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REGULATION OF CYTOTOXIC LYMPHOCYTE EFFECTOR FUNCTIONS

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Cover illustration: “Overexpression of mCherry-RHOG in a primary human NK cell that is engaged in killing a target cell”

Regulation of cytotoxic lymphocytes effector functions

Thesis for Doctoral Degree (Ph.D.)

By

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To Wallie, Vali and Aksel

“Dèyè mòn, gen mòn.”

"Beyond mountains, there are mountains."

(Haitian proverb)

Popular science summary of the thesis

The immune system maintains a delicate equilibrium between our internal environment and the external world. Picture your immune system as a tightrope walker, gracefully balancing high above ground. On one hand, our immune system must eliminate menacing viruses, bacteria, and cancer cells. On the other hand, it must not damage our healthy cells with too much activation. The immune system is usually well trained to do so, and constantly gets rid of defective cells and harmful pathogens without losing its balance. Still, none of us is born with the same immune system. Our inherited genetic variation makes us more or less susceptible to, for example, severe infections and cancer. Sometimes, hidden genetic surprises disrupt the balance of our immune system, and the immune tightrope walker stumbles. These genetic twists are what we call 'Inborn Errors of Immunity' (IEI). When we see a professional tightrope walker easily balancing, it may be difficult to understand how they are able to do so. However, if we see another walker stumbling, we can compare them to the first one and, for example, learn what type of pole the walker needs to balance successfully. By studying IEI in humans, we gain valuable insights on the regulation of our immune system in health and disease. In my thesis, I have explored the impact of several IEIs on the immune system function and human biology in general.

Immune cells are white blood cells and circulate throughout our body while checking that everything is functioning. Some immune cells eliminate defective cells that, for example, have been infected by viruses, or are becoming cancer cells. These immune cells are called “cytotoxic” (i.e., toxic for cells since "cyto" is the Greek word for cell). Together with other immune cells they participate in several immune responses, which often comprise inflammation. Inflammation is characterized by five signs: pain, heat, redness, swelling and loss of function in the affected areas. Even if these symptoms sound deleterious, in controlled settings, inflammation does help the immune system in eliminating pathogens and repairing damaged tissues. Once the original threat has been solved, cytotoxic cells also kill the other activated immune cells to turn down inflammation. Cytotoxic cells are therefore the damage control plan of inflammation. The major “weapon” used by our cytotoxic killers are “bullets” of toxic molecules that they "shoot" against their targets. In a rare IEI called familial hemophagocytic lymphohistiocytosis (FHL), cytotoxic cells cannot kill their targets anymore. When the bullets have stopped working, cytotoxic cells call up for help endlessly. Calling up for help means sending out tons of signaling

molecules that activate more immune cells and enlarge the initial inflammation out of control. In these individuals, more and more immune cells get activated and none eliminated. Eventually, the whole body is driven into inflammation. This "hyper"-inflammation requires emergency hospitalization and threatens the survival of these patients at very young ages. Therefore, it is important to understand how the bullets of cytotoxic immune cells work, and which genetic IEIs cause FHL. In **paper I** we discovered one novel gene causing FHL when missing. This discovery explained a fascinating molecular mechanism regulating cytotoxic cell killing. Above all, this new finding will be useful for FHL patient diagnostics.

Genetic variation makes some of us more susceptible to cancer. Since many of our blood cells are short-lived, they are constantly substituted by new cells coming from the bone marrow during a process called "hematopoiesis" (derived from the Greek words "haima", blood, and "poiēsis", to produce something). This process is inherently dangerous because it involves cell proliferation and DNA replication, whose errors are the starting point of cancer. Viruses are another threat to hematopoiesis, and any cell proliferation process in general, because they hijack the cell's machinery to produce their own DNA or RNA while pushing for dysregulated proliferation. Fortunately, our cells have evolved mechanisms against this hijacking. One of these mechanisms is the activation of two proteins called SAMD9 and SAMD9L, which detect viral infections and stop the stolen cell machinery in time. Nevertheless, we know little about how SAMD9 and SAMD9L function, or what different parts of these proteins, also known as "domains", do in this process. What we do know is that mutations in specific domains of SAMD9 and SAMD9L change the function of these proteins and they start to promote cancer during hematopoiesis. These mutations are often found in patients with hematopoietic disorders that can progress to cancer. We also know that some viruses have "viral factors" that block the action of normal SAMD9 and SAMD9L by binding to specific domains of these proteins. In **paper II**, we exploited these viral factors to see whether they could also block mutant cancer promoting versions of SAMD9 and SAMD9L. In this way, we gained useful insights into the regulation of SAMD9 and SAMD9L activities and found that both normal and mutant SAMD9 and SAMD9L were blocked by the viral factors studied. This approach can hypothetically be used to develop drugs that specifically targets the cancer promoting versions SAMD9 and SAMD9L.

Among the cytotoxic cells of our immune system, we have natural killer cells. Natural killer cells are called "natural killer" because they can eliminate

defective cells as soon as they have finished their own hematopoiesis. Other cytotoxic cells, namely $CD8^+$ T cells, need first to be specifically activated to do so. NK cell function is important because when missing, we are more susceptible to severe viral infections and cancer. Their discovery dates to the 1970s, and we still do not know some of their biology in humans. To be precise, it is unknown if all NK cells follow the same developmental trajectory and how the final steps of NK cell development are regulated. In these final steps, we see the rise of more cytotoxic NK cells that, accordingly, kill cancer cells better. Therefore, there is a huge interest in understanding how this terminal development is regulated in NK cells, so that we may exploit it in anti-cancer immunotherapies. Some IEIs affect NK cell development, which may be incomplete or blocked at intermediate steps. In **paper III**, we studied NK cells in patients with one of these IEIs. We discovered a gene, *DEF6*, that potentially regulates the terminal steps of NK cell development and is important for their cytotoxicity. Interestingly, this gene may not be required for all the NK cell developmental trajectories. These novel insights may help piece together the puzzle of NK cell development in humans.

To summarize, the diverse research articles of this thesis have delved into different aspects of human IEIs, from diagnosis to further understanding of the biology behind IEI manifestation. Particular attention was given to the mechanisms regulating cytotoxic cell function and development. These insights are anticipated to provide knowledge for the personalized treatment of patients with IEIs, and better understanding of immune system regulation in general. Our professional tightrope walker still keeps secrets from us, but we are beginning to understand some of their tricks.

Abstract

Studies of patients with inborn errors of immunity provide an important opportunity to understand the human immune system in a natural context. The articles in this thesis are a compilation of such investigations. With our work, we aimed to identify the cause of primary hemophagocytic lymphohistiocytosis (HLH) in an unexplained pediatric patient (**paper I**), explore the modulation of SAMD9 and SAMD9L gain-of-function (GOF) mutants (**paper II**), and understand natural killer (NK) cell biology in the context of DEF6 deficiency (**paper III**).

In **paper I**, we uncovered biallelic loss-of-function variants in *RHOG* in a 4-month-old patient presenting with HLH and displaying defective lymphocyte exocytosis. Deletion of *RHOG* in a human NK cell line abrogated exocytosis that could be rescued by constructs expressing wild type RHOG protein. Moreover, we found that MUNC13-4, associated with autosomal recessive familial HLH type 3, required RHOG interactions for recruitment to the plasma membrane during cytotoxic granule exocytosis. Thus, we demonstrated that RHOG is essential in cytotoxic granule exocytosis by human T and NK cells and proposed that biallelic loss-of-function mutations in *RHOG* are a novel cause for familial HLH.

In **paper II**, we examined a variety of pathogenic *SAMD9* and *SAMD9L* GOF variants associated with syndromes encompassing bone marrow failure, autoinflammation or selective loss of NK cells, B cells, and monocytes. We sought to understand whether viral host range factors, which are known to antagonize wild-type SAMD9 and SAMD9L proteins, could counteract the anti-proliferative and anti-translational activities of these pathogenic variants. SAMD9 or SAMD9L variants and viral factors were overexpressed in a cell line, followed by biochemical and functional analyses. Vaccinia virus K1 exhibited the highest inhibitory capacity but could not antagonize a truncated variant of SAMD9L that lacked the K1 binding site. The other factors (M062, C7 and KI) could interact with all the SAMD9 and SAMD9L mutants but displayed low capacity in antagonizing the anti-translational and anti-proliferative action of SAMD9/9L mutants. This study provided some novel insights into the structure and the regulation of SAMD9/9L proteins. Additionally, it showed that targeting pathogenic GOF SAMD9/L mutants via viral host range factors or, more broadly speaking, by protein-protein interaction is possible.

Autosomal recessive mutations in *DEF6* are associated with autoimmunity and severe herpes virus infections. While the latter is a hallmark of NK cell deficiencies, this cell type has not been thoroughly studied in DEF6-deficient patients. In **paper III,** we surveyed the role of DEF6 in NK cell development and function. We analyzed NK cell phenotypes in DEF6-deficient patients from two unrelated families and found a reduction in canonical $CD56^{dim}PLZF^{+}$ NK cells concomitant with the expansion of CMV-induced adaptive $CD56^{dim}PLZF^{-}$ NK cells. Deletion of *DEF6* in induced pluripotent stem cells gave rise to mature NK cells in an *in vitro* culture system. In primary human NK cells, deletion of *DEF6* reduced cell viability, degranulation and IFN-γ production. We therefore postulate that impaired canonical NK cell survival and function may contribute to the viral susceptibility of DEF6 deficient individuals.

To conclude, these studies have shed light on several cellular and molecular mechanisms behind IEI. These mechanisms enlarge our understanding of cytotoxic lymphocytes and of the immune system in general. Moreover, they have inspired us to pursue new directions and experimental approaches in our research.

List of scientific papers

I. Kalinichenko A, **Perinetti Casoni G***, Dupré L*, Trotta L*, Huemer J, Galgano D, German Y, Haladik B, Pazmandi J, Thian M, Yüce Petronczki Ö, Chiang SC, Taskinen M, Hekkala A, Kauppila S, Lindgren O, Tapiainen T, Kraakman MJ, Vettenranta K, Lomakin AJ, Saarela J, Seppänen MRJ, Bryceson YT, Boztug K: RhoG deficiency abrogates cytotoxicity of human lymphocytes and causes hemophagocytic lymphohistiocytosis. *Blood* (2021) PMID: 33513601

II. Gahr S, **Perinetti Casoni G**, Falk-Paulsen M, Maschkowitz G, Bryceson YT, Voss M: Viral host range factors antagonize pathogenic SAMD9 and SAMD9L variants. *Exp Cell Res*. (2023) PMID: 36894052

III. **Perinetti Casoni G**, Cabrerizo Granados D, Wahlen S, Kirchhof K, Campbell TM, Schlums H, Galgano D, Priftakis P, Sundin M, Walzer T, Belot A, Meeths M, Lundin V, Bryceson YT: DEF6 promotes canonical CD56dim NK cell survival and function. *Manuscript*

Relevant scientific publications, not included in the thesis

SI. Schmied L, Luu TT, Søndergaard JN, Hald SH, Meinke S, Mohammad DK, Singh SB, Mayer C, **Perinetti Casoni G**, Chrobok M, Schlums H, Rota G, Truong HM, Westerberg LS, Guarda G, Alici E, Wagner AK, Kadri N, Bryceson YT, Saeed MB, Höglund P: SHP-1 localization to the activating immune synapse promotes NK cell tolerance in MHC class I deficiency. *Sci Signal*. (2023) PMID: 37040441

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Contents

List of abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	acute myeloid leukemia
CAR	Chimeric antigen receptor
CG	Cytotoxic granule
CMV	Cytomegalovirus
DC	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
ExAC	Exome Aggregation Consortium
FHL	Familial hemophagocytic lymphohistiocytosis
GAP	GTPase activating proteins
GDI	Guanosine diphosphate dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factors
GnomAD	Genome Aggregation Database
GOF	Gain-of-function
GS2	Griscelli syndrome type-2
GTP	Guanosine triphosphate
HLH	Hemophagocytic lymphohistiocytosis
HSCT	Hematopoietic stem cell transplantation
IEI	Inborn errors of immunity
ILC	Innate lymphoid cells
iPSC	Induced pluripotent stem cell
IS	Immune synapse
ITAM	Tyrosine-based activation motif
KIR	Killer-cell immunoglobulin-like receptor
KO	Knock-out
LE	Late endosome

LOF	Loss-of-function
MCMV	Murine Cytomegalovirus
MDS	Myelodysplastic syndrome
MUNC	Mammalian uncordinate
mTORC2	Mammalian target of rapamycin complex 2
NGS	Next-generation sequencing
NK	Natural Killer
PBMC	Peripheral blood mononucleated cell
PID	Primary immunodeficiency
PMA	Phorbol 12-myristate 13-acetate
RE	Recycling endosomes
RNP	Ribonucleoprotein
SGR	Somatic genetic rescue
SJIA	Systemic juvenile idiopathic arthritis
SLE	Systemic lupus erythematosus
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
TF	Transcription factor
Treg	Regulatory T cells
WAS	Wiskott-Aldrich syndrome
WES	Whole exome sequencing
WGS	Whole genome sequencing
XLN	X-linked neutropenia
XLP	X-linked lymphoproliferative

Introduction

Patients with inborn errors of immunity (IEI) may present with severe infections, hyperinflammatory syndromes, autoimmunity, or cancer. As genomic sequencing techniques have progressed, increasing numbers of variants in the human genome have been associated with immune dysregulation. Identifying pathogenic variants can provide a diagnosis and potentially direct therapy. Still, most patients with suspected IEI lack a molecular diagnosis. This fact can be explained by genome sequencing efforts not identifying any known variant or finding candidate variants that remain of unknown significance. Functional evaluation of variants IEI-associated genetic variants is therefore an urgent need for the clinics. At the same time, these studies represent an unparalleled opportunity to better understand the principles of human immunology and hematology. The research work of my thesis thus hopefully has a circular evolution: instigated from studying patients with rare genetic variants, expanded into molecular and cellular studies of immune cells, and finally providing biological insights that can improve diagnostics and inspire the development of targeted therapies. Furthermore, my efforts have provided fundamental insights to cytotoxic lymphocyte biology, which are also key effectors in the immunosurveillance of cancer.

1 Literature review - Studying inborn errors of immunity to understand cytotoxic lymphocyte biology.

1.1 Inborn errors of immunity

Inborn errors of immunity: general features and manifestations

Inborn errors of immunity (IEI), also termed primary immunodeficiencies (PID), are a group of genetic conditions characterized by increased susceptibility to severe infections, autoimmunity, immune dysregulation, and cancers, and are caused by inherited pathogenic variants in genes regulating immunity (Tangye et al., 2022). Most severe IEIs have onset in childhood when the immune system is challenged for the first time by pathogens and allergens. Others may have a narrower window of manifestations later in life or upon the encounter of a specific pathogen. As such, it has been proposed that any lethal infectious disease can be considered the ultimate symptom of an underlying IEI (J. L. Casanova, 2015; J.-L. Casanova & Abel, 2021).

Mutations that severely impair essential functions of the immune system, such as the development or function of a major leukocyte line, display early clinical onset, and patients generally suffer from recurrent and/or severe opportunistic infections. These "classic" IEIs affect non-redundant molecular pathways, typically show complete penetrance, and were historically first molecularly explained (J. L. Casanova & Abel, 2018). More recently, many IEIs displaying later onset, increased susceptibility to selected microbes, and often incomplete penetrance have been associated with mutations in genes regulating innate immune responses (Bucciol et al., 2019). Besides infections, IEI patients may suffer from diverse severe immunopathologies (allergies, autoimmune and autoinflammatory diseases) and hematological conditions (Bousfiha et al., 2018). Moreover, some IEIs have syndromic features because the underlying mutations affecting genes that are required in cells and tissues beyond the immune system (Tangye et al., 2020). All these factors contribute to an increased susceptibility to malignancies, which is the second leading cause of death in IEI patients (Derpoorter et al., 2018). During carcinogenesis, the immune system plays a dual role in the process of cancer immunoediting, which is divided in three phases: elimination or immunosurveillance, equilibrium and escape (Schreiber et al., 2011). During the elimination phase, both innate and adaptive immune cells recognize the cancer as

detrimental and respond with several mechanisms apt to eliminate the cancer cells (Schreiber et al., 2011). Some rare cancer cells may overcome this phase and reach an equilibrium, especially with the adaptive immune cells, which keep the cells in a dormant/contained state and in the long run, shape the immunogenicity of the cancer cells(Schreiber et al., 2011). Eventually, some cancer cells evolve mechanisms to escape the control by the immune system such as loss of major histocompatibility complex (MHC) expression, and the cancer become aggressive again (Schreiber et al., 2011). Immunotherapy aims to eliminate cancer by re-establishing and improving the mechanisms of the immunosurveillance phase. Reduction of immunosurveillance due to IEIs affecting the immune cells specifically mediating this phase, such as natural killer (NK) cells, is probably a contributing factor to the elevated cancer risk in these patients, at least in some cancer types (Malmberg et al., 2017). Most malignancies in IEI are of hematologic origin or linked to other cell types intrinsically affected by underlying mutations that dysregulate cell metabolism and/or proliferation (Hauck et al., 2018). Hence, the study of IEIs is not limited to immunology but also streams into hematology as these two fields study cells with the same developmental origin. Besides, severe viral infections in IEI patients promote carcinogenesis by fueling inflammation and rapid cell replication accompanied by genetic instability (Hauck et al., 2018). Chronic inflammation caused by recurrent infections and autoinflammation promotes carcinogenesis, by fostering mutagenesis and providing proliferative and proangiogenic factors (Hanahan & Weinberg, 2011). Therefore, the increased susceptibility to cancer of IEI patients is multifactorial and is often linked to the underlying pathogenic variant.

