclease technologies may be associated with a lower risk of oncogenesis, they still carry the risk of off-target cutting, which has the potential to disrupt gene expression and promote transformation.[112] Although studies in mice and humans have demonstrated that low levels of donor chimerism are adequate to attenuate hyperinflammation,[123,124] it also remains to be seen whether existing gene therapy strategies can achieve adequate efficiency in a clinical context. It is known that active inflammation can make HSC less sensitive to viral transduction,[129] potentially impairing the efficiency of gene replacement in cells collected from patients with active HLH. Both viral gene replacement and gene editing require multiple steps that can significantly diminish cell viability,[112] which may make it challenging to obtain an adequate number of edited cells. Finally, unlike many other monogenic diseases, pHLH is characterized by significant genetic heterogeneity, with few recurring mutations across patient cohorts.[32] This may necessitate a high degree of personalization and, given the rarity of pHLH, this may pose considerable financial and regulatory challenges.[129]

Looking to the future

Looking back, it is encouraging to see how far the field has come over the last two decades. Prior to 2000, the diagnosis of HLH relied on basic clinical and laboratory investigations and its treatment was based exclusively on cytotoxic and

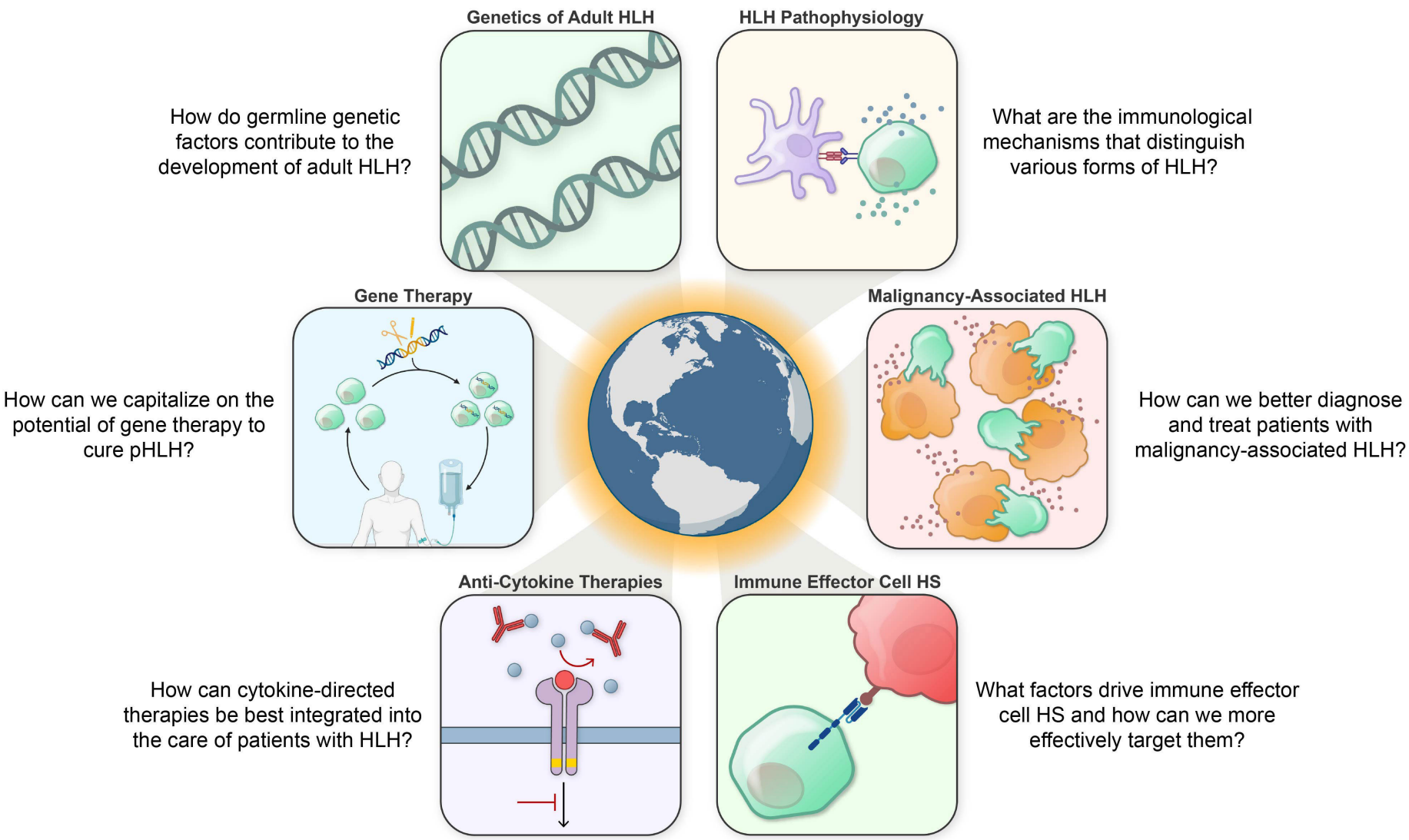

Figure 4. Blueprint for research. Future research will focus on addressing these six key thematic areas, which include establishing a deeper understanding of underlying disease pathophysiology across a diverse spectrum of etiologies, refining diagnostic tools to enable more prompt and accurate disease diagnosis, and developing precise, rationally-designed, and less toxic therapies. These efforts will require the coordinated activities of investigators and institutions from across the world, with guidance and infrastructure provided via local, national, and international cooperative groups, including the Histiocyte Society. HLH: hemophagocytic lymphohistiocytosis; pHLH: primary HLH; HS: HLH-like syndrome

immunosuppressive agents.[13] However, with the discovery of the pHLH genes, our ability to rapidly diagnose and treat HLH has evolved, in turn facilitating numerous avenues of basic, translational, and clinical research aimed at understanding the full spectrum of this heterogeneous disease. Building on these advances, there remain multiple opportunities to further advance the field.

Further understanding disease pathophysiology

As detailed above, major questions remain that are related to disease pathophysiology, including expanding our understanding of genetic predisposition to HLH and the similarities and differences in disease mechansism across diverse etiologies. A deeper understanding of disease pathophysiology will facilitate the development of more precise diagnostic tools and aid in identifying unique therapeutic vulnerabilities of each HLH subtype, paving the way for identification of novel treatment strategies and the development of prospective clinical trials to evaluate these strategies. In addition, the field as a whole now faces many challenges in disseminating the outcome of a research on a global scale, to ensure widespread availability of critical diagnostic tools and therapeutic modalities (Figure 4).

