From the Department of Medicine Huddinge,
Karolinska Institutet, Stockholm, Sweden

FUNCTIONAL ASSAYS FOR THE DIAGNOSIS OF PRIMARY DEFECTS IN LYMPHOCYTE CYTOTOXICITY

Samuel CC Chiang

Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet.
Printed by E-print AB

ISBN 978-91-7676-123-6

Back cover: 7-year collection of patient data. Image of files #1-24 holding patient information, consent forms, shipping invoices, and assay results from patient samples processed between 2008 and 2015. The open unfilled file #25 represents our anticipation of future cases.

Functional Assays for the Diagnosis of Primary Defects in Lymphocyte Cytotoxicity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Samuel Cern Cher, Chiang

Principal Supervisor:
Assistant Professor Yenan T Bryceson
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Co-supervisors:
Professor Hans-Gustaf Ljunggren
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Professor Jan-Inge Henter
Karolinska Institutet
Department of Women's and Children's Health
Childhood Cancer Research Unit

Opponent:
Associate Professor Mirjam van der Burg
University Medical Center Rotterdam
Department of Immunology

Examination Board:
Professor Qiang Pan Hammarström
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Immunology

Professor Ola Winqvist
Karolinska Institutet
Department of Medicine, Solna

Dr. Med. Hans Christian Erichsen
Oslo University Hospital
Department of Pediatric Research

For the life of the body is in its blood

Leviticus 17:11

ABSTRACT

Cytotoxic lymphocytes encompass natural killer (NK) cells and cytotoxic T lymphocytes (CTL). These cells detect and kill virus-infected as well as malignant cells. Primary defects in lymphocyte cytotoxicity are associated with development of a hyperinflammatory syndrome termed hemophagocytic lymphohistiocytosis (HLH). This thesis investigates cytotoxic lymphocyte function in HLH cases as well as the creation of new assays to improve diagnostics.

An NK cell degranulation assay has been developed to quantify NK cell responses as an alternative to the radioactive chromium release assay. CD107a is a transmembrane protein that in unstimulated cells is contained within the inner membrane of perforin-containing cytotoxic granules but is exposed on the cell surface upon cytotoxic granule exocytosis. In a pan-European effort, we established and validated a consensus protocol for the diagnosis of primary HLH patients with defective degranulation (**Paper II**). NK cell degranulation below 5% predicted a primary defect in exocytosis leading to defective lymphocyte cytotoxicity with 96% sensitivity and 88% specificity. We also provided further optimized protocols for NK cell phenotyping and degranulation (**Paper I**).

Highlighting the importance of reliable functional assays in primary immunodeficiency discovery, we described novel non-coding aberrations in *UNC13D* as a cause of HLH and defective degranulation (**Paper III, IV**). Point mutations were found in a highly conserved intronic region, while a 253kb inversion was identified as the most frequent cause of HLH in Swedish infants.

ORAI1 and STIM1 mediate store-operated Ca^{2+} entry in T cells, which is required for induction of cytokine expression. However, the role of store-operated Ca^{2+} entry was not clear. In **Paper V**, we studied NK cell cytotoxicity using ORAI1 and STIM1-deficient patients, confirming abrogated Ca^{2+} entry upon target cell stimulation. Importantly, ORAI1 and STIM1-deficient NK cells did not degranulate nor produced proinflammatory cytokines, demonstrating that store-operated Ca^{2+} entry is required for overall cytotoxic lymphocyte effector functions.

Primary HLH diagnostics have relied on NK cell functional assays but little had been done to evaluate T cell responses in these patients. In **Paper VI**, we described how the surface marker CD57 can be used to identify *bone fide* cytotoxic T lymphocytes, readily allowing identification of a T cell subset that expresses intracellular perforin and can efficiently kill target cells upon TCR engagement. The $CD8^{+}CD57^{+}$ T cell subset, similar to $CD56^{dim}$ NK cells, was found to display defective degranulation in FHL types 3-5, suggesting CTL and NK cell exocytosis has similar molecular requirements. In **paper VII**, we compared the established K562 cell-induced NK cell degranulation assay (**Paper II**) against the newly developed T cell degranulation assay (**Paper VI**) prospectively evaluating all primary immunodeficiency patients sent to our laboratory during a 3 year period. The T cell assay excelled with respect to sensitivity and specificity (97% and 95%, respectively) for predicting a primary defect in degranulation. Combining NK cell and T cell assays further increased assay specificity.

This thesis demonstrates the power of functional assays in evaluating cytotoxic lymphocyte activity and how this expands our understanding of their biological role and the primary HLH syndrome. These simple, rapid and accurate assays give us the power to quickly diagnose a life-threatening disease and initiate treatment, thus saving lives.

LIST OF SCIENTIFIC PAPERS

I. Measurement of NK cell phenotype and activity in humans.
Chiang SCC and Bryceson YT.
Detrick B, Hamilton RG, Schmitz J (ed), Manual of Molecular and Clinical Laboratory Immunology, 8th ed. ASM Press, Washington, DC, in press.

II. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes.
Bryceson YT, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, **Chiang SCC**, Marcenaro S, Meazza R, Bondzio I, Walshe D, Janka G, Lehmberg K, Beutel K, zur Stadt U, Binder N, Arico M, Moretta L, Henter J-I, and Ehl S.
Blood (2012). 119(12):2754-2763.

III. Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) caused by deep intronic mutation and inversion in *UNC13D*.
Meeths M, **Chiang SCC***, Wood SM*, Entesarian M, Schlums H, Bang B, Nordenskjöld E, Björklund C, Jakovljevic G, Jazbec J, Hasle H, Holmqvist BM, Rajic L, Pfeifer S, Rosthøj S, Sabel M, Salmi TT, Stokland T, Winiarski J, Ljunggren HG, Fadeel B, Nordenskjöld M, Henter J-I, and Bryceson YT.
Blood (2011). 118(22):5783-5793.

IV. Novel deep intronic and missense *UNC13D* mutations in familial haemophagocytic lymphohistiocytosis type 3.
Entesarian M, **Chiang SCC**, Schlums H, Meeths M, Chan MY, Mya SN, Soh SY, Nordenskjöld M, Henter J-I, and Bryceson YT.
British Journal of Haematology (2013). 162(3):415-418.

V. ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis.
Maul-Pavicic A*, **Chiang SCC***, Rensing-Ehl A, Jessen B, Fauriat C, Wood SM, Sjöqvist S, Hufnagel M, Schulze I, Bass T, Schamel WW, Fuchs S, Pircher H, McCarl CA, Mikoshiba K, Schwarz K, Feske S, Bryceson YT*, and Ehl S*.
Proceedings of the National Academy of Science U S A (2011). 108(8):3324-3329.

VI. Comparison of primary human cytotoxic T-cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production.
Chiang SCC*, Theorell J*, Entesarian M, Meeths M, Mastafa M, Al-Herz W, Frisk P, Gilmour KC, Ifversen M, Langenskiöld C, Machaczka M, Naqvi A, Payne J, Perez-Martinez A, Sabel M, Unal E, Unal S, Winiarski J, Nordenskjöld M, Ljunggren HG, Henter J-I, and Bryceson YT.
Blood (2013). 121(8):1345-1356.

VII. Efficacy of the T cell degranulation assay for the diagnosis of primary defects in cytotoxic lymphocyte exocytosis.
Chiang SCC, Tesi B, Schulms H, Meeths M, Nejati-Zendegani J, Mördrup K, Horne AC, Ljunggren H-G, Nordenskjöld M, Henter J-I, Bryceson YT.
Manuscript.

LIST OF SUPPLEMENTARY PAPERS

S.I. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity.
Wood SM, Meeths M, **Chiang SCC**, Bechensteen AG, Boelens JJ, Heilmann C, Horiuchi H, Rosthøj S, Rutynowska O, Winiarski J, Stow JL, Nordenskjöld M, Henter J-I, Ljunggren HG, and Bryceson YT.
Blood (2009). 114(19):4117-4127.

S.II. Spectrum of clinical presentations in familial hemophagocytic lymphohistiocytosis type 5 patients with mutations in *STXBP2*.
Meeths M, Entesarian M, Al-Herz W, **Chiang SCC**, Wood SM, Al-Ateeqi W, Almazan F, Boelens JJ, Hasle H, Ifversen M, Lund B, van den Berg JM, Gustafsson B, Hjelmqvist H, Nordenskjöld M, Bryceson YT, and Henter J-I.
Blood (2010). 116(15):2635-2643.

S.III. Sensitive and viable quantification of inside-out signals for LFA-1 activation in human cytotoxic lymphocytes by flow cytometry.
Theorell J, Schlums H, **Chiang SCC**, Huang TY, Tattermusch A, Wood SM, and Bryceson YT.
Journal of Immunological Methods (2011). 366(1-2):106-118.

S.IV. Hemophagocytic syndrome in a 4-month-old infant with biotinidase deficiency.
Kardas F, Patiroglu T, Unal E, **Chiang SCC**, Bryceson YT, and Kendirci M.
Pediatric Blood and Cancer (2012). 59(1):191-193.

S.V. Development of classical Hodgkin's lymphoma in an adult with biallelic *STXBP2* mutations.
Machaczka M, Klimkowska M, **Chiang SCC**, Meeths M, Müller ML, Gustafsson B, Henter J-I, and Bryceson YT.
Haematologica (2013). 98(5):760-764.

S.VI. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage.
Cohnen A, **Chiang SCC**, Stojanovic A, Schmidt H, Claus M, Saftig P, Janßen O, Cerwenka A, Bryceson YT, and Watzl C.
Blood (2014). 122(8):1411-1418.

S.VII. An N-Terminal missense mutation in *STX11* causative of FHL4 abrogates Syntaxin-11 binding to Munc18-2.
Müller ML, **Chiang SCC***, Meeths M*, Tesi B, Entesarian M, Nilsson D, Wood SM, Nordenskjöld M, Henter J-I, Naqvi A, and Bryceson YT.
Frontiers in Immunology (2014). 4:515.

S.VIII. Transcriptional regulation of Munc13-4 expression in cytotoxic lymphocytes is disrupted by an intronic mutation associated with a primary immunodeficiency.
Cichocki F, Schlums H, Li H, Stache V, Holmes TR, Lenvik T, **Chiang SCC**, Miller JS, Meeths M, Anderson SK, and Bryceson YT.
Journal of Experimental Medicine (2014). 211(6):1079-1091.

S.IX. Hematopoietic stem cell transplantation of an adolescent with neurological manifestations of homozygous missense *PRF1* mutation.
Hussein A, Hamadah T, Qandeel M, Sughayer M, Amarin R, Mansour A, **Chiang SCC**, Al-Zaben A, Meeths M, and Bryceson YT.
Pediatric Blood and Cancer (2014). 61(12):2313-2315.

S.X. Hemophagocytic lymphohistiocytosis in 2 patients with underlying IFN-γ receptor deficiency.
Tesi B, Sieni E, Neves C, Romano F, Cetica V, Cordeiro AI, **Chiang SCC**, Schlums H, Galli L, Avenali S, Tondo A, Canessa C, Henter J-I, Nordenskjöld M, Hsu AP, Holland SM, Neves JF, Azzari C, and Bryceson YT.
The Journal of Allergy and Clinical Immunology (2015). 135(6):1638-1641.

S.XI. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function.
Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, Han H, **Chiang SCC**, Foley B, Mattsson K, Larsson S, Schaffer M, Malmberg KJ, Ljunggren HG, Miller JS, and Bryceson YT.
Immunity (2015). 42(3):443-456.

S.XII. A case of XMEN syndrome presented with severe auto-immune disorders mimicking autoimmune lymphoproliferative disease.
Patiroglu T, Haluk Akar H, Gilmour K, Unal E, Akif Ozdemir M, Bibi S, Burns S, **Chiang SCC**, Schlums H, Bryceson YT, and Karakukcu M.
Clinical Immunology (2015). 159(1):58-62.

S.XIII. Spectrum of clinical presentations in atypical patients with biallelic *PRF1* missense mutations.
Tesi B, **Chiang SCC**, El-Ghoheimy D, Hussein AA, Langenskiöld C, Wali R, Fadoo Z, Silva JP, Lecumberri R, Unal S, Nordenskjöld M, Bryceson YT, Henter J-I, and Meeths M.
Pediatric Blood & Cancer (2015). 62(12)2094-2100.