The impact of inborn errors of immunity on human health

IEIs have a considerable impact on human health. In the past, IEIs were listed among rare diseases with an incidence of 1 in 10.000-50.000 live births. Nevertheless, improved sequencing techniques have tremendously accelerated the discovery of novel pathogenic gene variants causing immunodeficiencies and immune dysregulation since 2010, when the first paper using next-generation sequencing (NGS) to investigate the underlying cause of a novel IEI was published (Byun et al., 2010; Tangye et al., 2020). Compared to Sanger sequencing, NGS allows broader genetic analyses through the parallel sequencing of short fragments of DNA. Indeed, approximately 45% of all known disease-causing genes have been identified in the last 10 years by whole exome/genome sequencing (WES/WGS)(Tangye et al., 2020). Therefore, considering newly discovered

variants and a constantly improving patient diagnostics, the incidence of IEIs is now presumed to be much higher than what was previously hypothesized, and is probably around 1 in 1000-5000 live births (Tangye et al., 2020). Several of the newly discovered IEIs challenge the concept of Mendelian inheritance and complete penetrance of "classic" IEIs. Pandemics like the coronavirus one we recently experienced have revealed some "stealth" IEI in otherwise, apparently, healthy individuals. For example, autosomal recessive IRF7 deficiency was discovered in several patients with severe respiratory infections, such as SARS-CoV-2 and influenza, but no other major manifestations (Campbell et al., 2022). Deciphering the genetics behind suspected IEI cases is essential to improve diagnostics, treatment, and genetic advice for the patients' families. Moreover, understanding the molecular mechanisms causing IEI provides new knowledge about the immune system and human biology in general. Consequently, it has been an incentive to generate novel biological therapeutics specifically targeting pathogenic molecular processes pinpointed by IEI patients (Notarangelo & Fleisher, 2017). These drugs may also be useful to treat immunological conditions caused by the dysregulation of the same pathway. Finally, a better comprehension of the pathways sustaining immune cell function, survival and metabolism can be harnessed to develop novel drugs for the treatment of hematological malignancies. A milestone in this field has been the development of ibrutinib for the treatment of B cell malignancies, which was inspired by the study of X-linked agammaglobulinemia (Gayko et al., 2015).

Next-generation sequencing and identification of novel IEI candidate genes

The use of NGS in IEI diagnostics and research is nowadays routine in most high-income countries. According to the experimental design, NGS can be used in targeted gene panel approaches, WES or WGS. Each approach has both advantages and drawbacks in terms of result time, costs, types of detected variants and complexity (Chinn et al., 2020). WGS analyses are the most expensive and complex due to the large amount of generated data, but they are also the only ones able to identify copy number and structural variants, and variants in non-coding regions (Chinn et al., 2020). After sequencing, each variant must be scored and ranked according to its pathogenicity. Several *in silico* tools integrating biochemical data and phylogenic conservation are nowadays available to clinicians and scientists for variant prioritization (Chinn et al., 2020) (e.g., the Combined Annotation-Dependent Depletion (CADD) that integrates into a single measure conservation data, transcriptional and regulatory data and overall protein

structure data for any single nucleotide variant or insert-deletion (Kircher et al., 2014).

The analysis of 60.706 exomes by the Exome Aggregation Consortium (ExAC) identified 3230 genes intolerant to predicted protein truncating variants (PTVs) (Lek et al., 2016). Interestingly, 72% of these genes had not been associated with a human disease phenotype (Lek et al., 2016). As the authors suggest, loss-of function (LOF) variants in these genes are presumably subjected to strong negative reproductive selections, and disease phenotypes may only manifest because of milder monoallelic missense hypomorphic variants (Lek et al., 2016). Only broad genetic analyses by WES and WGS can find whether IEI patients with unknown etiology carry one or more of previously uncharacterized LOF variants, which likely arise as *de novo* variants in an extremely low number of individuals. An update of the ExAC database, the Genome Aggregation Database (GnomAD), was released in 2020, integrating 125,748 exomes and 15,708 genomes where the author further focused on the identification of LOF variants (Karczewski et al., 2020). As such, the ExAC database before, and the GnomAD database now, have proven to be a fundamental reference for interpreting candidate disease-causing variants found by NGS in patients with rare diseases and IEIs. Since several factors such as genetic and epigenetic heterogeneity, compensatory mechanisms, mosaicisms, and pathogen exposure influence the pathogenicity of candidate variants (Gruber & Bogunovic, 2020), even a single patient may be relevant for discovering novel gene functions and IEIs. Indeed, guidelines for single patient-studies are well established and represent a reliable approach to validate new findings suggested by NGS analyses (J. L. Casanova et al., 2014) (See Table I).

Table I. Criteria for assigning a clinical phenotype to a candidate genotype found in a single patient.

(1) The candidate genotype must not occur in healthy individuals (i.e. complete penetrance).
- a. The clinical phenotype is rare and distinctive, and the candidate genotype is monogenic.
- b. The candidate genotype is not shared by other non-affected family members (i.e. complete penetrance with a Mendelial model of inheritance).
- c. Population studies show that the candidate genotype does not occur in healthy individuals, and that the frequencies of the candidate variants and genotype are not higher than what predicted by the frequency of the clinical phenotype.
- d. In case of premature stop codon (nonsense, frameshift, or splice-site variants), other stop codon variants in the same gene are not more frequent in population studies than predicted by the frequency of the clinical phenotype.

(2) The functional consequence of candidate variants must be a severe impairement of the gene product.
- a. Protein-coding variants are nonsynonymous or, if synonymous, have a proven impact on mRNA structure or amount. A variant in an RNA gene must affect its function.
- b. Studies of cells carrying the variants (ideally in primary or iPSC-derived cell lines) document quantitative or qualitative changes of the gene transcript and of the encoded protein.
- c. *In silico* damage prediction algorithms support the pathogenicity of the candidate variants.
- d. The variants must be a loss- or gain-of-function for at least one biological activity.

(3) The causal relationship between the candidate genotype and the clinical phenotype must be established via a relevant cellular or animal phenotype.
- a. The candidate gene is expressed in the cell types relevant to the disease process.
- b. *In vitro* studies indicate that the cellular phenotype accounting for the clinical phenotype is explained by the candidate genotype.
- c. The cellular phenotype is rescued by a wild type allele or for dominant-negative mutations, by knockdown, knockout, or correction of the mutant allele.
- d. If the variants found in the candidate gene impede the development of the relevant cell type in the patient, or if the candidate gene governs a novel circuit and there is no relevant cellular phenotype, an animal model carrying the variant or lacking the gene of interest shall be used. This model must have a phenotype mimicking the patient's one.

Modified from (J. L. Casanova et al., 2014)

1.2 Hemophagocytic lymphohistiocytosis

General features of hemophagocytic lymphohistiocytosis

HLH is a potentially lethal hyperinflammatory syndrome clinically characterized by systemic inflammatory symptoms such as: fever, organomegaly, liver dysfunction, coagulopathy, and neurologic dysfunction (Al-Samkari & Berliner, 2018). When untreated, it leads to multi-organ failure and death. Due to the elevated levels of inflammatory cytokines found in patients at diagnosis, HLH is considered a cytokine storm syndrome (Cron et al., 2023). In 1997, the first international protocol for pediatric HLH treatment (HLH-94) was published (J. I. Henter et al., 1997) and proposed five diagnostic criteria: unremitting fever, splenomegaly, cytopenia affecting two or more lineages in the peripheral blood, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytes in bone marrow or lymphoid organs. Ten years later, the HLH-2004 protocol (J.-I. Henter et al., 2007) added three more criteria: low/absent NK-cell activity, hyperferritinemia and high-soluble IL-2 receptor levels (J.-I. Henter et al., 2007). Currently, five out the eight criteria need to be fulfilled to diagnose HLH (See Table

II below). Alternatively, the finding of biallelic LOF mutations in a gene causing the primary or familial forms of HLH (FHL) is sufficient for the diagnosis even if five criteria are not fulfilled (J.-I. Henter et al., 2007). HLH clinical manifestations are caused by the unchecked activation of cytotoxic lymphocytes and macrophages fueling the hypercytokinemia that in turns drives systemic inflammation (Al-Samkari & Berliner, 2018; Janka, 2012). Inherited LOF mutations in the genes regulating the killing machinery of cytotoxic lymphocytes cause FHLs, which commonly manifest during infancy (Sepulveda & de Saint Basile, 2017). Secondary or acquired forms of HLH are driven by external triggers such as severe infections, rheumatic diseases, and malignancies. Secondary HLH usually have onset during adulthood and lack a clear underlying genetic cause (Al-Samkari & Berliner, 2018).

Table II. HLH diagnostic criteria

A diagnosis of HLH can be established upon:
(A) a molecular finding consistent with HLH
(B) Fulfillment of five out of the eight criteria below:

1. Unremitting fever
2. Splenomegaly
3. Cytopenias (affecting ≥2 of 3 lineages in the peripheral blood):
   - Hemoglobin <90 g/L
   - Platelets <100 x 10^9/L
   - Neutrophils <1.0 x 10^9/L
4. Hypertriglyceridemia and/or hypofibrinogenemia:
   - Fasting triglycerides ≥3.0 mmol/L (i.e., ≥265 mg/dl)
   - Fibrinogen ≤1.5 g/L
5. Hemophagocytosis in bone marrow or spleen or lymph nodes
   No evidence of malignancy
6. Ferritin ≥500 mg/L
7. Soluble CD25 (i.e., soluble IL-2 receptor) ≥2,400 U/ml
8. Low or absent NK-cell activity (according to local laboratory reference)

(J.-I. Henter et al., 2007)

In both primary and secondary forms of HLH, overactivated lymphocytes secrete elevated amounts of inflammatory cytokines, which drive macrophages activation and additional production of inflammatory cytokines, as well as hemophagocytosis (Al-Samkari & Berliner, 2018). Involvement of the nervous system in the forms of seizure, irritability, ataxia, and coma is a bad prognostic marker (Al-Samkari & Berliner, 2018), and is more common in FHL3 (Sieni et al., 2011) and FHL5 (Pagel et al., 2012). Generally, neurological symptoms occur in one third of the pediatric patients (Janka, 2012). Differentiation between primary and secondary forms of HLH is central to determine the treatment regimen, and whether the patient should undergo allogenic hematopoietic stem cell transplant

(HSCT) (J.-I. Henter et al., 2007). The current therapy of FLH is based on the HLH-94 and 2004 protocols employing dexamethasone and etoposide prior to HSCT (J. I. Henter et al., 1997; J.-I. Henter et al., 2007). After HSCT, donor chimerism of more than 20%-30% is usually sufficient to protect against late disease reactivation (Hartz et al., 2016). Another therapeutical option that has been recently explored is the genetic correction of the mutations causing HLH in hematopoietic stem cells (HSC). Preclinical models have shown promising results, but further studies will be needed before this option is brought to the clinics (Dettmer-Monaco et al., 2023; Soheili et al., 2017; Topal et al., 2023). Secondary HLH is usually managed according to the underlying trigger: steroids in the presence of rheumatologic disorders, antimicrobial therapy during severe infections and cancer-specific therapy for malignant-HLH (Al-Samkari & Berliner, 2018). In the last few years, targeting the hypercytokinemia, which characterizes both familial and secondary HLH patients, has become an alternative for treating some genetic conditions causing HLH, and promising drugs antagonizing cytokine signaling are now being incorporated as a treatment prior to bone-marrow transplant or to treat adult patients. As IFN-γ is central to HLH pathology (Jordan et al., 2004), antibodies antagonizing this cytokine has proven being efficient in controlling inflammation prior to bone marrow transplant (Locatelli et al., 2020). Moreover, JAK/STAT inhibitors do not only target IFN-γ signaling, but also several other pro-inflammatory cytokines (e.g., interleukin (IL)-6 and IL-12) and cytokines driving $CD8^+$ T cell activation (e.g., IL-2). Ruxolitinib has showed promising results in pre-clinical studies (Albeituni et al., 2019; Meyer et al., 2020), but its therapeutical window and mode of use still needs to be refined (Keenan et al., 2021).

In most severe, neonatal onsets, defects in LAMP-1, a.k.a. CD107a, positive cytotoxic granule (CG) exocytosis and lymphocyte cytotoxicity reflect the genetic cause of the disease. Thus, functional evaluation of CG exocytosis and lymphocyte cytotoxicity by flow cytometry and chromium-release assays respectively are often used for the initial assessment of HLH patients and are key to guide sequencing efforts (Bryceson et al., 2013).

Familial HLH genetics

So far, five forms of "classic" early-onset FHL have been described, and four of them are caused by biallelic LOF mutations in genes regulating lymphocyte cytotoxicity. FHL type 1 has been mapped to a chromosomic region on chromosome 9, but the causative gene remains unidentified (Ohadi et al., 1999).

The first gene discovered as a cause of FHL was *PRF1* encoding perforin and mutated in FHL type 2 (FHL2) (Stepp et al., 1999). Cytotoxic lymphocytes release CGs against target cells to kill them (Kagi, Ledermann, et al., 1994). Perforin is a cytotoxic glycoprotein that creates pores in target cell membranes by Ca^{2+}-dependent polymerization (Baran et al., 2009). It is contained within the CGs together with pro-apoptotic granzymes and, upon pore formation, allows them to enter the cytoplasm of target cells. FHL3, 4 and 5 are caused by biallelic mutations in *UNC13D*, *STX11* and *STXB2*, respectively (Cote et al., 2009; Feldmann et al., 2003; zur Stadt et al., 2005, 2009). These three genes encode the proteins mammalian uncoordinate (MUNC)13-4, SYNTAXIN-11 and MUNC18-2 respectively, that are responsible for the trafficking, the tethering, and the exocytosis of perforin-containing CGs at the plasma membrane (de Saint Basile et al., 2010). Pathogenic variants may impair protein expression, function and interactions all resulting in severely reduced lymphocyte cytotoxicity (Hackmann et al., 2013; Müller et al., 2014). Human NK and cytotoxic T lymphocytes (CTLs) use the same molecular machinery to release CGs and kill target cells, and CG exocytosis is similarly impaired in both cell types in FHL patients (Chiang et al., 2013). Additionally, two hereditary syndromes displaying partial albinism, bleeding disorders and immunodeficiency are associated with a high risk of developing HLH: Griscelli syndrome type-2 (GS2) and Chediak-Higashi syndrome (Ménasché et al., 2000; Nagle et al., 1996). The genes mutated in these syndromes (*RAB27A* and *LYST* respectively) are required for vesicle biogenesis and trafficking of both melanosomes and CG, therefore causing albinism and impaired lymphocyte cytotoxicity (Sepulveda, Burgess, et al., 2015; Stinchcombe, Barral, et al., 2001). Therefore, they are also considered familial forms of HLH (see Table III) (Janka & Lehmberg, 2014).

Familial HLH pathogenesis

The pathogenesis of familial HLH has been studied using FHL gene knock-out (KO) animal models. Perforin-deficient ($Prf^{-/-}$) mice develop HLH upon Lymphocytic Choriomeningitis Virus (LCMV) infections confirming the causative role of impaired lymphocyte cytotoxicity in FHL onset (Jordan et al., 2004). In this model, cytotoxic-deficient activated $CD8^+$ T cells are the principal producers of cytokines, especially IFN-γ that drives the activation of macrophages, which in turn produce more pro-inflammatory cytokines and fuel systemic inflammation in the animals (Jordan et al., 2004). The contribution of NK cells in promoting inflammation by cytokine production was not significant in this model (Jordan et

al., 2004). Additionally, NK cells eliminate via perforin-dependent killing activated $CD4^+$ T cells that in turn promote $CD8^+$ T cell activation and immunopathology during LCMV infection (Waggoner et al., 2012). In the $Prf^{-/-}$ mouse model, restoring NK cell immunoregulatory function was sufficient to protect the animals from fatal HLH-manifestations and a concomitant reduction of hyperactivated antigen-specific $CD8^+$ T cells was observed (Sepulveda, Maschalidi, et al., 2015). Terrell and Jordan (2013) found that CTLs cells exerted a perforin-dependent immunoregulatory function, and restrained antigen-presentation by dendritic cells (DC) (Terrell & Jordan, 2013). Another study (Humblet-Baron et al., 2016) reported that hyperactivated $CD8^+$ T cells in $Prf^{-/-}$ mice consume large quantities of IL-2, undermining the homeostasis of regulatory $CD4^+$ T cells (Treg). Similarly, HLH patients had low numbers of Treg cells during hyperinflammation (Humblet-Baron et al., 2016). IL-2 was found sustaining hyperactivated T cell survival in $Prf^{-/-}$ mice (Humblet-Baron et al., 2019), arguing for the use of JAK/STAT inhibitors in HLH treatment (Meyer et al., 2020). Collectively, these studies suggest that, besides impairing the eradication of viral infections, the loss of perforin-based cytotoxicity disrupts several regulatory feedback loops based on the elimination of the immune cells actively promoting inflammation (figure 1). These regulatory loops are necessary not to incur in the uncontrolled expansion of IFN-γ-producing $CD8^+$ T cells, and the subsequent overactivation of the immune system in both physiological and pathological settings.

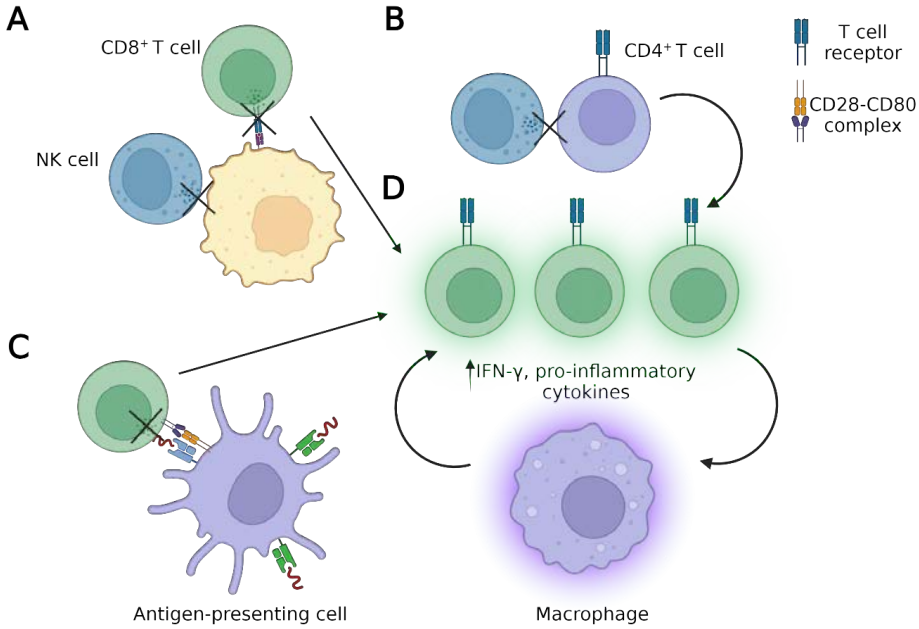

Figure 1. Immunoregulatory loops that depend on perforin-mediated cytotoxicity. A. When perforin-mediated killing is impaired or absent, CTLs and NK cells cannot eliminate infected or malignant target cells. B. NK cells cannot eliminate activated $CD4^+$ T cells that promote further activation and expansion of $CD8^+$ T cells. C. CTLs fail in killing antigen-presenting cells that promote further T cell activation. D. Hyperactivated $CD8^+$ T cells secrete high levels of IFN-γ and pro-inflammatory cytokines, which in turn activate macrophages to secrete more cytokines, infiltrate tissues and drive the inflammation systemically. Created with BioRender.com.