Carrying out clinical trials in hemophagocytic lymphohistiocytosis

Given the heterogeneity of HLH and the financial and regulatory complexities of running clinical trials, what should prospective HLH investigations look like and how should cooperative groups be involved in their development and execution? Prospective clinical trials are essential to systematically study candidate therapeutic agents and push the boundaries of cure for patients with HLH. Because HLH is rare, these trials must involve collaborations between multiple investigators and institutions. It is currently extremely difficult, if not impossible, to run large international trials involving hundreds of patients as was done in prior years. Therefore, future trials must rely on innovative designs that enable statistically sound outcomes derived from inclusion of fewer patients. Trials should incorporate standardized definitions of disease response and outcome to facilitate comparison between studies. Furthermore, trials should

include the collection of serial biospecimens to support research investigations aimed at enhancing our understanding of HLH biology and identifying biomarkers indicative of disease severity and response. Finally, HLH-directed therapies are associated with adverse sequelae that can have long-term impacts on quality of life. Thus, it will be important to include input from patients and their families in the design of clinical trials and collect patient-reported outcomes.

Increasing access to expertise and treatment of hemophagocytic lymphohistiocytosis on a global scale

Many patients with HLH around the world do not have access to centers with HLH expertise, including the technologies needed to perform specialized diagnostic testing and the latest therapies. Collaboration will be required to address these disparities. Forming multidisciplinary teams familiar with HLH at each institution will optimize communication and education of local providers about how to diagnose HLH. These teams can develop guidelines for the evaluation and treatment of HLH that are tailored to the resources available locally. Bringing together individuals globally to learn from one another will enhance understanding of alternative approaches to diagnosis and treatment as well as the latest advances in the field. Such meetings will also nucleate research efforts aimed at collecting clinical data and biospecimens, which will further enhance understanding of disease biology and treatment and ensure optimal care for patients with HLH worldwide. While these challenges may appear daunting, they are not insurmountable. Looking to the future, this blueprint serves as a guide to spur futher research and move the field ever closer to curing each and every patient with HLH.

Disclosures

No conflicts of interest to disclose.

Contributions

All authors prepared the manuscript and reviewed and approved the manuscript prior to submission.

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