S.XIV. Cancer risk in relatives of patients with primary defects in lymphocyte cytotoxicity: a retrospective cohort study.
Löfstedt A, **Chiang SCC**, Onelöv E, BrycesonYT, Meeths M, and Henter J-I.
Lancet Hematology (2015), in press.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
BRG1	Brahma-related gene 1
CD	Cluster of differentiation
CHS	Chediak-Higashi syndrome
CL	Cytotoxic lymphocyte
CMV	Cytomegalovirus
CRAC	Calcium release activated channel
CTL	Cytotoxic T Lymphocyte
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein–Barr virus
ELF1	E74-Like Factor 1
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FHL	Familial hemophagocytic lymphohistiocytosis
GS2	Griscelli syndrome type 2
HLH	Hemophagocytic lymphohistiocytosis
HPS2	Hermansky-Pudlak syndrome-2
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cells
ITK	IL-2-inducible T-cell kinase
KIR	Killer-cell immunoglobulin-like receptor
LAMP	Lysosomal associated membrane protein
LCMV	Lymphocytic choriomeningitis
LU	Lytic unit
LYST	Lysosomal trafficking regulator
MACPF	Membrane-attack complex perforin
MAS	Macrophage activation syndrome
MFI	Median fluorescence intensity

MHC	Major histocompatibility complex
MTOC	Microtubule-organizing center
NFAT	Nuclear factor of activated T-cells
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PID	Primary immunodeficiency disease
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPMI	Roswell Park Memorial Institute medium
SAP	SLAM associated protein
SCID	Severe combined immunodeficiency
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNP	Single nucleotide polymorphism
SOCE	Store-operated calcium entry
STAT	Signal transducers and activators of transcription
STIM1	Stromal interaction molecule 1
TCR	T cell receptor
TNF	Tumor necrosis factor
WBC	White blood cells
XIAP	X-linked inhibitor of apoptosis
XLP	X-linked lymphoproliferative disease

1 INTRODUCTION

Blood acts as a transporter and regulator for many crucial systems in the body including respiration, osmosis, heat transfer, nutrition, waste removal, regulatory molecules, clotting factors, and the circulating immune system. White blood cells, or leukocytes, are part of the immune system in blood and have continually been divided into smaller and smaller subsets as science races to discover novel functions for each new population. Broadly, leukocytes can be partitioned into polymorphonuclear cells, monocytes, and lymphocytes.

Polymorphonuclear cells make up the largest leukocyte subpopulation and recognize disrupted, opsonized, or cells expressing pathogen-associated molecular patterns (PAMPs) (1). This group is made up of neutrophils that respond by engulfing pathogens, basophils that excrete histamines, enzymes, and cytokines, and, eosinophils that produce reactive oxygen species as well as cytokines. Monocytes react to an infection by migrating to the site of injury where they differentiate into macrophages and dendritic cells. These are then named professional antigen presenting cells and function by engulfing and presenting foreign antigens on their surface. Dendritic cell can migrate to the lymph node in order to activate T cells, while macrophages efficiently clear off cell debris and dying cells. Besides functioning as a specialized phagocyte, macrophages can also kill target cells via antibody dependent cell-mediated cytotoxicity (2), and release cytokines (3).

The lymphocyte population consists of adaptive B cells and T cells that undergo RAG-dependent recombination of antigen receptors, as well as RAG-independent innate lymphoid cells (4). Upon recognition of an antigen through the B cell receptor, naïve B cells mature and produce specific antibodies against the recognized antigen and undergo somatic hypermutation to improve target recognition. Long-lived memory B cells can lay dormant until a secondary response is called for. T cells specifically recognize foreign protein antigens in the context of MHC, and will similarly expand and produce memory cells before the immune response is terminated by feedback mechanisms such as CTLA4 (5), or other regulatory cells, such as NK cells (6), leading to clonal contraction. Innate lymphoid cells (ILC) are lineage negative cells that are further categorized based on surface receptors and secreted cytokines. NK cells are the only subtype in ILC with cytotoxic potential (4) while other ILC groups are involved in the formation of secondary lymphoid organs, immunoregulation, and immunity against intracellular bacteria, parasites, and helminths.

The human body has a wide range of defenses to protect itself against the multitude of pathogens in the environment. With recent advancements and studies on patients with specific defects in their defense system, we are becoming more aware of the intricate overlapping function and inter-dependence between various members keeping us disease free.

1.1 NK cells

NK cell activity was first identified as a lymphocyte subset that could kill tumor cells without any prior sensitization (7). Their activity has later also been associated with control of virus infected and transformed cells (8-11). Because NK cells are in such an 'armed and ready to kill' state, strict controls over their function is necessary (12). Later, the observation that NK cells kill MHC class I deficient tumor cells (13) led to the 'missing self' hypothesis (14). Polymorphic inhibitory Ly49A and KIR Ig-like receptors were later identified on mice and human NK cells, respectively, thereby molecularly explaining "missing self" (15,16). Following which NKG2A was identified in both human and mice (17). All these inhibitory receptors bind MHC class I, inhibiting NK cell function. However, NK cell activation is not a default setting. Cellular activation requires specific signals from activating neceptors. A myriad of receptors with a capacity to activate NK cells have been identified. Some NK cell activating receptors bind ligands on activated immune cells, facilitating NK cell killing of activated macrophages and T cells (18). NK cells thereby play an important immunomodulatory role, attenuating immune cells once an infection has been cleared to minimize bystander damage. Moreover, reports on defective NK cells in cancer cohorts (19) and the proven usefulness in harnessing NK cells for cancer therapy (20) points to the fact that NK cells are important player in control of cancer. Not least, NK cells also produce large amounts of cytokines and chemokines, especially IFN-γ and TNF-α for recruiting other immune cells to the site of infection as well as to activate other cells in the vicinity (21).