Dysregulated inflammasome activation

The observation of HLH and MAS manifestations in patients and murine models that cannot respond to IFN-γ stimulation (Canna et al., 2013; Tesi et al., 2015) suggested the existence of additional pathological mechanisms that can drive hyperinflammation independently, or complementary, to IFN-γ. Dominant *NLRC4* GOF (Canna et al., 2014) and X-linked *XIAP* LOF (Rigaud et al., 2006; Wada et al., 2014) variants have been described in MAS and HLH patients. In these patients, the levels of free IL-18 were extremely high and resulted by excessive inflammasome activation (Canna et al., 2014; Wada et al., 2014). Recently, certain monoallelic missense mutations in *CDC42*, encoding for the small signaling and cytoskeletal regulator CDC42, have been associated with autoinflammation/HLH. Due to the excess of active IL-18 and IL-1β found in these patients, a still-not-

defined inflammasome dysregulation mechanism has been hypothesized (Bekhouche et al., 2020; Gernez et al., 2019; Lam et al., 2019).

Some recent studies have addressed the role of IL-18 in HLH pathogenesis (Landy et al., 2023; Tsoukas et al., 2020; Weiss et al., 2018). Weiss (2018) showed excessive IL-18 promoted experimental MAS in a murine model bearing a NLRC4 GOF variant(Weiss et al., 2018). In LCMV infected-mice, IL-18 and perforin deficiency drove hyperinflammation in a synergistic and independent manner each other (Tsoukas et al., 2020). A most recent publication suggests that defects in cytotoxicity and excessive IL-18 converge to antigen-driven $CD8^+$ T cell hyperactivation, which is central for HLH development (Landy et al., 2023). Interestingly, the authors were not able to pinpoint the antigens driving $CD8^+$ T cell activations, leaving a door open for speculation on their nature (Landy et al., 2023). Hence, excessive inflammasome activation and defective lymphocytes cytotoxicity are now to be considered as two independent and complementary pathological mechanisms predisposing to HLH.

Other EIEs and genetic conditions predisposing to HLH

Besides impaired cytotoxicity and dysregulated inflammasome regulation, several IEI patients have a predisposition to HLH. These include individuals with IEIs characterized by selective vulnerability to Epstein-Barr virus (EBV). On top of the list are X-linked lymphoproliferative (XLP) diseases 1, caused by LOF mutations in *SH2D1A* (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998), and XLP2, caused by LOF mutations in *XIAP* (Rigaud et al., 2006). In XLP1, lack of SAP impedes 2B4-mediated cytotoxicity of EBV-infected B cells (Sayos et al., 1998). Several others IEI predispose to increased susceptibility to EBV infections mostly by impairing initial T cell activation and expansion that are necessary to control EBV infection (Latour & Fischer, 2019). Still, the ones resulting most often in HLH are XLP1 and XLP2 as they significantly impair two major pathways converging into $CD8^+$ T cell uncontrolled activation (i.e., killing of target cells and inflammasome activation respectively.)

A compilation of pediatric IEI patients presenting with HLH in the absence of impaired lymphocyte cytotoxicity or X-linked lymphoproliferative disorders demonstrated that HLH manifestations can be the first sign of an underlining IEI (Bode et al., 2015). Most of the identified patients had either combined immunodeficiencies (CID) in T cell development and function, or chronic granulomatous disease (CGD) (Bode et al., 2015). The authors hypothesized that

in CGD patients, impaired autophagy is the main molecular mechanism contributing to HLH development because a previous study associated deficiency in reactive oxygen species (ROS) production to dysregulated autophagy and increased levels of IL-1β (Bode et al., 2015; De Luca et al., 2014). Also, patients with metabolic disorders may develop HLH, possibly due the accumulation of non-degraded substrates causing inflammasome activation (Taurisano et al., 2014). Recently, the systematic study of patients with neuroblastoma-amplified sequence (NBAS) syndrome showed defects in NK cell degranulation that could be corrected with IL-2 pre-stimulation (Lenz et al., 2021). Since then, biallelic LOF mutations in *NBAS* have been described in a cohort of children with HLH not bearing biallelic variants in known FHL genes (Bi et al., 2022). Based on NBAS protein role in Golgi to ER retrograde transport, disturbed vesicle trafficking upstream CG biogenesis release has been proposed (Bi et al., 2022), but more molecular studies are needed to clarify the role of this protein in CG release. Missense variants abrogating plasma membrane expression of the checkpoint inhibitor TIM-3 (encoded by *HAVCR2*) were found in patients with subcutaneous panniculitis-like T cell lymphomas and HLH (Gayden et al., 2018). Gayden (2018) suggested that both uncontrolled IFN-γ production by $CD4^+$ T helper 1 subset, and macrophage activation may contribute to HLH disease in these patients. Finally, defects in cytoskeleton regulation may also predispose to HLH in some patients with actinopathies by affecting actin dynamics at the IS and CG-mediated cytotoxicity. For example, LOF variants in *DOCK2*, *DOCK8*, encoding CDC42 regulators, impair NK cell function and immune synapse organization (Dobbs et al., 2015; Ham et al., 2013; Mizesko et al., 2013; Q. Zhang et al., 2009). Possibly pathogenic *DOCK8* variants were found in pediatric HLH patients without a molecular diagnosis and in MAS patients (Chinn et al., 2018). Deficiency of another regulator of CDC42, DOCK11, has been recently reported in patients with anemia and systemic inflammation (Block et al., 2023). In this case, the authors did not observe any abnormality in immune synapse formation and did not find either any indication of inflammasome regulation (i.e., elevated IL-18 and IL-1β) (Block et al., 2023). These studies emphasize the myriad of molecular mechanisms connected to actin regulation.

These findings reinforce the notion that HLH may be caused by multiple pathological mechanisms ultimately leading to impaired cytotoxicity, hyperinflammation and uncontrolled T cell activation. Hence, WES and WGS

analyses are key in identifying the presence of a genetic predisposition in these patients.

Table III. Genetic predisposition to HLH

Pathogenic mechanism	Gene Defect	Mode	Inheritance
Defective lymphocyte granule-mediated cytotoxicity (familial HLH)	*PRF1*	Loss-of-function	Autosomal recessive (AR)
	UNC13D		
	STX11		
	STXBP2		
Defective lymphocyte granule-mediated cytotoxicity	*RAB27A*		
	LYST		
	NBAS		
Dysregulated inflammasome activity	*XIAP*	Loss-of-function	X-recessive (XL)
	NLRC4	Gain-of-function	Autosomal dominant (AD)
	CDC42	Gain-of-function Dominant negative	AD
EBV- HLH	*SH2D1A*	Loss-of-function	XL
	XIAP		XL
EBV susceptibility disorders (impaired T cell activation and expansion)	*RASGRP1*	Loss-of-function	AR
	MAGT1		XL
	ITK		AR
	CTPS1		AR
	CD70		AR
	CD27		AR
	TNSFR9		AR
Chronic granulomatous disease (impaired autophagy)	*CYBB*	Loss-of-function	XL
	NCF1		AR
	CYBA		AR
	NCF4		AR
	NCF2		AR
	CYBC1		AR
Dysregulated metabolism and accumulation of non-degraded substrated	*LIPA*	Loss-of-function	AR
	MVK		AR
	SLC7A7		AR
	Other inborn errors of metabolism		
Other inborn error of immunity (IEI) (cytoskeletal defects)	*HEM1*	Loss-of-function	AR
	DOCK8	Loss-of-function	AR
	WAS	Loss-of-function	XL
	Other IEI of actin dysregulation		
Other IEIs	SCIDs		
	CIDs		
	HAVCR2	Loss-of-function	AR

References (Canna & Marsh, 2020; Chinn et al., 2018; Planas et al., 2023)

MAS and secondary HLH

Macrophage-activation syndrome, MAS, is a form of HLH that arises as a severe complication in circa 10% of patients with systemic juvenile idiopathic arthritis (SJIA) (Crayne et al., 2019). Other rheumatic conditions predisposing to MAS are systemic lupus erythematosus (SLE), Kawasaki disease and adult-onset Still disease (Schulert & Cron, 2020). Since the application of the 2004-HLH criteria has been found too restrictive for the clinical diagnosis of MAS in SJIA, other diagnostics criteria were published in 2016 (Reported in (Ravelli et al., 2016)).

Nevertheless, the pathologies of MAS and FHL have much in common: systemic inflammation, hyperactivation of T cells and macrophages, hypercytokinemia, and hemophagocytosis in some cases (Schulert & Grom, 2015).

Other secondary forms of HLH usually occur in adults and are associated with severe infections, cancer, autoinflammatory and autoimmune diseases and, less frequently, with medical procedures such as allogeneic HSCT (Al-Samkari & Berliner, 2018). Treatment with novel immunotherapies such as chimeric antigen receptor (CAR)-T cells and anti-checkpoint inhibitors, unleashes powerful T cell responses against cancer cells. In some patients, they cause cytokines release syndromes that mirror some aspects of HLH pathology (Cron et al., 2023). Like primary HLH, severe infections, followed by malignancies, were the most common triggers for secondary HLH in a collaborative study of HLH in adults in Germany (Birndt et al., 2020).

The distinction between primary and secondary HLH is sometimes blurred by the existence of polymorphic variants in FHL genes in adults with late onset HLH (K. Zhang et al., 2011), and of variants with variable penetrance in healthy individuals (e.g., a hemizygous *SH2D1A* mutation was recently identified in both a 21-years-old HLH patient and his healthy siblings (Torralba-Raga et al., 2020)). Still, targeted gene sequencing may overestimate the presence of significant HLH variants in heterozygosity, and we may look at NGS studies to understand whether enrichment in FHL gene variants predispose to late onset/secondary HLH. A WES study (Kaufman et al., 2014) reported enrichments in monoallelic rare variants in FHL genes (*LYST, UNC13D* and *STXBP2*) in patients with SJIA-MAS compared to patients with SJIA but not MAS episodes (Kaufman et al., 2014). In fatal cases of H1N1 influenza Schulert (2016) reported finding FHL gene variants (*PRF* and *LYST*) in 36% of the patients (Schulert et al., 2016). These and other studies corroborate the hypothesis of a genetic predisposition acting onto environmental factor unleashing MAS development (Schulert & Cron, 2020) (figure 2). The stronger the inflammatory stimuli driving immune cell activation, the less the accumulation of detrimental variants in pathways regulating perforin-mediated cytotoxicity may be tolerated (Steen et al., 2021). The impact of polygenic inheritance in FHL is still under investigation, with one murine study suggesting cooperative accumulation of monoallelic LOF mutation in *Prf1, Stx11* and *Rab27a* predisposing to LCMV-induced HLH(Sepulveda et al., 2016), and one study finding a cumulative effects of heterozygous variants in FHL gene in adult HLH (Bloch et al., 2023). Still, more studies will be needed to fully understand the contribution of these variants to

adult HLH. Functional validation of novel variants in FHL genes identified in heterozygosity in one or more FHL genes is difficult because the pathological phenotype may emerge only with a strong inflammatory stimulus (i.e., an animal model is probably necessary in this case).

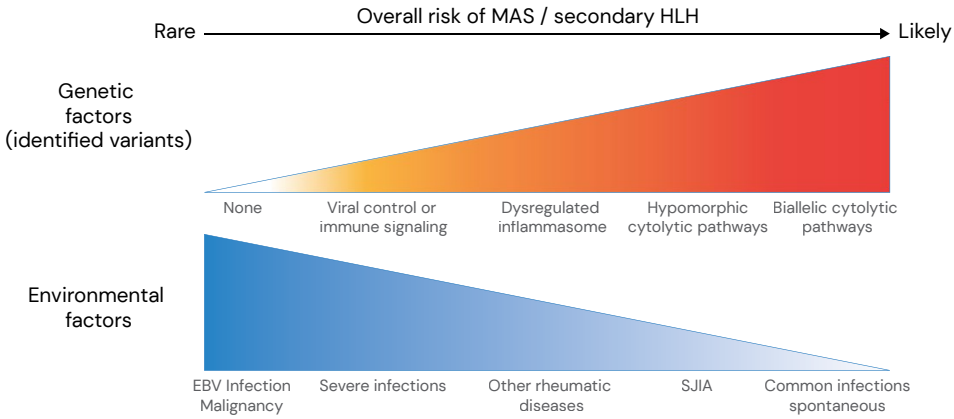

Figure 2. Complementarity of genetic and environmental factors to the risk of developing macrophage activation syndrome (MAS)/secondary HLH. Epstein-Barr virus, EBV; systemic juvenile idiopathic arthritis, SJIA. Adapted from (Schulert & Cron, 2020)

Strikingly, mutations in HLH-associated genes are associated with to augmented occurrence of lymphomas and HPV-associated cancers (Chaudhry et al., 2016; Chia et al., 2009; Löfstedt et al., 2015; Löofstedt et al., 2019; Machaczka et al., 2013). This underscores the importance of lymphocyte cytotoxicity in the immunosurveillance of infected and most likely, neoplastic cells, and argues for intensified screenings for malignancies in the individuals carrying these variants.

The use of NGS for HLH diagnostics

The heterogeneity of genetic predisposition to HLH complicates patients' genetic diagnosis. With the implementation of NGS sequencing, the use of single-gene tests for HLH genetic diagnosis has diminished drastically, particularly if prior immunological screenings do not clearly point towards a specific FHL-causing gene (e.g., perforin deficiency can be identified by flow cytometry intracellular staining in patient's cells (Kogawa et al., 2002)). Nonetheless, even with genome sequencing, a molecular diagnosis is not achieved in many HLH cases.

A recent retrospective study by Gadoury-Levesque (2020) showed how the adoption of a NGS panel for 15 known HLH-associated gene outperformed single-gene tests at the Cincinnati Children's Hospital Medical Center (CCHMC) (Gadoury-Levesque et al., 2020). Still, Gadoury-Levesque (2020) and their

colleague were able to molecularly diagnose only 10.4% individuals in a cohort of 1892 patients with suspected HLH analyzed between 2013 and 2018 (Gadoury-Levesque et al., 2020). In another study, Chinn (2018) compiled genomic data from 101 children presenting with HLH and found FHL biallelic mutations only in 19% of the patients (Chinn et al., 2018). WES was performed in 48 subjects, and a variety of putative disease-causing variants were identified in 58% of the patients (Chinn et al., 2018). In 10% of the patients, the authors found novel candidate genes regulating cytotoxicity, cell activation and proliferation, and therefore, possibly implicated in HLH pathogenesis (Chinn et al., 2018). Still, in the remaining 42% of the patients that underwent WES sequencing, no genetic explanations were found except for some digenic FHL variants with uncertain significance (Chinn et al., 2018). The paucity of "classic" FHL patients found in the cohorts of Gadoury-Levesque (2020) and Chinn (2018) underlines that targeted sequencing of FHL genes is often insufficient to find if there is a genetic predisposition for HLH (particularly in patients have more than 1 year of age). Many immunoregulatory genes are often excluded by FHL gene panels. As reported by Chinn (2018), suspected pathological variants in these genes were identified through WES in HLH patients (Chinn et al., 2018). However, even this approach may ultimately also not be successful because of intronic variants, variants in guanosine-cytosine-rich regions, copy number and structural variants that will not be detected by WES (Chinn et al., 2020). Such variants are relevant also for classic HLH genes, as exemplified by *UNC13D* intronic mutations found in FHL3 patients (Entesarian et al., 2013; Meeths et al., 2011).

The multitude of molecular mechanisms leading to HLH suggests that many disease-associated genes have not been described yet. Consequently, in depth, mechanistic and functional studies of novel candidate genes are needed alongside more comprehensive sequencing methodologies. Assigning of causality to novel variants and candidate genes found by NGS in HLH patients ultimately relies on their biological validation in relevant cellular and/or animal models (J. L. Casanova et al., 2014).

1.3 Cytotoxic lymphocyte biology illuminated by IEIs

Cytotoxic lymphocytes

Cytotoxic lymphocytes are key effector cells of the immune system, capable of rapidly killing target cells. Cytotoxic lymphocytes play several key and non-redundant roles in immunity and maintenance of immune homeostasis. Beyond

their primary function in eradicating tumor cells and combating viral infections, cytotoxic lymphocytes are implicated in immune responses against different fungal and intracellular bacterial infections (Mody et al., 2019). They produce large amounts of cytokines and chemokines that influence the activity and the differentiation of other immune cells (Böttcher et al., 2018). Additionally, they exert several regulatory functions by killing activated immune cells (Terrell & Jordan, 2013; Waggoner et al., 2012). Consequently, IEIs impairing these cells exposes patients to serious acute and chronic conditions such as severe infections, hyperinflammatory diseases, increased risk of malignancies and autoimmunity (Meeths et al., 2014). Cytotoxic lymphocytes utilize two molecular mechanisms to kill target cells: granule-mediated cytotoxicity, and the expression of death receptor ligands, such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligands (Kagi, Vignaux, et al., 1994). Death receptor ligands are also found inside CGs and become a predominant mechanism of NK cell-meditated killing after the usage of perforin and granzyme CGs (Prager et al., 2019). Here, I will focus on the two most frequent cytotoxic lymphocyte cell lineages: cytotoxic $CD8^+$ T cells and NK cells, and on the regulation of CG exocytosis because characterizing novel genes in this pathway is one of the principal topics of my doctoral studies.

Cytotoxic $CD8^+$T lymphocytes

CTLs are effector $CD8^+$ T cells and represent the largest subset of cytotoxic lymphocytes among adaptive immune cells. Each cell possesses a unique, somatically rearranged T cell receptor (TCR) that recognizes a specific peptide, or antigen, mounted on MHC class I molecules. Like $CD4^+$ T cells, $CD8^+$ T cells develop in the thymus upon a strict selection that eliminates self-reacting cells, and enter the blood stream as naïve or antigen-inexperienced cells. Naïve cells constantly recirculate through the blood and the lymphatic system until they are activated in secondary lymphoid organs (SLOs) (van den Broek et al., 2018). There, mature DCs present antigenic peptides derived from intracellular pathogens, or extracellular mounted on MHC-class I molecules through antigen cross-presentation. Importantly, they provide the co-stimulatory signals necessary for cell expansion and differentiation. Upon antigen recognition and the following differentiation, naïve CD8+ T cells give rise to a variety of effector and memory cells (Henning et al., 2018). CTLs emerge as effector CD8+ T cells that have acquired cytolytic capabilities and can then migrate to peripheral tissues to eliminate infected and malignant cells.

Natural killer cells

Natural killer (NK) cells are innate lymphoid cells (ILC) representing 5-15% of circulating lymphocytes in humans. These cells do not express somatically rearranged receptors and are regulated by the integration of signals received from germline-encoded activating and inhibitory surface receptors (Long et al., 2013). The first reports of NK cell activity date to the 1970s when a study observed lymphocyte cytotoxicity against leukemia cells isolated from sick twins by parents, siblings, and healthy identical twins (Rosenberg et al., 1972). Subsequently, studies in both murine models and humans further proved the existence of "natural" cytotoxicity against cancer cells that was not dependent on T cell activity or prior immunization (Herberman et al., 1973; Takasugi et al., 1973). The cells mediating this phenomenon were discovered in 1975 and were named "natural killer" cells after their ability to spontaneously eliminate cancer cells (Herberman et al., 1975; Kiessling et al., 1975). Since then, NK cells have been appreciated also in the control of viral and other microbial infections (Björkström et al., 2022; Mody et al., 2019), and in the regulation of adaptive immunity (Schuster et al., 2016). Furthermore, it is now clear that NK cells can themselves mediate memory-like "adaptive" immunity upon exposure to inflammatory cytokine and chemical haptens, and following cytomegalovirus infection (CMV) (Mujal et al., 2021).

In humans, two major subtypes of NK cells are found: a $CD56^{dim}$ population, which is predominant in peripheral blood, and $CD56^{bright}$ NK cells, which are less than 10% of NK cells in peripheral blood but constitute the largest fraction of the NK cells residing in other organs such as lymph nodes and tonsils (Björkström et al., 2016; Freud et al., 2017). Functionally, peripheral blood $CD56^{dim}$ cells are more cytotoxic and express the activator receptor CD16 that enables them to kill opsonized target cells in antibody-dependent cell-mediated cytotoxicity (ADCC). Peripheral blood $CD56^{brigh}$ are more proliferative and responsive to immuno-regulatory cytokines. A linear model of NK cell development proposes the differentiation of $CD56^{bright}$ NK cells from $CD34^{+}$ committed precursors in lymph nodes and SLOs like lymph nodes and tonsils (Cichocki et al., 2019; Freud et al., 2005). This process has been recapitulated *in vitro* with several protocols using both peripheral blood-, bone marrow- and cord blood-derived $CD34^{+}$ hematopoietic stem cells (Hao et al., 2001; Miller et al., 1992).

According to the linear model, $CD56^{dim}$ NK cells emerge from the proliferation of the $CD56^{bright}$ subset (Chan et al., 2007; Romagnani et al., 2007), and the

existence of an intermediate population was suggested(Yu et al., 2010). Still, the $CD56^{bright}$ to $CD56^{dim}$ transition of human NK cells has been difficult to model *in vitro*, and mounting evidence suggests a certain degree of independence between these two distinct subsets. In rhesus macaque experiments with adoptively transferred, genetically barcoded $CD34^{+}$ progenitor cells, $CD56^{+}CD16^{-}$cells, corresponding to human $CD56^{bright}$ NKs, and $CD56^{-}CD16^{+}$ cells, corresponding to human $CD56^{dim}$ NKs, have showed distinct self-renewal capacity(Wu et al., 2018). These studies suggest a generation of the two subsets by different precursor cells in rhesus macaques(Wu et al., 2018). Moreover, the $CD56^{dim}$ is characterized by a multitude of different sub-subsets and maturation states (Freud et al., 2017). Strikingly, CMV infection drives clonal expansions of adaptive NK cells, which parallel the epigenetic regulation of terminally differentiated cytotoxic T cells (Guma, 2004; Rückert et al., 2022; Schlums et al., 2015; Truitt et al., 2019). Loss of transcription factor PLZF expression in $CD56^{dim}$ NK cells demarcates the acquisition of adaptive properties in NK cells characterized by the stochastic loss of intracellular signaling adaptors FcεRγ, SYK, EAT-2 and the receptors for immunoregulatory cytokines IL-12 and IL-18 (Schlums et al., 2015). Hereafter, I will use the term "canonical" NK cells to refer to $CD56^{dim}PLZF^{+}$ NK cells and "adaptive" NK cells for $CD56^{dim}PLZF^{-}$ NK cells. Another transcription factor, Bcl11b, is instrumental in promoting adaptive NK cell differentiation, in part by silencing and antagonizing PLZF(Holmes et al., 2021). Adaptive NK cells elicit strong functional responses against CMV infected cells, but lack immunoregulatory abilities (Schlums et al., 2015). Also, ADCC and IFN-γ production via CD16 and NKG2C stimulation are enhanced in adaptive compared to canonical NK cells (Luetke-Eversloh et al., 2014; Schlums et al., 2015). In adaptive NK cells lacking FcεRγ, CD16 is associated exclusively with CD3ζ, which has more activating tyrosine-based activation motifs (ITAM) than FcεRγ, and potentially elicits stronger activating signaling (W. Liu et al., 2020). Moreover, adaptive NK cells exhibit long-term persistence, as demonstrated by the studies of Schlums and Jung (2017) and Corat and Schlums (2017) (Corat et al., 2017). Strikingly, adaptive NK cells seem to be more suited for the elimination of cancer cells in NK-based immunotherapy (A. B. Bigley et al., 2021). On top of these diversification mechanisms, educating NKG2A and killer-cell immunoglobulin-like receptors (KIR) interacting with cognate MHC-class I molecules permit NK cells responses against cells missing self-MHC-class I molecules, which are often downregulated by viral infections and cancerogenesis (Anfossi et al., 2006; Kim et al., 2005). Therefore, NK cells are an important complementary cytotoxic subset to the MHC-class I-dependent

activity of T cells and are thoroughly studied for the development of immunotherapy strategies against cancer (Wolf et al., 2023).

In conclusion, the pronounced diversity of phenotypes found among NK cell subsets (both in blood and other tissues), the plasticity of NK cell subsets and the recent characterization of intermediary subsets in tissues other than SLOs, suggest a branched developmental model where environmental cues and infections drive the diversification of terminal NK cell development (Cichocki et al., 2019) (figure 3).

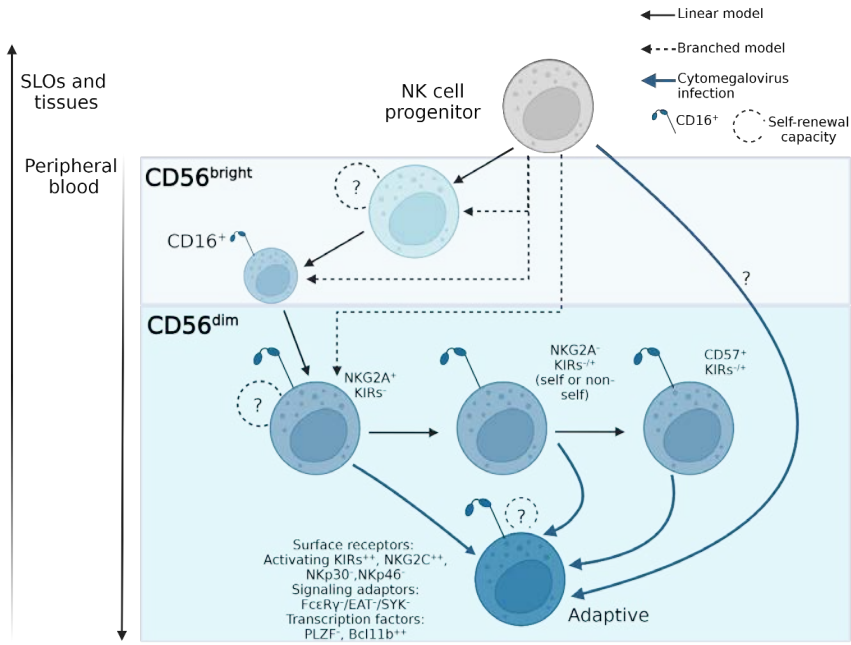

Figure 3. Human NK cell terminal development and subsets found in peripheral blood. Circulating NK cells are prevalently CD56^{dim}, while CD56^{bright} NK cells are found in secondary lymphoid organs (SLO) and tissues. The linear model of NK cell terminal development theorizes the differentiation of CD56^{dim} cells from CD56^{bright} cells, and further differentiation from NKG2A^{+} to CD57^{+} subsets. The branched model of NK cell development proposes that CD56^{bright} and CD56^{dim} subsets may originate from NK cell progenitor cells separately. Cytomegalovirus (CMV) infection drives the differentiation of adaptive NK cells that are characterized by clonally distributed surface receptors, signaling adaptors and transcription factors. "?" indicates current hypotheses about subset self-renewal and developmental pathways. References (Cichocki et al., 2019; Holmes et al., 2021; Tesi et al., 2016). Created with BioRender.com.

NK cell deficiencies

Studying IEIs affecting NK cells may uncover some requirements for NK cell development and function in humans. Even though many IEIs impact the NK cell line, some IEI have an NK cell deficiency (NKD) as their principal immunological manifestation (Mace & Orange, 2019). A common trait for NKD patients is the increased susceptibility to herpes viruses, and perhaps to certain types of cancer such as EBV-associated cancers (Mace & Orange, 2019). So far, 9 IEI have been grouped together as NK cell deficiencies (Table IV). Three of them, GATA2 (Mace et al., 2013), IRF-8 (V. Bigley et al., 2018; Mace et al., 2017) and ELF4 (Salinas et al., 2022) deficiency, are caused by LOF mutations in transcription factors (TF) genes, which regulate important transcriptional programs necessary for hematopoiesis (GATA2 (de Pater et al., 2013)) and NK cell maturation and function. Notably, adaptive subsets of $CD56^{dim}$ NK cells persisted in patients GATA2 haploinsufficiency despite the lack of $CD56^{bright}$ NK cells and the loss of hematopoietic progenitors (Schlums et al., 2017). This denotes the long-term survival of this subset and, perhaps, a renewal capacity that does not depend on continuous hematopoietic stem cell differentiation. The largest group of NKDs is constituted of CMG helicase deficiencies, namely MCM4 (Casey et al., 2012; Gineau et al., 2012; Hughes et al., 2012), GINS1 (Cottineau et al., 2017), MCM10 (Mace et al., 2020) and GINS4 (Conte et al., 2022). A homozygous mutation in another gene important for DNA repair and telomerase maintenance, *RTEL1*, was found in a patient that suffered from severe varicella infection (Hanna et al., 2015). The variants found in these patients are usually more or less hypomorphic, but all results in low numbers of circulating NK cells, especially within the subsets of $CD56^{dim}$ cells (Table IV). Thus, it has been hypothesized that this subset requires the expansion of $CD56^{bright}$ NK cells for its generation (Mace & Orange, 2019). The more these variants impair DNA replication, the more syndromic the patient phenotype is (e.g., some MCM4-deficient patients showed adrenal insufficiency and short stature (Gineau et al., 2012; Hughes et al., 2012). The reasons why NK cells suffer from in CMG helicase component haploinsufficiency more sensitive than T cells are unknown. Higher baseline transcription of helicase genes in T cells, and faster NK cell replication limiting the availability of CMG helicase and a sensitivity different from T and B cells to DNA damage are some of the hypotheses currently ongoing in the field (Conte et al., 2022). Moreover, there is an interest in understanding whether this sensitivity to DNA replication is stage specific during human NK development. As mentioned above, the molecular regulation of NK cell terminal differentiation, especially from Step 4 $CD56^{bright}$ to Step 5 $CD56^{dim}$ are not

completely understood yet. Therefore, the careful examination of patients susceptible to herpes virus infections with abnormal NK cell subsets in peripheral blood may help provide gainful insights. Finally, the only purely functional NKD described so far comprises LOF variants in *FCGR3A* encoding the CD16 activating receptor (De Vries et al., 1996; Grier et al., 2012; Jawahar et al., 1996). Other defects in NK cell synapse signaling and activation most often also affect T cell responses since these lymphocytes share several components of intracellular signal transduction.

Table IV. NK cell deficiencies

NKD	Gene Defect	Mode	Inheritance	Infections	NK cell amount (PB)	NK cell phenotype	References
CNKD	*GATA2*	Haploinsufficiency	AD	VZV, HSV, CMV, HPV, mycobacterial and fungal infections	↓↓↓	$CD56^{dim}$ (adaptive)	(Mace et al., 2013; Spinner et al., 2014)
CNKD	*IRF8*	Loss-of-function	AR	EBV, mycobacterial, pneumonia, viral respiratory infections	↓↓↓	$CD56^{bright}$	(V. Bigley et al., 2018; Mace et al., 2017)
CNKD	*ELF4*	Loss-of-function	XL	VZV, respiratory infections	↓↓	$CD56^{bright}$	(Salinas et al., 2022)
CNKD	*MCM4*	Hypomorphic	AR	EBV, HSV, VZV, respiratory infections	↓↓↓	$CD56^{bright}$	(Casey et al., 2012; Gineau et al., 2012; Hughes et al., 2012)
CNKD	*GINS1*	Hypomorphic	AR	VZV, HSV, CMV, RSV, adenovirus,	↓↓↓	$CD56^{bright}$	(Cottineau et al., 2017)
CNKD	*MCM10*	Loss-of-function	AR	CMV (fatal)	↓↓↓	$CD56^{bright}$	(Mace et al., 2020)
CNKD	*GINS4*	Hypomorphic	AR	VZV, HSV, CMV, pneumonia	↓↓↓	$CD56^{bright}$	(Conte et al., 2022)
CNKD	*RTEL1*	Loss-of-function	AR	VZV (fatal)	↓↓↓	ND	(Hanna et al., 2015)
FNKD	*FCGR3A*	Loss-of-function	AR	HSV, EBV, HPV, respiratory infections	Normal	Normal	(De Vries et al., 1996; Grier et al., 2012; Jawahar et al., 1996)

CNKD, classical NK deficiencies; FNKD, functional NKD; AD, autosomal dominant; AR, autosomal recessive; VZV, varicella zoster virus; HSV, herpes simplex virus; CMV, cytomegalovirus; HPV, human papillomavirus; EBV, Epstein-Barr virus; RSV, respiratory syncytial virus; PB, peripheral blood; ND, not determined.

SAMD9L

NK deficiency has also been described in patients with heterozygous GOF mutations in *SAMD9L*(Tesi et al., 2017). These patients have a syndrome characterized by ataxia, pancytopenia, variable neurological dysfunctions, immunodeficiency, and predisposition to myelodysplastic syndrome (MDS) and myeloid malignancies (D. H. Chen et al., 2016; Davidsson et al., 2018; Tesi et al., 2017). Predisposition to MDS has also been observed in patients with GATA2 haploinsufficiency, which besides isolated NKD in some patients, manifests as progressive immunodeficiency in monocytes, B, NK cells, and dendritic cells, and a variegated phenotype of clinical symptoms (Dickinson et al., 2014; Spinner et al., 2014). As immunodeficiency advances, patients become more and more susceptible to several infections, as listed in Table IV., and progress to MDS by the age of 60 (Collin et al., 2015). Deficiency in the same leukocyte lines have also been observed in SAMD9L GOF patients (Tesi et al., 2017). *SAMD9L* encodes a tumor suppressor that is upregulated following type I IFN signaling during viral infections

(Meng et al., 2018; Nagamachi et al., 2013; Schoggins et al., 2011). Ectopic expression of the heterozygous missense GOF mutations was found to negatively affect cell proliferation (Tesi et al., 2017). Further studies found that the anti-proliferative effect was due to SAMD9L-mediated translation block (Russell et al., 2021). Truncating GOF mutations in *SAMD9L* exacerbate SAMD9L anti-proliferative function potentially by abrogating regulatory domains yet to be defined (Russell et al., 2021). Some truncating GOF mutations downstream of the central p-loop domain of SAMD9L cause a different syndrome characterized by autoinflammatory features and termed SAMD9L-associated autoinflammatory disease (SAAD) (de Jesus et al., 2020). Cells with mutant GOF *SAMD9L* alleles tend to rescue the mutated allele, by either eliminating chromosome 7 (monosomy 7, -7), acquiring somatic *SAMD9L* LOF mutations in *cis*, or by uniparental isodisomy 7q (UPD7q) (Tesi et al., 2017). Monosomy 7 causes the predisposition to MDS and myeloid malignancies and is also found in patients with GATA2 haploinsufficiency (Sahoo et al., 2021; Wlodarski et al., 2016). Germline LOF mutations in SAMD9L have been identified in patients with late onset/adult MDS (Nagata et al., 2018). The loss of one allele of *SAMD9L* induces HSC proliferation, on top of which somatically acquired additional mutations may promote carcinogenesis (Nagata et al., 2018). Similarly, *Gata2* haploinsufficiency promotes HSC proliferation (Abdelfattah et al., 2021). Truncating GOF and LOF mutations in *SAMD9L* tend to be spatially segregated. Truncating LOF mutations are most frequently found at the N-terminus while a series of truncating mutations downstream the predicted NTPase domain have strong GOF effects (Allenspach et al., 2021; Russell et al., 2021). This is not an absolute rule as illustrated in figure 4. The protein domains of SAMD9L and their function are not fully characterized yet, but first insights have come from evolutionary conservation studies and predictions (Mekhedov et al., 2017). Investigating the impact of various SAMD9L variants may reveal the molecular mechanisms behind SAMD9L-mediated inhibition of mRNA translation and cell proliferation during anti-viral responses, shedding light on potential connections to hematological cancers. Due to the high sensitivity of NK cells to mutations affecting the cell cycle (Mace & Orange, 2019), it is possible that the NK cell deficiency found in these patients is a direct consequence of the antiproliferative activity of pathogenic SAMD9L GOF variants. Allegedly, similar defects in NK cells may also be observed in patients carrying GOF variants in *SAMD9*, a paralogous gene of *SAMD9L*, which has a similar function and has been found in patients with the MDS, infection, restriction of growth, adrenal hypoplasia, genital phenotypes,

and enteropathy (MIRAGE) syndrome (Narumi et al., 2016), but none has been reported so far.