NK cells express a range of activation receptors. CD16 is a low affinity receptor for Fc expressed on NK cells, thus facilitating activation towards antibody coated target cells. Other NK cell stimulating receptors include activating KIRs, NKp30, NKp46, NKG2C, NKG2D, DNAM-1, and 2B4. Some require co-stimulation to initiate NK cytotoxicity. NKG2D and 2B4 for example, when stimulated together leads to a synergistic effect with high percentages of NK cell that degranulate, kill target cells, and secrete cytokines (22). However, when either receptor is activated alone, poor responses are generated.

NK cells are traditionally categorized as part of the innate system because they have preformed defense mechanisms in place before an infection occurs and because NK cells respond in essentially the same way to repeated infections (23). It has been however pointed out that the maximal potential for NK cell killing is only realized after 'priming' via cytokines (24), and while NK cells rely on germline encoded receptors that do not undergo somatic recombination, there is a high individual variability in the number and combination of KIR receptors (25). NK cells are also able to clonally expand (26) and very recently were shown to display memory-like features in primates (27). Both arms of the immune system are however deeply intertwined. The innate system controls most infections well and holds out until the adaptive cells have had time to clonally expand and mature. Once an infection has been cleared, NK cells help regulate other activated cells (28,29). Another possible route NK cells could take in order to perform such varied tasks is through reprograming at the

epigenetic level (30,S.XI). As such, NK cells occupy a unique niche by having features of innate immunity but showing traits of adaptability and memory (24).

1.2 T cells

T cells represent the largest lymphocyte subpopulation and require a maturation step in the thymus giving them their name. A result of somatic recombination generating a vast diversity of unique, clonally distributed receptors, the T cell receptor (TCR) is the main activating receptor, and works alongside a few co-receptors. Naïve T cells receiving a signal through both TCR and a co-receptor would divide and be activated to produce cytotoxic proteins while cells that only receive the TCR signal may undergo anergy (23). T cells can be generally divided into two fractions: CD4 and CD8. CD4 T-helper cells function as regulators and exert their effect through collaboration with other immune cells namely B cells to proliferate and differentiate, and macrophages to break down phagocytized microbes. CD4 cells also release cytokines that help regulate other immune cells.

CD8 cytotoxic T lymphocytes (CTL) can exert cytotoxic responses. The CTL compartment together with NK cells are known as cytotoxic lymphocytes (CL), and are involved in cell-mediated immunity. CTLs control intracellular microbes by recognizing non-self peptides attached to MHC I on cell surfaces and immediately targets them (31). Should MHC I be downregulated to escape T cell detection as in the case of many cancers (32), NK cells will instead be sensitized. Once the infection is controlled, a subset of memory T cells will persist and produce a more rapid and efficient response upon a repeat intrusion of the same pathogen.

The immune system is thus highly complex and regulated. NK and T cells occupy separate niches in cellular immunity and play complimentary roles. Generally, our immune system protects against threats from the outside but in some persons, a defect inside the genetic code disrupts this delicate balance. Those bearing a deficiency or overactivation of a particular immune component could present with a wide range of symptoms. Less severe errors might be undetected due to redundancy of the immune system, while at the other end of the spectrum severe alterations could lead to death.

1.3 Primary immunodeficiencies

Primary immunodeficiencies (PID) are diseases caused by inborn errors in genes required for immune function. Genetic replication and repair is inherently imperfect, with errors either leading to a better adapted species, or more commonly being detrimental and leading to disease. The first article describing a possible PID is attributed to a German publication in 1922 where a group of patients presenting with severe neutropenia was reported (33). Then in 1952, Bruton described a male patient who had suffered 18 episodes of pneumonia and later

found to totally lack gammaglobulin. The patient was administered gammaglobulin injections and responded well (34).

There are more than 250 described PIDs today (35). These have been divided into various categories including combined immunodeficiency, defects in innate immunity, antibody deficiency, complement deficiency, and immune dysregulation. Estimating the PID population incidence rate worldwide is difficult as there is a large variability in the level of healthcare facilities and reporting, an ever increasing number of new genes implicated in PID, specific endemic mutations in particular geographical gene pools, and differing social practices e.g. consanguinity (36,37). An extrapolation estimated that up to six million people worldwide live with a PID with just 0.6% of these diagnosed (38). Data on the number of diagnosed persons in various countries however are sparse and vary tremendously per 100000 living inhabitants, with 83 in USA (39), 19 in Iceland (40), 4.6 in Australia and New Zealand (41), 2.3 in Japan (42), and 1.5 in Germany (43).

Many PIDs are passed down through Mendelian inheritance patterns, i.e. autosomal recessive but other forms do exist including dominant negative (44), hemizygous (X-linked) (45), hypomorphic (46), and gain of function (47) mutations. In this thesis, we focus on a particular family of PIDs and its specific effect on CL function. Rare individuals have been found with NK cells that fail to kill MHC class I deficient cells or opsonized targets. These patients may carry mutations in one of the genes involved in the synthesis of lytic granule content, the transport of lytic granules to the cell surface, or the release of lytic granule content towards the target cell (48).

1.4 Hemophagocytic lymphohistiocytosis

Hemophagocytic lymphohistiocytosis (HLH) is a disease of immune dysregulation, according to the International Union of Immunological Societies classification (35). This condition is currently recognized by a molecular diagnosis or the fulfillment of at least 5 out of 8 of the criteria presented in Table 1 (49-51). Although named ‘hemophagocytic’, the detection of hemophagocytosis which entails the excessive engulfment of cells by activated macrophages usually only comes late in disease progression (52). In fact, criteria 1 through 7 can be found individually in other diseases (53-58). Hence, it is suggested that the HLH acronym be renamed “hyperinflammatory lymphohistiocytosis” (59). When first suggested, only the first five criteria were listed and fulfilling all five was required for diagnosis (50). This underscores the severity and unique disease clinical pattern (60). HLH is usually incited by an infection or malignancy. Macrophages and other leukocytes are activated to clear the non-self antigens but due to an ineffective immune response, there is an overproduction of inflammatory cytokines and expansion of T cells but no disease resolution (61). Thus, activated histiocytes, including macrophages, are activated and can be found accumulated in various organs. One obvious reason why this happens is because of malfunctioning CL. As would be expound on, low or absent CL function could be used as an accurate diagnosis of a

group of primary HLH, as confirmed by molecular results, and represents the only pathognomonic criteria in Table 1.