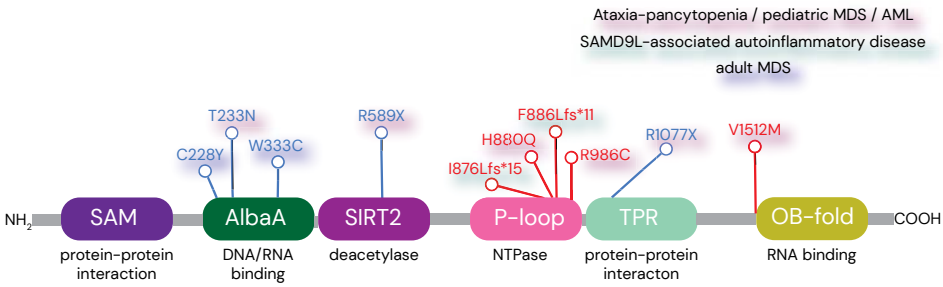

Figure 4: Predicted SAMD9L domain architecture and some pathogenic and revertant variants found in patients with *SAMD9L*-associated diseases. GOF variants are indicated in red, LOF variants in blue. SAM= Sterile alpha motif; SIRT2= silent information regulator 2; P-loop= predicted NTPase domain; TPR= tetratricopeptide repeats; OB-fold= oligonucleotide/oligosaccharide-binding. MDS= myelodysplastic syndrome, AML= acute myeloid leukemia. References (D. H. Chen et al., 2016; de Jesus et al., 2020; Mekhedov et al., 2017; Nagata et al., 2018; Pastor et al., 2018; Tesi et al., 2017)

Regulation of the immune synapse formation

Granule-mediated cytotoxicity requires the exocytosis of vesicles containing perforin and granzymes against a target cell. Central to this process is the formation of a functional cytolytic immune synapse (IS) by an activated cytotoxic lymphocyte adhering to a target cell. The IS is a nanoscale organization of adhesion and activating receptors and has been classically described as divided in three zones in T cells. A lamellar branched actin structure is formed during the initial spread of the T cell onto an antigen-presenting cell (APC) (J. A. Hammer et al., 2019). This structure is then centrally cleared to make space for the peripheral supramolecular activation cluster (pSMAC) where T cells accumulates LFA-1 integrin molecules to firmly adhere to the APC, and which enclose the central SMAC (cSMAC) where TCRs and activating receptors cluster (de la Roche et al., 2016; J. A. Hammer et al., 2019; Ritter et al., 2015). An ulterior structure of branched actin filaments surrounding the pSMAC is called distal SMAC (dSMAC) is the residue of the initial spreading of the T cells onto its target (J. A. Hammer et al., 2019). In CTLs, the CGs are recruited and secreted against the targets in correspondence of the cSMAC (Stinchcombe, Bossi, et al., 2001). Likewise, NK cells adhere to target cells by forming organized structures promoted by the adhesion molecule LFA-1 that coordinates the segregation of different synergistic coactivation receptor-ligands pairs, and the establishment of a central area where

exocytosis of CGs occurs (D. Liu et al., 2009). The so organized IS is called mature, and it has been reported that CTLs necessitate of circa ten complexes of stimulatory peptides mounted on MHC molecules to achieve full activation and to form a mature IS (Purbhoo et al., 2004). Still, release of CGs may happen also in the presence of only three TCR- peptides MHC complexes without the formation of a mature IS (Purbhoo et al., 2004). Another study suggested the existence of a dual activation threshold regulating the faster formation of cytolytic synapses and the more organized synapses necessary for cytokine production (Faroudi et al., 2003). Such a distinction may explain the ability of CTLs to rapidly kill multiple targets simultaneously while engaging stronger interactions necessary for cytokine production (Wiedemann et al., 2006). Similarly, the cytolytic IS induced by CD16 and LFA-1 does not require the organized distribution of these two receptors to induce the formation of a fusion permissive area in NK cells and rapid CG secretion in NK cells has also been reported (D. Liu et al., 2009).

Signalling from the mature IS directs a cytoskeletal reorganization within the cells that is necessary for: a) the polarization at the IS of the microtubule-organizing centre (MTOC) where the CGs converge b) the creation of a fusion permissive actin environment at the cSMAC where the granules pass through before fusing in correspondence of the plasma membrane (Brown et al., 2011; Mace et al., 2014; Rak et al., 2011). For example, in *DOCK8* deficiency the integrin-mediated signalling from the IS and the polarization of actin at the IS are impaired, resulting in altered MTOC polarization and CGs recruitment (Ham et al., 2013). In *CORO1A* deficiency, the lack of the actin regulator Coronin 1A impairs the creation this hypodense actin areas (Mace & Orange, 2014). CG convergence is important for efficient target cell killing and for preventing unspecific elimination of healthy cells nearby (Hsu et al., 2016). Notably, signals regulating MTOC polarization and CGs exocytosis can be uncoupled in NK cells, since the engagement of synergistic coactivation receptors or CD16 alone is sufficient to induce unpolarized secretion of CGs while LFA-1 signalling directs MTOC relocation at the IS (Bryceson et al., 2005). This property can be exploited to study the relative contribution of novel candidate proteins to CG recruitment and exocytosis.

Inborn errors of immunity affecting actin regulation and lymphocyte cytotoxicity

IEIs impairing the remodelling of the cytoskeleton often impair lymphocyte cytotoxicity. The archetype of actin regulation immunodeficiencies is the Wiskott-Aldrich syndrome (WAS), an IEI with many clinical manifestations

such as immunodeficiency, haemorrhages, thrombocytopenia, atopy, autoimmunity, and increased susceptibility to cancer (Candotti, 2018). In 1994, the gene causing WAS was isolated and named *WASP*, WAS protein (Derry et al., 1994). WASP is an actin polymerization regulator that activates ARP2/3, which in turn nucleates actin in branched structures. The conditions caused by inborn-mutation in *WASP* are multiple, going from the "classic" most severe symptoms, where LOF mutations severely compromise, or abrogate, WASP function and expression, to milder X-linked thrombocytopenia, where the gene is expressed but carries deleterious missense mutations (Candotti, 2018). GOF variants in *WASP* causes X-linked neutropenia (XLN) characterized by early onset of bacterial infections, lack of mature neutrophils and impaired lymphocyte activation accompanied by genomic instability (Westerberg et al., 2010). In both WAS and XLN patients, NK cell cytotoxicity is reduced, and the cells have difficulties in forming conjugates with target cells and accumulate actin at the IS (Gismondi et al., 2004). WASP-mediated actin regulation in the IS creates actin foci in the peripheral and distal SMAC, which are important for the organization of signalling molecules such as LFA-1 and TCR micro-clusters (J. A. Hammer et al., 2019). Despite cytotoxicity is diminished in WASP-deficient NK cells (Orange et al., 2002), IL-2 activation partly rescues this defect thanks to the activation of a ARP23/3 homologous protein, WAVE2 (Orange et al., 2011), which is also important for the creation of the branched dSMAC in T cells immune synapse (J. A. Hammer et al., 2019). HEM1 deficiency (which is part of the WAVE complex) has been recently described by three independent studies (Castro et al., 2020; Cook et al., 2020; Salzer et al., 2020). Despite defects in actin accumulation at the IS, degranulation in HEM1-deficient NK and T cells was not impaired, but uncontrolled because of cortical actin structures as described above (Castro et al., 2020; Cook et al., 2020). Still, some HEM1-deficient patients presented with HLH-like symptoms (Castro et al., 2020). This may be due to the dysregulated inflammasome activation as observed in patients with *CDC42* (Castro et al., 2020). Interestingly, Cook (2020) also found an actin independent role for HEM1 in promoting the activation of the mammalian target of rapamycin 2 complex (mTORC2) that is important for T cell proliferation and cytokine production that were found reduced in patients' derived T cells (Cook et al., 2020). These findings highlight that multiple and diverse cellular processes can be impaired by actin dysregulation, and that proteins regulating actin may also take part to other important processes. Regardless of the multitude of molecular mechanisms affected, some common themes have been identified in actin regulation

deficiencies. Namely, cytopenia in the myeloid compartment, low absolute numbers of diverse lineages of leukocytes, dysfunctions in lymphocyte function, recurrent infections, autoimmunity and autoinflammation (Papa et al., 2021). Small Rho GTPases are important hubs for actin regulation during lymphocytes responses. They undergo cycles of activation and de-activation where they bind or are released from intracellular membranes (figure 5). In the active form, they transmit signals to effector proteins directly organizing actin such as WASP. Several mutations in Rho GTPases, and their regulators, expressed in the hematopoietic system have been recently linked to syndromes with hyperinflammation and defective cytotoxicity (El Masri & Delon, 2021) Importantly, missense mutations of single residues may result in different protein localization, regulation and consequently, a variegated clinical presentation. For instance, this has been seen in patients with different missense GOF/dominant negative variants in *CDC42* (Coppola et al., 2022).

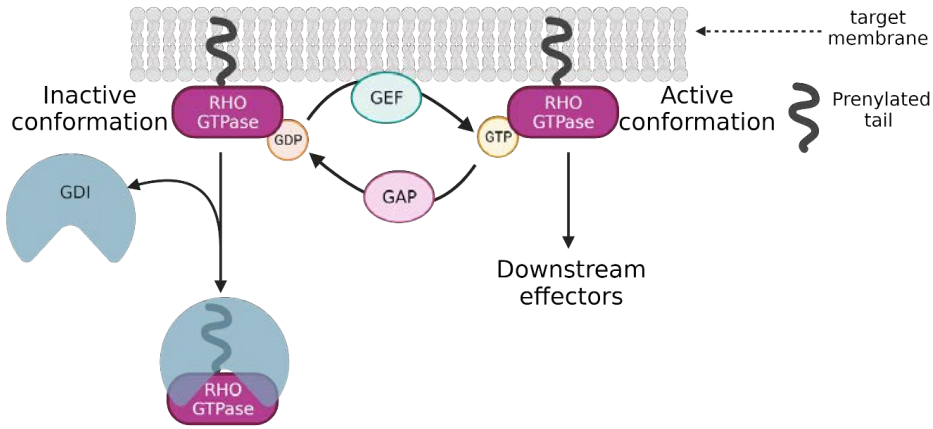

Figure 5. Regulation of Rho GTPases. Rho GTPases can bind the membrane of several organelles and vesicles thanks to their prenylated C-terminal CAAX motif. Like other classic monomeric GTPases, Rho GTPases cycle continuously from a guanosine diphosphate (GDP)-bound inactive conformation to an active one bound to guanosine triphosphate (GTP). Guanosine nucleotide exchange factors (GEFs) activate Rho GTPases to release the GDP and bind to GTP. On the other hand, GTPase activating proteins (GAPs) inactivate Rho GTPases by promoting the hydrolysis of GTP in GDP. In the inactive conformation, Rho GTPases can be extracted by GDP dissociation inhibitors (GDI) from the target membranes and kept in the inactive conformation in the cytosol. Created with BioRender.com.

Cytotoxic granule exocytosis

The fusion of CG at the PM in the centre of the IS is a finely regulated process, of which we know less than exocytosis in other cells like neurons. The core machinery promoting membrane fusion is the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complex. This complex is formed by 2 N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins located on the target membrane (t-SNARE) and one on the vesicle membrane (v-SNARE) (Thorn et al., 2016). The SNARE complex formation is assisted by several proteins mediating SNARE complex components trafficking, assembly, and calcium-depending activation, which ultimately triggers membrane fusion. In neurons, the identity of all the SNARE proteins and most of proteins regulating synaptic vesicle fusion is known, and the structure of the pre-fusion assembled SNARE complex has been recently crystallized (Brunger et al., 2019; Zhou et al., 2017). Notably, the identity of the v-SNARE protein found on the membrane of cytotoxic lymphocytes is debated, because different vesicle-associated membrane proteins (VAMPs) have been found on CGs in mice and human cytotoxic lymphocytes. VAMP2, the one proposed in mice, is not expressed in human cytotoxic lymphocytes, though it is the v-SNARE found on neuronal synaptic vesicles (Spessott et al., 2017). Divergence seems to exist between mice and human, and more than one VAMP protein regulates GC fusion at the PM in NK and T cells. Recently, one study suggested the requirement of VAMP7 in human CTLs and the same VAMP has been found to regulate cytotoxic granule release in a NK cell line (Chitirala et al., 2019; Marcet-Palacios et al., 2008).

Remarkably, many of the proteins regulating CG exocytosis have been identified by studying FHL patients (figure 6). SYNTAXIN-11 is a t-SNARE protein participating to the SNARE complex in cytotoxic lymphocytes and LOF mutations in the *STX11* gene cause FHL4 with CG polarizing at the IS, but unable to fuse (Bryceson et al., 2007; zur Stadt et al., 2005). Still, NK cells from FHL4 patients that have been stimulated with IL-2 partially recover their ability to degranulate and kill target cells (Bryceson et al., 2007). A study by Hackmann (2013) suggests that the upregulation of SYNTAXIN-3 upon IL-2 stimulation can compensate the lack of SYNTAXIN-11 (Hackmann et al., 2013). Another t-SNARE protein proposed as part of the SNARE complex in cytotoxic lymphocytes is SNAP-23, as it has been shown to interact with SYNTAXIN-11 (Hellewell et al., 2014; Spessott et al., 2017).

Priming renders the docked CG vesicles fusion competent by opening SYNTAXIN-11 during the assembly of the SNARE complex. In neurons, the protein

Munc13-1 and Munc18-1 have been identified as crucial priming factors for Syntaxin-1 (Rizo, 2022). Therefore, a similar function has been hypothesized for their homologous proteins expressed in cytotoxic lymphocytes: MUNC13-4 and MUNC18-2. MUNC18-2 is a chaperon protein for SYNTAXIN-11 regulating its stability and assembly in the SNARE-complex (Spessott et al., 2017). Inborn mutations abrogating MUNC18-2 protein expression or interaction with SYNTAXIN-11 compromise SYNTAXIN-11 stability and result in FHL5 (Cote et al., 2009; zur Stadt et al., 2009). Alike FHL4, an IL-2-dependent compensatory mechanism has been proposed in MUNC18-2 deficiency (zur Stadt et al., 2009). The loss of MUNC13-4 function instead completely blocks CG exocytosis and advocate for an irreplaceable function of this protein in this molecular process. Accordingly, FHL3 is the second most severe FHL following FHL2 (Sepulveda et al., 2013). LOF mutations in *UNC13D* where first identified as the cause of FHL3 where CG are polarized at the IS but were unable to fuse (Feldmann et al., 2003). Moreover, MUNC13-4 was found to regulate CG biogenesis by regulating the $RAB11^+$ recycling endosomes and $RAB27A^+$ late endosome fusion (Ménager et al., 2007). The interaction between RAB27A on CG and MUNC13-4 is specifically required for CG arrest at the IS, and mutations impairing this interaction did not abrogate CG polarization, but impaired docking at the PM (Elstak et al., 2011). LOF mutations in *RAB27A* cause GS2 characterized by albinism and HLH (Ménasché et al., 2000). The regulation of pigmentation in melanocytes depend indeed by the interactor between RAB27A and its downstream effector protein melanophilin. Still, in some patients with HLH due to missense mutations in *RAB27A*, pigmentation is normal (Ohishi et al., 2020; Zondag et al., 2022). This is due to diverse interaction sites between *RAB27A* and its cell type-specific downstream effectors (Ohishi et al., 2020; Zondag et al., 2022). Many questions regarding MUNC13-4 trafficking and regulation at the PM of cytotoxic lymphocytes still stand. LOF mutations in the first intron of *UNC13D* causing HLH have indeed led to the identification of two isoforms of this protein in hematopoietic cells (Cichocki et al., 2014; Meeths et al., 2011). The second isoform is predominantly expressed in lymphocytes and platelets but has equivalent intracellular localization and function to the conventional one (Galgano et al., 2020). The CNS dysfunctions observed in in FHL3 (Sieni et al., 2011) potentially imply an unexplored role for MUNC13-4 in the nervous system. Finally, it is unsure how MUNC13-4 binds CGs to the PM because, unlike other MUNC13 family members, it lacks a C1 lipid-binding domain (Phatarpekar & Billadeau, 2020). **Paper I** proposes that MUNC13-4 is assisted by RHOG during the exocytosis of CGs.

After priming of the SNARE complex, an influx of extracellular calcium promotes membrane fusion and release of CG content in the synaptic cleft (Phatarpekar & Billadeau, 2020). Indeed, impaired lymphocytes cytotoxicity have been described in patients with mutations in *ORA1* and *STIM1*, encoding for a sensor of intracellular calcium deposit in the ER that opens the extracellular calcium channels in cytotoxic lymphocytes (Maul-Pavicic et al., 2011). Synaptotagmins are the calcium sensor of the neuronal SNARE complex by inhibiting fusion in the absence of a calcium stimulus, but the lack of Synaptotagmin-7 in mice CTLs did not impair CG fusion (Sleiman et al., 2020). Instead, a recent study proposed Munc13-4 as the calcium sensor regulating CG fusion dynamics and the activation of the SNARE complex (Bin et al., 2018).

After fusion, perforin is released in the synaptic cleft and creates transient pores in the PM of the target cell allowing the entrance of granzymes in the cytoplasm (Lopez et al., 2013). Once inside the cytoplasm, granzymes trigger target cell apoptosis by both caspase-dependent and -independent mechanisms (Darmon et al., 1995; Martinvalet et al., 2005).

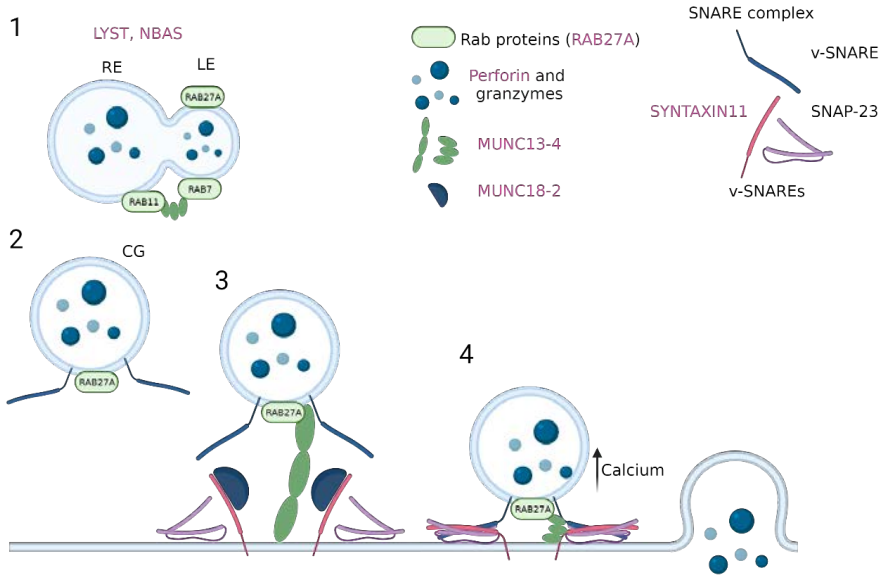

Figure 6. Cytotoxic granules (CG) exocytosis at the immune synapse. The proteins associated with familial forms of HLH are indicated in pink. 1. The biogenesis of cytotoxic granules is still poorly defined, but mutations in some proteins controlling vesicular trafficking and lysosome function presume their involvement in this process. MUNC13-4 promotes RAB11^{+} recycling endosomes (RE) and late endosomes (LE) fusion during CG biogenesis. 2. CGs are then trafficked to the plasma membrane via microtubule first and actin filaments later (not shown). 3. At the plasma membrane, CGs are docked and tethered by the interaction between MUNC13-4 and RAB27A. The t-SNARE proteins SYNTAXIN-11 and SNAP23 are already there. SYNTAXIN-11 is trafficked to the plasma membrane via RE that fuse prior the CG and position the protein in the sites where the SNARE complex will be formed (not shown). MUNC18-2 stabilizes SYNTAXIN-11 and keeps it in a close conformation. 4. MUNC13-4 primes the SNARE complexes for membrane fusion by opening the conformation of SYNTAXIN-11. Membrane fusion happens following local upregulation of calcium and the granule content is released in the synaptic cleft (de Saint Basile et al., 2010; Ham et al., 2022; Marshall et al., 2015). Created with BioRender.com.

2 Research aims

In the Yenan Bryceson laboratory, we study samples from patients with suspected HLH or IEIs, or with phenotypes that are interesting for our research purposes in cytotoxic lymphocyte biology. Initially, we use patients' samples to assess cytotoxic lymphocyte functions and development, and we screen for the absence of known disease-causing proteins. By doing so, we may speed up or confirm patients' diagnosis. When our immunological screenings do not pin-point any of the known proteins suspected to cause a patient's disease, we send patient's DNA for NGS sequencing. Possible candidate genes are then arranged according to their role in cytotoxicity, and in other relevant processes during immune responses. In some cases, the published evidence is not sufficient to validate the top candidate genes as disease-causing, and laboratory work is needed to confirm their role in disease pathogenesis. Alternatively, we select patients with known molecular defects and further explore how these deficiencies impact cytotoxic lymphocytes.

The specific research aims of **paper** I were to functionally validate *RHOG* as a novel gene causing familial HLH. In **paper II**, we focused on studying the interactions between pathogenic SAMD9/9L GOF mutants and poxviral host range factors to establish how much the GOF activity of these variants was modulated by factors known to antagonize wild type SAMD9/9L functions. Having observed a peculiar NK cell phenotype in a DEF6-deficient patient, we investigated the role of DEF6 in NK cell biology in **paper III**.

Overall, the goal of my thesis was to boost the present knowledge of cytotoxic lymphocyte biology with a focus on function and development in humans. This knowledge will improve patients' diagnostics in the contexts of hyperinflammation and severe infections and may be capitalized in cancer immunotherapy approaches based on lymphocyte cytotoxicity.

3 Ethical considerations

From an ethical point of view, research with human and patients' material poses some issues that I will try now to explain relative to the four "default" principles of medical research ethics stated by the American philosopher Jim Childress and Tom Beauchamp in their book "Principles of medical bioethics", New York, Oxford University Press, 1979.

1. **"Doing good" or the principle of beneficence.**
   The final aim of medical scientific research is to provide a benefit for the patients and the humanity in general. In our research, I can identify a short- and a long-term application of this principle. The short-term application is that the data obtained by assessing patients' immune system function and by DNA sequencing is quite often fundamental for HLH diagnosis and therapy, helping the treating doctors in tailoring the regime of anti-inflammatory drugs and deciding whether or not to proceed with bone marrow transplant. Since the FLH patients are usually very young children, the finding of damaging mutations inhereted by the patient's parents is also a foundation for genetic counseling. In the long-term, studying patients' clinical history and data will be used to gain further knowledge in HLH pathophysiology and cytotoxic lymphocytes in general. This will benefit both HLH patients and immunotherapy research that relies on cytotoxic lymphocyte to kill cancer cells. Nevertheless, "doing good" for both patients and society has sometimes opposing interests. As mentioned above, it is difficult in many cases to obtain a definitive molecular diagnosis for HLH and often the cause cannot be identified. For the patient's own benefit it might be optimal to always perform WGS analyses, but this has a cost in terms of time and money and it could be counterproductive for our laboratory to analyze every patient we screen in this way. Therefore, we may not proceed with in depth genetic analyses unless the patient's phenotype and initial findings are relevant for our research purpuses. Still, we always refer to the treating doctor all our observations, which may help the doctor in finding the correct diagnosis.
2. **"Avoiding harm" or the principle of non-maleficence.**
   Almost all the screening assays we run are performed on peripheral blood. Draining blood is always performed by qualified healthcare professionals and is associated with very little risk for the patient. Occasionally, we may use other types of specimens (saliva, skin biopsies, and tissue material from operation). Also in this case, the healthcare professionals will be responsible for collecting

the material, and we do not require biological tissue such as skin biopsies unless there an utter evidence of its importance in the study. Especially when dealing with sequencing data, protecting patients' and families' privacy is mandatory. All the samples are coded and cannot be linked directly to the individuals. The genetic sequences may be stored in databases that are accessed by other researchers. Still, only the people directly involved in patients' studies can retrieve the identifying codes.

The purpose of the DNA sequencing we perform is to identify the damaging mutation responsible for patients' symptoms and data analysis is focused in studying genes involved in immune function. Nevertheless, collateral findings are not unthinkable and we must refer them to the treating doctor, especially if they have clinical relevance for the patient. The treating doctors are responsible for the genetic counseling of the family and we do not directly interact with them. A particular care must also be put also in explaining the results of DNA sequencing. Findings may be not always conclusive and we may only provide a conservative assessment of which genes can contribute to the disease. To avoid instilling patients and families with too much hope, they need to be well informed about the possible inconclusive outcome of our investigations.

3. **Respect for autonomy.**

In our research we are obviously obliged to collect informed consent from the patients or their relatives in case of small children. Informed consents have evolved along with national ethical regulations and guidelines. The information stresses out the possibility of inconclusive results from and aims to make participants understand the complexity of NGS. We also provide information about how the privacy protection and we affirm the voluntariness of participation. The subjects and their families are entitled to withdrawal from the study whenever they want, and the genetic material will be destroyed from our database. Even though I personally find the content of the letter suitably informative and balanced, the autonomy of a parent whose child has a life-threatening condition is questionable due to the heavy emotional distress. There might not be a definitive way to solve this issue. Still, our group is contacted by the treating doctors, and we do not actively recruit patients for our studies. Therefore, I think respecting the parents' autonomy is, for a large part, up to the treating doctors.

4. **Justice.**
   The justice principle affirms that "equals must be treated equally". Therefore, researchers should not perform research on vulnerable subjects to help a more powerful group.
   Our patient samples do not come only from Sweden. Sometimes, they are from countries where the healthcare system is different, or more limited, than Sweden. There is definitively a possibility that some patients coming from poorer countries will not receive the same level of healthcare assistance that a Swedish patient gets. Still, our research is not aimed to favor a particular group, it is meant for all HLH patients notwithstanding of their origin. Scientific publications are, or should be, globally accessible to help clinicians anywhere in treating their patients. As such, Karolinska institutet actively supports open access policies. Finding new disease-causing genes can significantly speed up the diagnosis and direct primary HLH patients to bone marrow transplant, which is nowadays available in all the countries we are working with.

In these pages, I listed some ethical considerations, which are relevant for the research I pursued at Karolinska Institutet. Some concerts may still stand. Even if our ethical permit allows us to process samples coming from abroad, the Swedish regional committee cannot approve research on patients located outside Sweden and, jurisdictionally speaking, we will need a permit from each healthcare center we are collecting patient material from. Nevertheless, these samples are referred to us for diagnostics from the treating doctors who may not have access to the same expertise and the technologies we provide. For many of these patients, the severity of their conditions requires immediate intervention, and we can supply some results within weeks from obtaining the blood samples. In doing this, we have a strong commitment to respect at all steps of the process the principals of medical research ethics.

4 Results and discussion

4.1 Improving HLH diagnostics: *RHOG* is a novel FHL disease-causing gene.

The study of **paper I** began with a 4-month-old child who came to the clinic with severe hyperinflammation. The patient fulfilled the following HLH criteria: unremitting fever, hepatosplenomegaly, cytopenias (low hemoglobulin and low leukocyte absolute number), elevated serum ferritin, hypertriglyceridemia, and elevated soluble IL-2 receptor. Hemophagocytosis was found in the patient's bone marrow. When we examined patient's derived NK cells, we found decreased exocytosis and cytotoxicity compared to parents and controls. Overnight incubation with IL-2 was able to improve both functions, as it has been demonstrated in some FHL patients(Bryceson et al., 2007; Pagel et al., 2012), but patient's cells never achieved the same capability of the controls. As reported in **paper I**, perforin was found expressed at normal level in the patient's $CD3^{-}CD56^{+}$ cells by intracellular flow cytometry. We did not find any variant in known FHL genes by targeted sequencing. By immunoblot on patient's expanded T cells, we found normal expression of known FHL-causing genes (*UNC13D*, *STX11*, *STXBP2*, *RAB27A*) and therefore excluded both coding and non-coding biallelic LOF variants in these genes. The DNA from the patient and his parent were subsequently subjected to WES and a rare missense variant in *RHOG* (c.511G>A; p.Glu171Lys with a CADD score of 31, the variant was not reported in the population studies available at the time (Lek et al., 2016)) was ranked as the most likely disease-causing variants. Further investigations determined that the patient had inherited this variant from his mother, while the other parent carried a 33 kb deletion (Chr11:3,848,730-3881730) that span the whole *RHOG* locus and was inherited by the patient too. Therefore, *RHOG* remained the only gene carrying candidate LOF biallelic variants. Hence, the next studies in **paper I** focused on confirming the deleterious effects of the c.511G>A; p.Glu171Lys missense variant (hereafter referred to as p.E171K) on one side, and on investigating the physiological role of RHOG in CG exocytosis on the other.

From a structural point of view, the p.E171K variant resulted in the change of one evolutionary conserved amino acid with one with an opposite charge. The mutated site was within the C-terminal α5 chain that is important for RHOG protein-protein interactions (Prieto-Sánchez & Bustelo, 2003), and close to a region that in small Rho GTPases is important for oligomerization (B. Zhang &

Zheng, 1998). Incorporation of an amino acid with an opposite charge into a folded domain often causes protein misfolding and instability, which was reflected by the high CADD score of this variant (31). Indeed, we found no expression of RHOG in patient's derived cells. By overexpressing the mutant protein in HEK293FT cells, we saw reduced protein expression levels compared to the wild type. These experiments confirmed that *RHOG* expression was lost in the patient. On the other hand, a *RHOG* KO NK cell line (NK92) was established and displayed defect in exocytosis too. Importantly, knocking down RHOG expression with small interfering RNA (siRNA) in cytotoxic $CD8^+$ T cells isolated from healthy blood donors, reduced degranulation without affecting cytokine production.

RHOG regulates actin dynamics with functions depending and not depending on other small Rho GTPases downstream its activation (Gauthier-Rouvière et al., 1998; Katoh & Negishi, 2003; Prieto-Sánchez & Bustelo, 2003; Wennerberg et al., 2002). Its action regulates cell migration (Katoh et al., 2006) and lymphocyte cell morphology(Vigorito et al., 2003). Therefore, we explored the dependence of RHOG function for CDC42, RHOA and RAC1 mediated actin regulation in cytotoxic lymphocytes. We found that pharmaceutically promoting RAC1 activity restored the decrease in filamentous actin observed in *RHOG* KO cells. The remodeling of cortical actin at the IS was also impaired in RHOG-deficient NK92 cells, with the loss of low-density actin zones that are necessary for CG access to the IS plasma membrane and subsequent fusion (Brown et al., 2011; Rak et al., 2011). Such remodeling could be recovered upon promoting RAC1 function. Still, the degranulation defects of RHOG-deficient cells were only partly rescued by promoting Rac 1 activation. Therefore, some RHOG-dependent functions in cytotoxic lymphocytes are Rac1- and actin polymerization-dependent, and some are not.

In mouse platelets, RhoG plays a role in the exocytosis of α-granules (Goggs et al., 2013), but a clear molecular mechanism for this function was unknown at the time our study began. To determine how RHOG regulates CG exocytosis, we performed a proteomic screening following affinity purification of Strep-hemagglutinin-tagged RHOG. We found and confirmed an interaction between RHOG and MUNC13-4, whose biallelic LOF mutations cause FHL3 (Feldmann et al., 2003). MUNC13-4 is an important regulator of SNARE complex formation in cytotoxic lymphocytes and other immune cells (Elstak et al., 2011). It belongs to the MUNC13 family of synaptic priming proteins, where neuronal MUNC13-1, bridges synaptic vesicles and plasma membrane while opening SYNTAXIN-1 during

neuronal SNARE complex priming (Rizo, 2022). Importantly, biallelic LOF mutations in *UNC13D*, the gene coding for MUNC13-4, causes FHL3 (Feldmann et al., 2003). Its neuronal homologous, MUNC13-1, has an additional diacylglycerol (DAG)/phorbol ester-binding C1 domain that enables this protein to directly bind cellular membranes (Brose et al., 2000). This domain is not present on MUNC13-4 (Feldmann et al., 2003), therefore leaving open the question of whether this protein can bind phospholipid membranes directly. Being RHOG a classic Ras family member, it can interact with cellular membranes thanks to its C-terminal prenylated CAAX motif (M. Wang & Casey, 2016). We therefore speculated that the interaction between RHOG and MUNC13-4 is necessary for MUNC13-4 carrying vesicles to attach to the plasma membrane prior to CGs release. Full-length MUNC13-1 ectopic expression overcame the degranulation defect in *RHOG* KO NK92 cells, but a MUNC13-1 without the C1 domain could not. We further verified RHOG-assisted binding of MUNC13-4 to cellular membrane with protein-lipid overlay assays.

Paper I adduced a function for RHOG in lymphocyte cytotoxicity and supported a role of this gene in FHL (figure 7). No HLH patients carrying biallelic LOF mutation in *RHOG* had been identified prior to this study, and none has been identified so far. A possible explanation is that being the gene very small (one intron and one coding exon for a total of 1295 bp corresponding to 191 amino acids), the likelihood of two-hits mutations is reduced compared to other larger FHL-associated genes. Moreover, this protein is reported in gnomAD v2.1 with a pLI of 0.43 and an o/e score of 0.19, which implies some negative selection for LOF mutations in *RHOG*. Therefore, the chance of biallelic LOF inheritances in non-consanguineous settings will carry biallelic LOF variants is quite low. The onset of FHLs positively correlates with the severity of the defect in lymphocyte cytotoxicity, with *PRF1* complete LOF (FHL2) and FHL3 (LOF in *UNC13D*) being the earliest, while FHL4 (*STX11*) and FHL5 (*STXBP2*) usually manifest later in life(Pagel et al., 2012; Sepulveda et al., 2013). Since the patient identified in **paper I** presented with HLH at 4 months of age, we hypothesize that the defect in cytotoxicity in patients lacking RHOG function is very severe, and the few patients with this genotype may have not been sequenced before death. To conclude, our experimental approach in **paper I** fulfilled the requirements for single-patient studies by demonstrating the complete penetrance, the deleterious functional consequences, and the causal relationships between biallelic LOF variants in

RHOG and HLH (J. L. Casanova et al., 2014). Therefore, we propose *RHOG* as a novel FHL-causing gene.

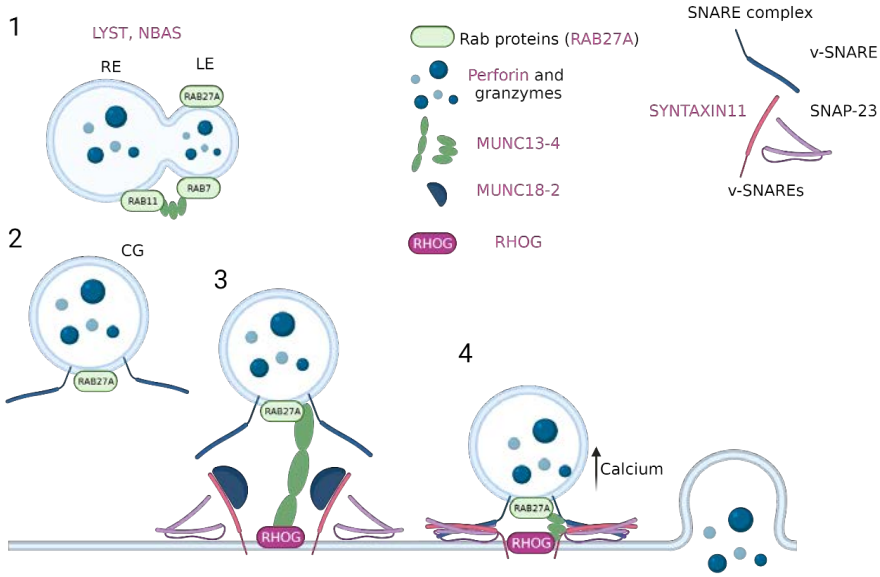

Figure 7. Cytotoxic granules (CG) exocytosis at the immune synapse. The proteins associated with familial forms of HLH are indicated in pink. In the model proposed in **paper I**, RHOG helps MUNC13-4 in anchoring at the plasma membrane by directly attaching to the bilayer with its prenylated tail.