Table 1. Diagnostic guidelines for HLH from the HLH-2004 protocol.

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
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)

No evidence of malignancy

At the point of the HLH-2004 publication (49), as it still is today, the method of choice for analyzing cytotoxic cell killing activity is the chromium release assay. First published from Karolinska in 1965 (62) and refined over the years (63,64), it has remained the widely accepted 'gold standard' for assessing CL killing although no systematic study on the sensitivity and specificity of the chromium release assay in discriminating HLH cases has been published. As early as 1977, patients with HLH were found to be deficient in lymphocyte killing via the chromium release assay (65). Later on, sporadic reports found absent NK degranulation in primary HLH cases via flow cytometry (66-70). This new assay analyzed effector cell response, unlike the chromium assay where target cell lysis was measured. However, there is no consolidated effort to study large cohorts of primary HLH and neither was there a consensus protocol on performing NK degranulation assays. What was then needed was a study to thoroughly evaluate NK cell degranulation for the diagnosis of primary HLH.

1.4.1 Primary HLH

HLH is traditionally divided into two groups: primary and secondary (71). Primary HLH, also known as familial HLH (FHL) represents patients with an inherited autosomal recessive deficiency in genes of which many are associated with defective lymphocyte cytotoxicity (Table 2). When manifest but left untreated, the disease would ultimately lead to death (65). Secondary or sporadic cases on the other hand are thought to be incidental occurrences

secondary to a primary malignant, infectious, autoimmune disease, or complications due to therapy, with no clear genetic determinant established (72). This taxonomy is important as current treatment varies between the two groups. Hematopoietic stem cell transplantation (HSCT) is for the moment the only curative treatment for primary HLH. This is performed according to the HLH-94 protocol based on chemotherapy and immunotherapy followed by transplantation (51,73). Although transplantation is associated with considerable risks, a delay increases the chance of neurological symptoms in primary HLH patients (74). For secondary cases however, patients undergo cytotoxic and immunomodulatory treatment but would not normally proceed with HSCT as most remain disease free after initial treatment (75,76). As such, there is a need for rapid, sensitive, and simple assays for the differentiation of these two subgroups as patient counseling becomes simpler once a diagnosis is achieved. Taking this one step forward, patients found with defective NK cell function can be evaluated for genetic alterations. This could be one route for the discovery of new mutations leading to defective degranulation.

The first familial HLH case report was published in 1952 (77), but only in 1996 was the *LYST* gene, linked to Chediak-Higashi syndrome identified (78). Then in early 1999, perforin and the associated apoptotic components were suggested to be involved in familial HLH (79). This was quickly followed that same year by a seminal paper describing perforin gene defects in familial HLH cases (80). A few years on, other familial HLH patients with defects in *UNC13D*, *STX11*, *STXBP2*, *RAB27A*, and *AP3B1* were described in quick succession (Table 2) (68,81-86). Long before the identification of these genes, CL abnormalities were already reported in HLH patients and suggested to be the cause of disease (65,87,88). To note, those harboring defects in *RAB27A*, *LYST*, and *AP3B1* almost always display partial albinism as these genes are involved in melanin storage and transport (89,90). Care should be taken when evaluating patients of Caucasian background as fair skin could mask the underlying albinism (91).

With time, although primary HLH is a rare disease with an estimated incidence rate of 1.2:1,000,000 in Swedish children <15 yo (92,93), or 1.07:10,000 children in Texas (94), hundreds of primary HLH cases have been documented. Amongst these, reports of patients succumbing to HLH with monoallelic mutations or multiple heterozygous mutations in different primary HLH genes are raising questions on the inheritance pattern of primary HLH as well as the environmental factors leading to disease (95-99). Monoallelic mutations in primary HLH genes are also being linked with cancer (100,101). It remains to be proven if monoallelic mutations in HLH genes do in fact lead to disease or there exists mutations on the second allele that are not picked up with regular sequencing. Alternatively in these patients, there is a possibility that yet to be described gene(s) linked to HLH are defective. Additionally, are other carrier relatives healthy, especially the parents?

Table 2. List of major PIDs studied in this thesis.

Gene	Protein	Syndrome	OMIM
PRF1	Perforin	FHL2	170280
UNC13D	Munc13-4	FHL3	608898
STX11	Syntaxin 11	FHL4	605014
STXBP2	Munc18-2	FHL5	613101
AP3B1	AP-3 complex subunit beta-1	HPS2, Hermansky-Pudlak syndrome 2	608233
RAB27A	Rab27a	GS2, Griscelli syndrome type 2	607624
LYST	Lysosomal-trafficking regulator	CHS, Chediak-Higashi syndrome	214500
SH2D1A	SAP	XLP1	308240
XIAP	XIAP	XLP2	300635
ORAI1	CRAC channel protein 1	IMD9	610277
STIM1	Stromal interaction molecule 1	IMD10	612783

Patients fulfilling HLH criteria but displaying normal CL function have also been documented including *ITK* (102), *CD27* (103), *GATA2* (104), *BIRC4* (105,106), and *SH2D1A* deficiency (107). As such, 'low or absent NK cell activity' cannot be used as one of the diagnostic guidelines for these primary HLH cases. Moreover, new diagnostic assays need to be designed (108-111) and the HLH nomenclature clarified.

While patient evaluation has focused on NK cells from primary HLH cases, few have worked on patient CTL (112) as they generally require a long stimulation and clonal expansion step before gaining cytotoxic potential. Mice studies have recognized the different roles played by NK and CTL in maintaining immune homeostasis (12,113). It is thus of interest to evaluate both arms of the CL in primary HLH patients. We addressed this issue by designing a T cell degranulation assay to evaluate cytotoxic T cell function without requiring any prior activation. By juxtaposing the established NK assay against this T cell degranulation assay,

we hope to draw conclusions on the similarities between these two arms of CL and improve diagnostics.

1.4.2 Secondary HLH

Great strides have been made in the understanding and diagnosis of primary HLH. However, it has been more challenging to study secondary HLH. Also known as adult onset or sporadic HLH, searching Pubmed would return case reports of patients with very varied diseases fulfilling HLH criteira. This includes spider bites (114), inborn errors of metabolism (115), Gaucher's disease (116), rheumatic diseases (117), macrophage activation syndrome (118), malignancy (119), dengue virus (120), Ebola virus (121), HIV (122), leishmania (123), Lyme disease (124), leprosy (125), tuberculosis (126), and various other zoonoses (127).