4.2 Molecular regulation of SAMD9/SAMD9L activity: viral host range factors antagonized both wild-type and mutant SAMD9 and SAMD9L variants

Mutations in *SAMD9* and *SAMD9L*(*SAMD9/9L*) cause a plethora of human diseases, among which MDS and predisposition to myeloid leukemia. *SAMD9/9L* are IFN-regulated genes and encode two tumor suppressor proteins with anti-viral activity. SAMD9 and SAMD9L (SAMD9/9L) have emerged as anti-viral factors particularly against poxviruses infection (J. Liu & McFadden, 2015; Meng et al., 2018), but interaction with other viral proteins have been documented too (J. Wang et al., 2016). Reciprocally, many viruses have evolved proteins that can directly bind and antagonize SAMD9/9L activity (Meng et al., 2018). In **paper II**, we decided to exploit these interactions to examine whether viral proteins known to antagonize wild type SAMD9/9L could still bind pathogenic SAMD9/9L. On one hand, we hoped that this approach would allow new insights into the molecular mechanisms underlying SAMD9/9L regulation. On the other, we wanted to explore whether regulation of mutant SAMD9/9L was possible. Of the well-known poxviral anti-SAMD9/L factors, we chose the myxoma virus derived M062 and Vaccinia virus K1 and C7. M062 is known to antagonize SAMD9/9L by binding to their N-termini, specifically to the predicted SAM and AlbaA domains (Nounamo et al., 2017), while M064 is unable to antagonize SAMD9/L and was chosen as negative control (Meng et al., 2015). From the Vaccinia virus we chose K1 and C7 that inhibit wild type SAMD9/L (Meng et al., 2018). For SAMD9L, we chose to test the following missense variants: the missense GOF variants p. R986C falling in the C-terminus of the p-loop in the NTPase domain and the p. V1512M falling in the OB-domain. Both GOF variants have been often reported in patients with an ataxia pancytopenia phenotype and familial predisposition to MDS (D. H. Chen et al., 2016; Pastor et al., 2018; Tesi et al., 2017). Among the truncating variants found downstream the p-loop of the NTPase domain, which cause autoinflammatory SAAD syndrome and are the ones with the highest transcription repression activity (Allenspach et al., 2021; de Jesus et al., 2020; Russell et al., 2021), we picked the frame shift mutation p.F886Lfs*11 (de Jesus et al., 2020). We included a disease attenuating LOF variant p.T233N, which was inherited in cis to the GOF p.R986C in mildly affected individuals (Tesi et al., 2017). The same variant is adjacent to variants acquired somatically in patients with in late-onset MDS (Nagata et al., 2018). For SAMD9, we selected the GOF MIRAGE variants p.R489Q, p.P1280L, p.R1293W (Narumi et al., 2016). As potential LOF variants we included the variant p.K1495E that was identified in a family with autosomal recessive

normophosphatemic familial tumoral calcinosis (NFTC) (Topaz et al., 2006). Moreover, the variant p.D800N was generated as LOF mutation potentially abrogating the activity of the NTPase domain (Mekhedov et al., 2017) (figure 8).

First the poxvirus factors were cloned into a bicistronic vector containing a sequence for EGFP reporter followed a by P2A-linker. The factors were cloned downstream of the P2A-linker so that a minimal number of residues from the P2A-linker would be attached to their N-terminus. As these proteins are quite small (18-30 kDa), a fusion with a reporter fluorescent protein (e.g., a 26kDa EGFP) may interfere with their interaction with SAMD9/9L. Instead, the viral factors were tagged C-terminally with a V5 tag. The different SAMD9/9L variants were cloned in a plasmid with an N-terminal mCherry reporter. Such N-terminally tagged reported constructs have been widely used to evaluate the function of wild type SAMD9/9L, and of clinically relevant variants (Peng et al., 2022; F. Zhang et al., 2023). Different combination of viral factors and SAMD9/9L were then co-transfected in human HEK293 cells, which are an established cell line for studying mutant SAMD9/9L growth inhibition (Russell et al., 2021; Tesi et al., 2017). Immunoprecipitations experiments of the epitope-tagged viral factors followed by immunoblotting for the co-expressed SAMD9/9L variants were carried out to assess the interactions between the viral proteins and the SAMD9/9L variants. Growth inhibition of HEK293 was assessed in co-transfected cells (i.e., expressing both EGFP and mCherry) by click chemistry-based flow cytometric quantification of Edu incorporation. Similarly, translation repression instigated by ectopically expressed wild type and mutant SAMD9/9L proteins was assessed by a flow cytometry-based assay of O-propargyl-puromycin (OPP) incorporation followed by click chemistry.

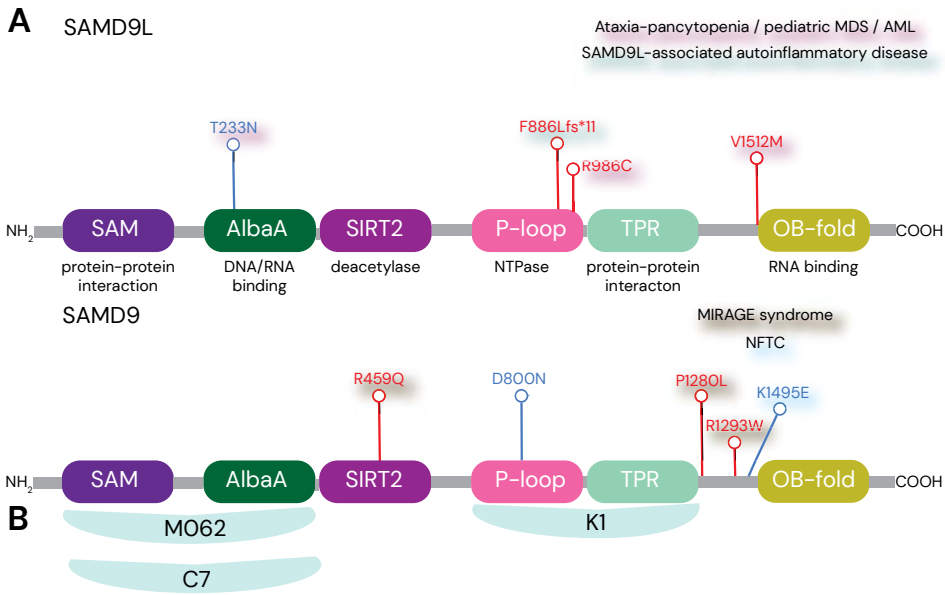

Figure 8. A. SAMD9L and SAMD9 predicted structure with the location of the variants studied in **paper II**. GOF variants are indicated in red, LOF variants in blue. SAM= Sterile alpha motif; SIRT2= silent information regulator 2; P-loop= predicted NTPase domain; TPR= tetratricopeptide repeats; OB-fold= oligonucleotide/oligosaccharide-binding. MDS= myelodysplastic syndrome; AML= acute myeloid leukemia; MIRAGE= MDS, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, and enteropathy syndrome; NFTC= normophosphatemic familial tumoral calcinosis References (D. H. Chen et al., 2016; de Jesus et al., 2020; Mekhedov et al., 2017; Narumi et al., 2016; Pastor et al., 2018; Tesi et al., 2017; Topaz et al., 2006)**.** B. Binding sites of the viral host factors studied in **paper II**. Binding site of C7 is predicted based on its structural homology with M062 References (Meng et al., 2015; Nounamo et al., 2017; F. Zhang et al., 2019)

Generally, all the factors examined could antagonize the anti-translational and anti-proliferative activity of SAMD9/9L wild type and GOF variants to some degree, except for the truncating F886Lfs*11 mutant in SAMD9L that also had the most dramatic antiproliferative and translation-repressing phenotype. Excluding this variant, K1 was the strongest anti-SAMD9/9L factor. All truncating *SAMD9L* GOF variants are located downstream of the Walker B motif of the predicted P-loop domain, while variants introducing stop codons more N-terminally result in LOF mutants (Allenspach et al., 2021). This indicates that the predicted NTPase domain of the protein is essential to SAMD9L antiproliferative functions as identified by previous studies (Allenspach et al., 2021; Russell et al., 2021). K1, our most potent inhibitor for SAMD9/L, binding to SAMD9/9L was mapped to the NTPase site (F. Zhang et al., 2019), explaining why it failed to interact with SAMD9L

p. F886Ls*11 in our immunoprecipitation assays. These findings are in line with a recently proposed theory of SAMD9/9L regulation, which presumes an autoinhibited conformation due to the binding of the C-terminal domains of the protein to the central p-loop NTPase region (Russell et al., 2021). We found that a more N-terminal SAMD9 GOF variant (p.R459Q) was still bound and moderately antagonized by all host factors, indicating that the missense mutation in the predicted SIRT2 domain was irrelevant to the binding of the poxviral factors examined here. This is consistent with the predicted binding sites of M062 (Nounamo et al., 2017) and likely C7, that has a homologous structure (Meng et al., 2015), also binds the same region of SAMD9/9L (figure 8). In the future, it will be interesting to examine more N-terminal missense GOF variants, like those falling before the p-loop Walker B motif, and specifically in the SAM and AlbaA predicted domains. Even if uncommon, some of these variants have recently been reported (e.g., p.Y72C and p.D169H in *SAMD9L* (Sahoo et al., 2021)).

A major limitation of this study concerns the use of an overexpression system in a non-hematological cell line that cannot recapitulate the endogenous expression levels of *SAMD9* and *SAMD9L*. An alternative option would be using an inducible system for SAMD9/9L expression in HEK293 cells to achieve a physiological expression of mutant SAMD9/9L proteins. Presumably, the expression levels of pathogenic GOF SAMD9/9L will be substantially lower than what we used in our study, and the inhibitory capacity of the viral host factors higher. Still, the flow cytometry assays used in **paper II** are a good system to quickly screen the effects of *SAMD9/9L* gene variants found in the clinics on cell proliferation. Relevant ones may then be selected and studied further with other methods.

4.3 NK cell deficiency: DEF6 is needed for the homeostasis of canonical NK cells.

DEF6, a.k.a. IRF4 binding protein (IBP) (Gupta, Lee, et al., 2003) or SWAP-70-like adaptor of T cells (SLAT) (Tanaka et al., 2003), belongs to the SWEF family. DEF6 and SWAP-70 are the only two known members of this family. They are Guanosine nucleotide exchange factors (GEF) with an inverted domain architecture where a pleckstrin homology (PH) domain precedes a DH-like (DHL) domain with GEF activity (figure 9) (Bécart & Altman, 2009; Mavrakis et al., 2004). DEF6 is most highly expressed in T cells and NK cells (Gupta, Lee, et al., 2003; Tanaka et al., 2003), while high levels of SWAP-70 are found in B cells (Borggrefe et al., 1998). Alike to SWAP-70 (Shinohara et al., 2002), DEF6 is a GEF that can bind actin filaments directly and organize actin cytoskeleton in several processes (Mavrakis et al., 2004; Mehta et al., 2009). In T cells, several studies have shown that TCR activation recruits DEF6 at the IS where it contributes to actin reorganization during synaptic signaling (Bécart et al., 2008; Fanzo et al., 2006; Gupta, Fanzo, et al., 2003; Singleton et al., 2011). Def6-deficient murine models from different research groups displayed diverse, and sometimes opposing, types of immunopathology. In most cases, the mice had a tendency for autoimmunity with a SLE-like (Fanzo et al., 2006) or a rheumatoid arthritis-like disease when crossed with mice carrying a I-A^d-restricted TCR (Q. Chen et al., 2008). These features affected specifically female mice (Q. Chen et al., 2008; Fanzo et al., 2006). Dysregulation of Irf4, normally bound and restricted in its transcriptional activity by Def6, was proposed as the mechanism driving autoimmunity (Q. Chen et al., 2008). A different murine mouse had instead defective T cell responses and was protected against experimental autoimmune encephalomyelitis (EAE) (Bécart et al., 2007, 2008; Canonigo-Balancio et al., 2009; Feau et al., 2013). Still, in both models, T cell signaling at the IS was suboptimal and could be corrected with phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation (i.e., by bypassing IS proximal signaling and augmenting intracellular calcium) (Bécart et al., 2008; Fanzo et al., 2006). Since these early studies, a consistent amount of evidence suggests that the dysregulation of Irf4 and Irf5 in the absence of SWEF proteins alters follicular T helper – B cell interactions resulting in the autoimmune phenotypes observed in the murine models (Biswas et al., 2012; Manni et al., 2017). Moreover, several functions downstream TCR activation have been described for DEF6. For example, the promotion of Ca^{2+}-NFAT signaling (Bécart et al., 2007, 2008; Fos et al., 2014) and inside-out signaling mediating cell adhesion(Cote et al., 2015).

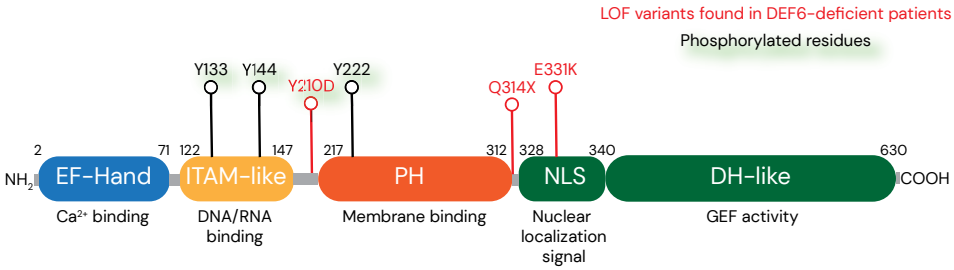

Figure 9. DEF6 domain architecture. ITAM= immunoreceptor tyrosine-based activation motif, PH= pleckstrin homology, DH = Dbl homology (Bécart & Altman, 2009; Hey et al., 2012). The LOF variants found in DEF6-deficient patients are indicated in red (Fournier et al., 2021; Serwas et al., 2019).

The description of two unrelated family with individuals bearing biallelic homologous *DEF6* LOF mutations by Serwas (2019), and the description of one family by Fournier (2021) opened a door for understanding the biology of DEF6 in humans(Fournier et al., 2021; Serwas et al., 2019). In Serwas (2019), two families with patients bearing homozygous LOF variant Is *DEF6* were described(Serwas et al., 2019). One with a missense function (p.E331K) that reduced DEF6 expression and function, and one with a missense function (p.Y210Y) that abrogated DEF6 expression (Serwas et al., 2019). The patients of the first family had an additionally pathogenic homozygous variant in *SKIV2L*, whose deficiency causes trichohepatoenteric syndrome and therefore, complicated the phenotype of these patients (Fournier et al., 2021; Serwas et al., 2019). Fournier (2021) described another family where patients had a homozygous truncating variant (p.Q314X) that abrogated DEF6 protein expression(Fournier et al., 2021) (figure 9). In the complete absence of DEF6, patients suffer from autoimmunity and susceptibility to herpes virus infections (Fournier et al., 2021; Serwas et al., 2019). Serwas (2019) found that in T cells, DEF6 regulates the trafficking of RAB11^{+} recycling endosomes (RE) back to the plasma membrane (Serwas et al., 2019). These REs carry CLTA-4 molecules, which are essential for the suppression of T cell activation by regulatory T cells (Treg) and cause a severe immune dysregulation when missing (Friedline et al., 2009; Kuehn et al., 2014; Schubert et al., 2014; Tivol et al., 1995). Lack of DEF6 caused a reduction of CTLA-4 on the plasma membrane of Treg cells and contributed to autoimmunity in DEF6-deficient patients (Serwas et al., 2019). Still, this novel finding could not explain the increased susceptibility of DEF6-deficient patients to herpes viral infections. We noticed low numbers of circulating NK cells, and a peculiar phenotype of increased CD56^{bright} NK cells in the peripheral blood our DEF6-deficient index patient (Serwas et al., 2019), A:II-1 (figure 10A).

Being abnormal NK cell phenotypes and increased susceptibility to herpes virus infections distinctive signs of NKD, we decided to thoroughly study the role of DEF6 in human NK cell biology. We found that DEF6 expression is upregulated upon NK cell maturation in the peripheral blood of healthy individuals, with $CD56^{dim}$ subsets expressing higher levels of the protein than $CD56^{bright}$ NK cells. Still, *DEF6* KO clones of induced pluripotent stem cells (iPSC) clones differentiated normally in an in vitro culture model. We studied the phenotype of our index-patient bearing a missense variant in *DEF6* (p. Y210D) originally described by Serwas (2019) and three of the patients from Fournier (2021) that carried a truncating variant (p.Q314*)(Fournier et al., 2021)(figure 10A) All patients lacked DEF6 expression. We found reduced fractions of canonical $CD56^{dim}$ $PLZF^{+}$ NK cells concomitant to the expansion of adaptive $PLZF^{+}$ subsets. In patient A:II-1, this was accompanied by high frequency of immature $CD56^{bright}$ NK cells, which is extremely uncommon among both CMV^{+} and CMV^{-} individuals(Schlums et al., 2017) (figure 10B-C). In patients B:II-1, II-2, and II-3 we observed a lack of conventional $CD57^{+}$ $CD56^{dim}$ NK cells and expansions of adaptive NK cells, but no increased fractions of $CD56^{bright}$ NK cells (figure 10B and 10D).