Large overlaps in clinical features and genes involved in primary and secondary HLH have blurred the distinction between those two groups. Primary defects lead to increased susceptibility to infections for example EBV in X-linked lymphoproliferative diseases (128-130). Thus, seemingly secondary cases could in fact be due to a primary cause. As DNA in every person contains unique variations, minor changes in one or several genes could lead to increased disease susceptibility given the right environmental factor or pathogen. It has been suggested that everyone has at least one mutation affecting the immune system (131). However, it is almost never detected as a very specific antigen needs to be encountered and the high immune redundancy normally compensates for the deficiency. Studying intronic mutations, regulatory elements, epigenetic changes, multiple gene effects, and somatic mutations would feature in the next generation of PID research but would require exponential effort to fully comprehend.

There have been efforts made to differentiate primary HLH, secondary HLH, and other hyperinflammatory diseases using laboratory parameters. Adult HLH cases were found to have skewed CD3 populations (132) while ferritin levels are distinguishable between primary HLH and virus-associated HLH (133). In an intensive care unit setting, it was possible to differentiate HLH cases solely based on ferritin levels (134). Another study found the soluble IL2 receptor/ferritin ratio a good marker to differentiate pediatric primary HLH from other pediatric hemophagocytosis fulfilling HLH criteria (135). Thus, noticeable differences can be found between these syndromes giving us reason to believe there are unique genetic or environmental backgrounds in the various groups of diseases. However, the disease spectrum of HLH is more continuous rather than discrete (136) and vast overlaps between what is currently known as primary and secondary HLH could confound efforts. Still, there is a need to develop more sensitive functional assays and genetic screenings to try to tweeze apart these closely related phenotypes.

1.5 HLH Therapy

Patients with primary HLH will, as a first-line treatment, be subject to intense immunosuppression and anti cytotoxic drugs. This is to suppress the underlying life threatening inflammation (51). After the initial therapy, immunosuppressive drugs will be administered to maintain the patient in a stable resolution of disease and be ready for HSCT when a donor becomes available. For secondary HLH, only anti inflammatory or immunosuppressive drugs would be prescribed (75). In 1983 where specific HLH therapy was non-existent, only 3% of patients with HLH survived long term (65). This improved to 55% long term survival with the HLH-94 protocol (137) which is the current standard of care, confirmed in an independent retrospective report (94).

In an effort to reduce patient mortality in the pre-transplant phase as well as the high levels of central nervous system complications, various studies have considered other drugs and combinations (49,138,139). Many patients bear infections at pre-transplant or are susceptible to infections because of the immunodeficiency they carry. Reduced intensity conditioning would hopefully reduce infection rates as well as the toxicity associated, leading to higher numbers of long term survivors.

Gene therapy is an option in the early stages of experimentation. Eleven patients with SCID were treated with a corrective lentiviral vector (140). Nine patients were cured but one developed a lymphoproliferative disorder (141). As such, effort in translating similar technology to the HLH field (142) should be done with utmost care and caution. Another interesting HLH treatment option currently in clinical trials is with an anti-IFN-γ antibody. This idea stemmed from reports showing blockade of IFNγ could increase the survival of perforin and Rab27a deficient mice (143,144). However, other models have shown that high IFN-γ does not necessarily lead to a HLH phenotype (18). Moreover, a recent report described two patients with IFN-γ receptor 2 deficiency but yet fulfilled HLH criteria (S.X). As such, further study is required to understand these observations.

As the literature on HLH improves, primary HLH patients have been found with functional deficiencies in other cell types or organs not usually associated with host defense but could still contribute indirectly to the HLH pathology. For example, in some primary HLH patients, the gastrointestinal tract, erythrocytes, and platelets (145-147) have been shown to be affected. HSCT replaces not just CLs descended from the common lymphoid progenitor, but also macrophages, neutrophils, platelets, and red blood cells from the common myeloid progenitor. As such, other HSC derived cells affected by primary HLH (145,146,148,149) will be corrected, possibly leading to better overall standard of living post-transplant. Studies on the non-immunological functions of primary HLH genes are lacking, but it can be postulated that other cell types could utilize similar molecules for intracellular vesicle transport and exocytosis. Examples would include neurotransmission, excretion of digestive enzymes, and hormones from various organs/glands. Alternatively, hematopoietic cells could play important roles in various organ systems of the body that is yet unappreciated. Still, CL

most likely play a big role in HLH pathology and we next describe their function in relation to the work herein.

1.6 The cytotoxic lymphocyte exocytosis pathway

Natural killer cells and cytotoxic T lymphocytes can kill target cells through the secretion of lytic granules or via the FAS ligand pathway (150). In the case of granule secretion, which is the focus here, the effector cell first recognizes stress markers on the target cell through various receptors. Engagement of an activating receptor (Figure 1A) rapidly leads to 'inside-out' signaling, altering lymphocyte function-associated antigen 1 (LFA-1) from a closed to an open conformation (151) (Figure 1B). Extended LFA-1 mediates adherence to the target cell and initiates the formation of an immune synapse (152), or to a vascular wall for extravasation and onwards to various targeted tissues (153). This event has been recently found to be potassium dependent (154). Engagement of the open conformation integrin then leads to 'outside-in' signaling that further activates the lymphocyte leading to polarization (155).