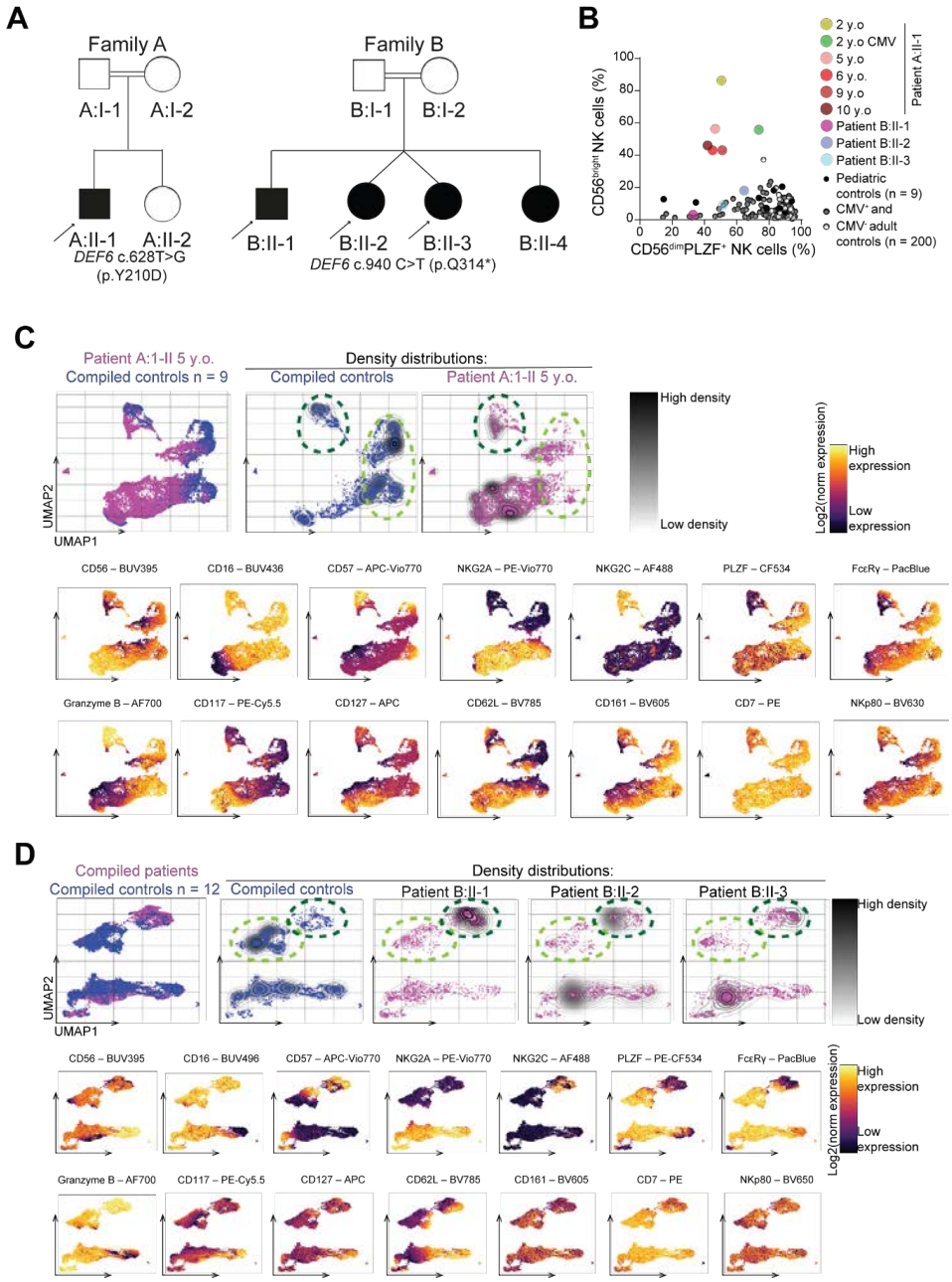

Figure 10. NK cell phenotype in DEF6-deficient patients. A. Family diagrams of the patients studied in paper III. The arrow indicates the patients for whom NK cells were characterized in frozen PBMCs samples. B. Plotting of the percentage of $CD56^{bright}$ NK cells within the total NK cell population (gated as $CD19^{-}CD14^{-}CD3^{-}NKp80^{+}CD56^{+}/CD16^{+}$ lymphocytes) as a function of the frequency of PLZF-expressing $CD56^{dim}$ NK cells; $CMV^{+/-}$ individuals were previously reported by(Schlums et al., 2017). C. Unsupervised clustering of NK cells from patient A: II-1 (time point: 5 years of age) and controls visualized by UMAP

projections. Terminal conventional NK cell subsets and adaptive NK cell subsets are circled in light and dark green respectively. Cell density in the different clusters and expression of the 14 parameters used in the UMAP analysis is depicted. D. Unsupervised clustering of NK cells from patient B: II-1, II-2 and II-3 and controls visualized by UMAP projections. Cell density in the different clusters and expression of the 14 parameters used in the UMAP analysis is depicted.

Given the importance of DNA replication during NK cell expansions and homeostasis, we examined whether NK cell division was affected by DEF6 absence. Strikingly, KO of *DEF6* with ribonucleoprotein complexes (RNP) of Cas9 and guides targeting *DEF6* in primary human NK cells did not affect proliferation. We found instead a reduction in cell viability. We also functionally interrogated these cells and found lower degranulation and IFN-γ production in KO cells compared to wild type. Similarly, DEF6-deficient patients' NK cells displayed lower degranulation and IFN-γ production. Specifically, the NK cells from three patients from Fournier *et al.* had lower degranulation, but normal IFN-γ production. Our index patient's NK cells were slightly impaired in both functions. The defect in degranulation and IFN-γ in *DEF6* KO NK cells may be due to defective proximal signaling at the IS, since DEF6 is known to coordinate this pathway. In humans, TCR-directed cell responses in T cells derived from DEF6-deficient patients were not severely impaired though. Reduced ERK and AKT phosphorylation were reported by Serwas *et al.*, but T cell proliferation and calcium flux were unaltered (Serwas et al., 2019). Also, Fournier *et al.* did not find defect in T cell calcium flux, proliferation and IS proximal proteins phosphorylation (Fournier et al., 2021). Even so, they noticed reduced NK cell function. In our hand, NKp30 and CD16 receptor triggering with PMA co-stimulation compensated for the defective functionality of *DEF6* KO NK cells, but ionomycin did not (unpublished observation). Considering these results, the role of DEF6 in calcium regulation may be less relevant or redundant in human lymphocytes. Still, DEF6 appears important for the proximal signaling regulating degranulation and cytokine production in NK cells. Small defects in these pathways reduce the cytotoxic potential of lymphocytes and can become significantly deleterious during severe infections, like the ones DEF6-deficient patients experienced. Therefore, we think that defective effector responses in DEF6-deficient NK cells, and to some degree in T cells, can explain the susceptibility of these patients to herpes virus severe infections. Additionally, suboptimal T cell signaling is a known cause of autoimmunity, and this probably contributes to the immunopathology of DEF6 deficiency (Notarangelo, 2013).

In our experiments, DEF6 was important for the survival of canonical NK cells. IL-15 is essential for NK cell differentiation, survival, and function (Eidenschenk et al., 2006; Kennedy et al., 2000). Being DEF6 a protein regulating actin and adhesion (Cote et al., 2015), we reasoned that eventual defects in adhesion and IL-15 uptake from DCs and other supporting cells could be an explanation for the NKD in DEF6-deficient patients (Koka et al., 2004; Lucas et al., 2007; Luu et al., 2016). After *DEF6* KO in primary human NK cells, we incubated the cells with K562 cells genetically modified to express fluorescently tagged IL-15Rα receptors (Anton et al., 2020). This system allows to study IL-15 internalization by endocytosis of shed IL-15/IL-15Rα complexes, or by trans-endocytosis of IL-15/IL-15Rα directly from plasma membrane of the feeder cells (Anton et al., 2020). Strikingly, we did not find any impairment in IL-15 internalization by both modalities (unpublished observation). The experimental protocols to generate primary human KO cells by CRISPR/Cas9 require high levels of IL-15 stimulation to promote NK cell division and thus KO efficiency. In these settings, DEF6 protein expression is lost progressively as the generation of new cells requires new gene transcription. Nevertheless, this loss was not accompanied by a stop in cell division as we observed upon the KO of the cyclin-dependent kinase 6 (*CDK6*) in the same settings. To study the effects of DEF6 complete absence in NK cell survival and development, we generated *DEF6* KO induced pluripotent stem cell (iPSC) clones. iPSCs are a good model to recapitulate *in vitro* the early stages of human NK cell development and can be put in cultures promoting NK cell development after the induction of hematopoiesis (Euchner et al., 2021). In NKDs with haploinsufficiency in CMG helicase genes, similar models showed defect in NK cell development (Conte et al., 2022; Mace et al., 2020). In our case, induction of hematopoiesis and NK cell development were unaffected in *DEF6* KO clones. Since upon *DEF6* KO in primary human NK cells we did not observe any defect in cell division either, we are quite confident in excluding a defect in DNA replication in DEF6-deficient NK cells.

These findings still do not explain why *DEF6* KO in primary NK cells reduced cell survival in our experiments, or why DEF6-deficient patients lacked canonical NK cells. Possibly, as the primary NK cells lost DEF6, they became more prone to apoptosis in our culture conditions. Further experiments will be needed to understand which pathways promoting survival and/or NK cell terminal development are affected in DEF6-deficient NK cells.

Primary CMV infection drives the differentiation and the clonal expansion of adaptive NK cells in both human and barcode models in Rhesus Macaque (Rückert et al., 2022; Wu et al., 2022). A strong ITAM signaling from NKG2C, or CD16 coupled with CD2 co-stimulation, and inflammatory cytokines drive the initial expansion during adaptive NK cell differentiation (Q. Hammer et al., 2018; L. L. Liu et al., 2016). The mechanisms regulating the subsequent establishment and persistence of oligoclonal long-lived populations of adaptive NK cells are less clear. Increased levels of the anti-apoptotic Bcl-2 (T. Zhang et al., 2013), resistance to reactive oxygen species (ROS) (Sarhan et al., 2016), and a metabolism resembling long lived memory T cells (Cichocki et al., 2018) are some of the features that have been observed in these cells and may explain their better fitness and persistence. Since these cells emerge after several rounds of expansions and contractions during CMV primary infection and (potentially) reactivation, it is possible that the adaptive NK cells found in DEF6-deficient patients have been selected for better functionality and survival, while patient's short-lived canonical NK cells lack the ability to compensate for DEF6 absence. These findings are in line with finding of Schlums (2017), who showed that in patients with GATA2 haploinsufficiency, adaptive NK cells persisted despite the progressive loss of HSCs and progenitor cells (Schlums et al., 2017).

5 Conclusions

In this doctoral thesis, I have had the chance to contribute personally to the discovery and the further characterization of variants and genotypes causing IEIs. These studies concerned different aspects of IEI research, from diagnostics to further understanding of patients' diseases and inspiration for future therapeutical approaches. A summary of the conclusion of each paper is reported below.

Paper I demonstrated that *RHOG* is a FHL disease-causing gene. The encoded protein RHOG is essential for cytotoxic granule exocytosis by assisting MUNC13-4-mediated fusion of the granules at the plasma membrane. Hence, screening for pathogenic variants in *RHOG* should be prioritized in patients with suspected HLH.

By studying whether SAMD9/9L GOF mutations were inhibited by poxvirus derived factors in **paper II**, we found that the Vaccinia virus host range factor K1 is the most effective antagonist of SAMD9/L wild type and GOF, except for variants truncating the putative NTPase domain where K1 binds. Molecular targeting of SAMD9/9L is possible, and its potential therapeutical application warrant further investigation.

In **paper III**, we found a predominant adaptive phenotype in the NK cells of DEF6-deficient patients. Upon KO of *DEF6* by CRISP-Cas9, primary human NK cells displayed impaired survival and effector functions, but no defects in cell proliferation. Based on the known role of DEF6 in signaling, we propose that defective signaling from the IS was responsible for the diminished degranulation and IFN-γ production in *DEF6* KO cells. On top of this, reduced NK cell survival may be due to a novel form of NK cell dysregulation, which is different from the defects in DNA replication previously found in patients with NKDs.

In the realm of diagnostics, my research has led to the identification of a novel HLH-causing gene. This not only enhances patient care by accelerating FLH diagnosis but may also be useful for genetic counseling. Moreover, a better understanding of HLH pathology will guide treatment and, and perhaps, prevent cytokine storm syndromes following T cell-directed immunotherapies. Regarding modulation of pathogenic variants found in MDS patients, my work has explored the development of targeted treatments to antagonize pathogenic SAMD9/9L variants. This approach holds the potential to improve patient outcomes and

quality of life. In my pursuit of further understanding the pathological mechanisms behind NK cell deficiency, my research has provided valuable insights into the intricate molecular processes regulating NK cell homeostasis. The culmination of my PhD work represents not only a significant contribution to the field of immunology but also a step forward in the quest to improve the lives of individuals affected by IEIs. This doctoral thesis serves as a testament to the ongoing dedication and collaborative efforts of the scientific community in the pursuit of better diagnoses, treatments, and ultimately, a deeper understanding of these complex and challenging conditions.

6 Points of perspective

For each article of this doctoral thesis, I will now discuss some future research work and points of perspective.

Elucidating the molecular regulation of RHOG activation in cytotoxic lymphocytes is important for diagnostic purposes. We tested the effect of a previously described inhibitor of a RHOG GEF, TRIO (Bouquier et al., 2009), and found reduced killing of target cells. The interplay between these two proteins could be explored further in cytotoxic lymphocytes, also considering the intricate regulation of RAC1/RHOG and RHOA-dependent activities by TRIO in other cell types (Bateman & Van Vactor, 2001). To further characterize other RHOG activators and downstream effectors for CG exocytosis, proteomic studies of RHOG taking advantage of proximity-dependent biotinylation assays will be of help, like Bagci (2020) did in a recent studying examining the network of Rho GTPases in human cell lines (Bagci et al., 2020). A challenging, but important, experiment would be to study this network in primary cytotoxic lymphocytes such as NK cells. Even so, transfection of large DNA constructs in these cells is notoriously difficult. Such difficulties may be overcome by using transient mRNA nucleofection that is efficient for transient ectopic expression experiments in primary human NK cells (Carlsten et al., 2016). Finally, patients carrying missense mutations in *RHOG* may selectively abrogate the interaction of RHOG and specific downstream interactors, like what has been described for *RAB27A* (Ohishi et al., 2020; Zondag et al., 2022). Finding and studying these patients may be interesting to further understand the RHOG-regulated network in cytotoxic lymphocytes. RHOG deficiency has not caused any neurodevelopmental disorder in our patient, but missense heterozygous variants in a related small GTPase, CDC42, do (Martinelli et al., 2018). Of note, *de novo* variants in *TRIO* cause syndromic neurodevelopmental disorders (Ba et al., 2016; Barbosa et al., 2020; Pengelly et al., 2016), and one study reported that patients suffered from recurrent infections (Ba et al., 2016). Accordingly, the dysregulation of Rho GTPases gives rise to syndromes where immunodeficiency may aggravate the clinical picture of the patients. Recognizing the importance of these proteins for immune responses will guide clinicians in taking care of the patients that may be first referred to neurology.

Understanding the precise requirements of SAMD9/9L inhibition opens novel therapeutical doors for targeting the functions of these proteins in

pathological settings. Targeting SAMD9/9L activities is specifically relevant to modulate the detrimental effects of GOF mutants. Still, this approach would probably be unfeasible using the anti-SAMD9/9L viral factors screened in **paper II**. These factors also inhibit wild type SAMD9/9L compromising anti-viral responses and normal cell growth control. Specific SAMD9/9L modulators should preferentially hamper GOF mutants over the wild type proteins. Hence, designing an inhibitor for truncating GOF mutants of SAMD9L may be feasible by taking advantage of the structural differences between wild type and truncated proteins. First, screenings of several anti-SAMD9/9L viral factors, or endogenous human proteins, could be performed to find antagonists for the truncating SAMD9/9L variants using the methods of **paper II**. After selecting candidate proteins, an optimal inhibitor could be generated by rational design or directed evolution methods combined with functional validations *in vitro* (Packer & Liu, 2015). A recent largescale study of a pediatric MDS cohort identified that the somatic genetic rescue (SGR) found in patients with germline *SAMD9/9L* GOF mutations gave rise to a mosaicism of benign (UPD7q and somatic LOF variants in *SAMD9/9L*), and maladaptive (-7, with a propensity for additional cancer mutations) mechanisms (Sahoo et al., 2021). Remarkably, most individuals (95%) had maladaptive SGR in a portion of their cells (Sahoo et al., 2021). Therefore, it is compelling to explore if any anti-mutant SAMD9/9L inhibitor can tilt SGR towards more benign outcomes. This aspect may be studied in *in vitro* models of hematopoiesis that undergo spontaneous gene dosage rescue, such as iPSC cells (Kotini et al., 2015). Nevertheless, we still know too little about the overall structure and functions of SAMD9/9L proteins to directly jump into similar studies. Since the architecture and the function of the different domains in SAMD9/9L are still hypothetical, we may use the assays of **paper II** to further explore clinically relevant and artificially generated SAMD9/9L variants. In this way, we may discover several residues important for SAMD9/9L activities, and further understand the role of the predicted domains in this protein.

The molecular mechanisms behind NKD deficiency in DEF6-deficient patients have not been completely understood yet. Future efforts will be dedicated to pinpointing which specific pathways are dysregulated in primary human *DEF6* KO NK cells. In this regard, we have initiated RNA-sequencing experiments in primary human NK cells after *DEF6* KO with CRISPR/Cas9 RNPs. Another compelling question is whether *Def6*-deficient mice harbor defects in NK cell development. After studying the NK cell phenotype in these mice at steady

state, infections with murine CMV (MCMV) may provide insights into the requirement of *DEF6* during the generation of Ly49H$^+$ memory NK cells. Indeed, GATA2-regulated *Nfil3* which is essential for NK cell survival and development in mice, was conversely found to be non-essential in allowing development of antiviral NK cells against MCMV (Firth et al., 2013; Kamizono et al., 2009). This suggests that some parallel trajectories of NK cell development may exist during complex immune responses. For all these reasons, it will be interesting to explore the role of Def6 in murine models. These studies may point us towards which pathways ensure human NK cell survival and terminal differentiation. Furthermore, better understanding of NK cell development and regulation could be harnessed to promote NK cell activation, function and persistence in anti-cancer immunotherapies. Since higher levels in *DEF6* expression have been associated with malignant behavior of non-hematological cancer cells (Li et al., 2009), a better understanding of the pathways regulated by DEF6 may also highlight important therapeutic targets for cancer treatment.

As we have seen in this last section, studying these phenomena is a never-ending quest. In science, we will always encounter new challenges and opportunities. However, the results we achieve every day give us the strength, knowledge and experience to go forward and climb the next peak. After all, "beyond mountains, there are mountains."

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