Besides the LFA-1 activation, a phosphorylation signal cascade from the activated receptor would cumulate at the endoplasmic reticulum (ER) where Ca^{2+} is released from the ER into the cytoplasm. Lowering of the ER calcium concentration is sensed by stromal interaction molecule 1 (STIM1) and results in its aggregation. Aggregated STIM1 gathers ORAI1 monomers on the cell surface, which bind into tetramers to form functional ion channels. Calcium fluxes into the cell in a process termed store-operated calcium entry (SOCE) (Figure 1C). The Ca^{2+} influx activates calcinurin, a phosphatase which dephosphorylates NFAT, allowing this transcription factor to migrate into the nucleus where it mediates transcription of FasL, IFN-γ, TNF-α, IL-2, and chemokines (156,157). Besides lymphocyte signaling, ORAI1 is crucial for the proper function of airway epithelial cells (158), microglia (159), pancreatic acinar cell (160), and possibly many other cell types. ORAI1 deficiency has been identified in humans and its function heavily studied in *Drosophila* and mice (157,161). Work on human T cell lines demonstrated a role for ORAI1-mediated Ca^{2+} influx for induction of cytokine and chemokine expression. However, little is known about how crucial calcium signaling is in granule exocytosis by CL in general or NK cell function in particular. We had a chance to study NK cell signaling and function in a rare ORAI1 deficient patient and presented the results in Paper V.

Signaling through activating receptors also leads to Fyn and ADAP activation, which control diverse downstream cell functions including cytotoxicity, proliferation, and cytokine production (162). Lytic granules in T cells are upon activation gathered to the MTOC via microtubules mediated by the motor protein dynein (163) (Figure 1D). Then the granules are transported to the polarized region on both microtubules and actin (164) via a complex of kinesin-1/Rab27a/Slp3 (165) (Figure 1E). Along the way, perforin-containing granules colocalize with vesicles containing Munc13-4 (S.I). The Rab27a/Munc13-4 complex is

required for granule tethering at the plasma membrane (166) (Figure 1F). Other molecules including Stx11 and Munc18-2 can then interact with the lytic granule (167) and most likely regulate the SNARE complex formation. This large complex mediates the fusion of lytic granule membrane and the cell membrane (Figure 1G).

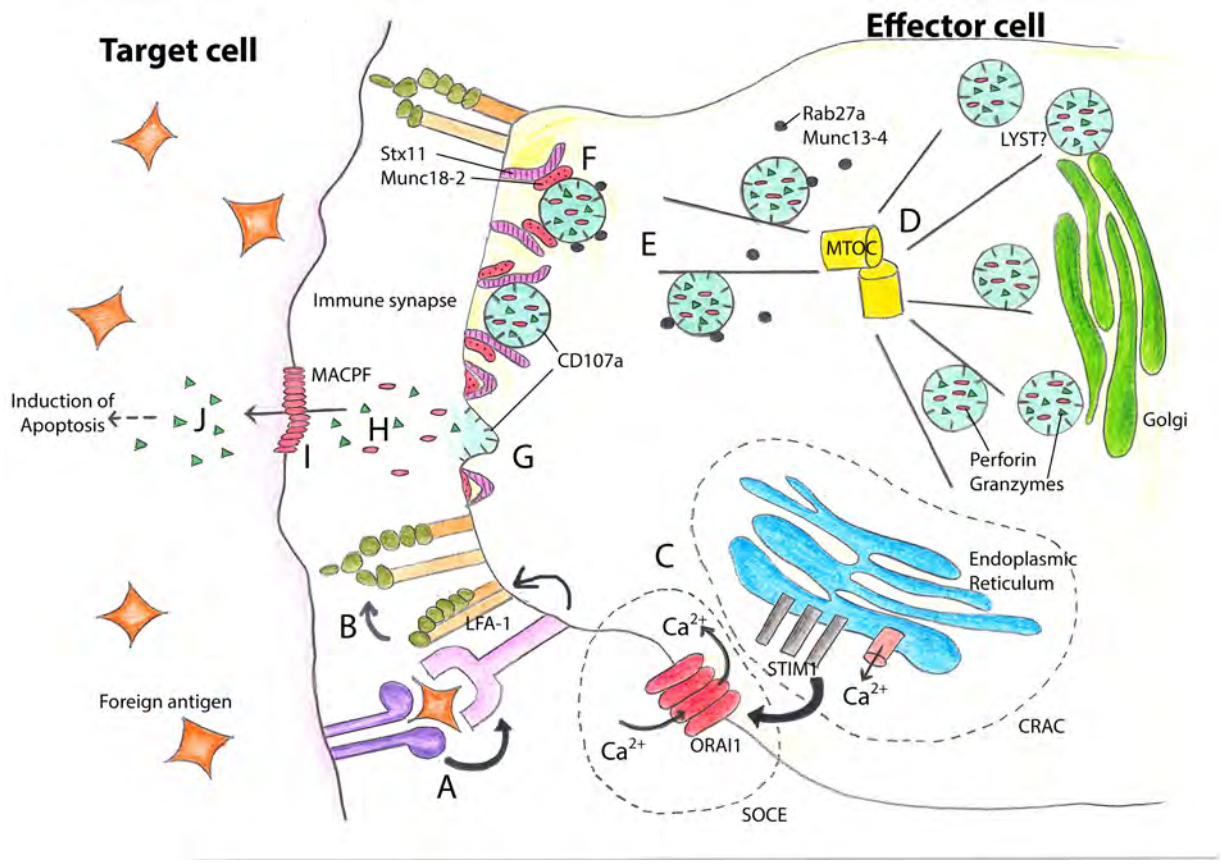

Figure 1. The cytotoxic cell response to a target cell. A virus infected 'target' cell triggers a 'effector' cytotoxic lymphocyte via an activating receptor leading to multiple responses (A). LFA-1 molecules through an inside-out activation would extend (B) to link to the target, forming the immune synapse. Calcium flux mediated CRAC and SOCE pathways (C) lead to production of cytokines and other stimulating factors. Lytic granules would be pulled towards the MTOC (D) and directed to the polarizing cell end (E) during which it associates with other factors including Rab27a and Munc13-4 , which are required for tethering (F). The actions of the SNARE complex including Stx11 and Munc18-2 (G) lead to docking and fusion of membranes. CD107a can then be found on the effector cell surface while perforin and granzymes are released into the immune synapse (H) where perforin acquires an active form and binds to the target cell membrane (I) and forms the MACPF. Granzymes enter the target cell through the perforin pores (J) and initiates target cell apoptosis. Illustration by Jin Ming Tee.

Once the two sets of membranes fuse, the lytic granule content is released into the immune synapse and CD107a molecules attached to the inner membrane of lysosomes diffuse on the effector cell surface and can be detected by antibody staining (Figure 1H). This event is termed degranulation and represents a measure of CL activity (168,169). The whole multistep degranulation pathway is tightly regulated and removing one component collapses the whole

cascade. Target cell killing is thus abrogated, leading to disease progression.

The immune synapse stably links the effector and target cell, allowing for precise unidirectional delivery of cytotoxic mediators directly aimed at specific target cells (170), minimizing bystander effect. This channel is most likely leaky but rapid dilution of perforin and granzymes outside the immune synapse renders them ineffective as these need to be colocalized and at a high concentration to be functional (171).

The heavily glycosylated Lysosome Associated Membrane Protein (LAMP) family consists of CD107a (LAMP1), CD107b (LAMP2), and CD63 (LAMP3). The first two members together make up 50% of lysosomal membrane proteins (172). Upon exocytosis, the LAMPs diffuse onto the cell membrane in the vicinity of the immune synapse. Because they are found in such abundance, it has been suggested to be important in preventing perforin and granzymes from attaching and killing the parent cell (173). In addition, low lysosomal pH alters perforin structure rendering it inactive (174). CD107a has also been specifically found to be important for the maintenance of lysosomal compartment structure (175). In mice, knocking out CD107a did not result in significant pathology (176), possibly due to the redundancy offered by CD107b, which was upregulated. Knocking down both CD107a and b resulted in non-viable mice. CD107a could also play a role in the transport of lytic granules and the stability of perforin (177). In S.VI, where human cells were used, target cells forced to express surface CD107a were shielded from granule-mediated killing due to the inability of perforin to bind to the cell surface. Additionally, siRNA knock-down of CD107a led to poor NK cell cytotoxicity and increased effector cell apoptosis demonstrating that LAMP proteins do indeed confer protection against proteolytic damage. Multiple cancer lines (178) express surface CD107a and CD107b, and highly metastatic cells were found to be correlated with higher levels of surface CD107a and CD107b (179), suggesting again the protective nature of LAMPs.

After traversing the immune synapse, perforin monomers change in conformation due to the more physiological extracellular pH and similar to the C9 complement component (180), oligomerize to form the membrane attack complex perforin (MACPF) on the outer surface of the target cell, similar to complement (181) (Figure 1I). Perforin would bind multiple calcium ions leading to a structural change that facilitates lipid binding (182). Once the pore forms, granzymes freely diffuse into the target cell and are able to directly induce apoptosis by activating caspases (183) (Figure 1J).

The whole cytotoxic process happens relatively quickly with NK cells possibly being slightly faster compared to CTL (170). TCR recognition requires approximately two minutes (184) while cells targeted by NK cells show propidium iodide uptake in under two minutes (170). The whole process from initial target identification to target cell detachment has been estimated to last between six to eight minutes (61,185). It has been noted however that not all CL degranulate and are in fact very heterogeneous in terms of function. A minority of NK cells performs the majority of cytotoxic function while half of the NK cells observed did not kill any targets (186). Another study found CTLs have very varied killing rates and have long

moments of inactivity followed by 'burst killing' where multiple targets are killed in quick succession (187). As such, there is a need to study why certain cytotoxic cells function better than others and what leads to the vast heterogeneity. Cytotoxic cells with knocked-out perforin are incapable of mounting a killing response. When challenged, these CL have been found adhered up to 40 minutes to a target and repeatedly fluxed calcium. During this period, excessive amounts of cytokines was produced but no targets were killed (61). This possibly leads to the cytokine storm characteristic of HLH.

Besides the established chromium release assay and the CD107a degranulation assay, many other measures of lymphocyte cytotoxicity have been developed. Amongst these, a granzyme B Enzyme-Linked ImmunoSpot (ELISPOT) assay detects the level of granzyme B secreted from effector cells after the addition of CL (188) while the GranToxiLux kit measures granzyme B directly in target cells. Another family of flow cytometric methods mimics the chromium release assay requiring a pre-labeling of target cells with a lipophilic dye before the addition of effector cell. Later, staining for dead cells allows separation of live and dead targets from effectors (189-191). The addition of other markers would allow for the discrimination of degranulating CL, early, and late apoptotic cells. All these methods have been tested to be 'equivalent' to the standard chromium assay, but there is currently no broad acceptance of any one technique which makes data comparison difficult. Moreover, most methods have not been tested in a diagnostic setting with primary cytotoxicity deficient patients.

NK and T cells play different roles in the pathology of HLH as elucidated by mouse models. Knock-outs with a deficiency in one of the genes important for exocytosis leading to both defective NK and T cells are susceptible to severe HLH-like disease when challenged with LCMV (144,192), while mice lacking either cytotoxic NK or T cell did not show severe HLH symptoms when challenged (18). T cells specifically, have been found to be important in the controlling of antigen presenting cells and a major source of IFNγ secretion in HLH (144,193). NK cells on the other hand are important for the removal of inflammatory macrophages and hyperactivated T cells (18). Interestingly in mice with normal NK cells and perforin negative T cells, IFN-γ levels were elevated but HLH did not develop. This shows that both NK cells and T cells play different and non-redundant roles in the pathogenesis of HLH.

In summary, the lack of NK cell and CTL cell cytotoxicity would lead to a susceptibility to malignancy, infections, and inadequate modulation of immune response. Hence, precise regulation of these killer cells, their various pathways, and activity is crucial for the well being of an organism. Herein we present work done on optimizing and creating new assays for the rapid, simple, and sensitive diagnosis of primary diseases associated with a defect in NK cell and cytotoxic T cell function. Starting off with an up-to-date step-by-step protocol for testing NK cell degranulation and perforin content (Paper I), we then present a multicenter European investigation into the sensitivity and specificity of the K562-NK cell degranulation assay in diagnosing primary HLH (Paper II). This diagnostic assay has been instrumental in

the discovery of novel noncoding mutations (Paper III, IV) and biological characterization of rare PID cases (Paper V). Finally, in an effort to study CTL cell function, a new degranulation assay was designed and the requirements for NK cell degranulation compared to CTL, in the context of primary HLH. This was performed with resting state cells without any prior activation (Paper VI). The accuracy of this T cell degranulation assay was then compared against established NK cell degranulation assays for the diagnosis of primary HLH leading to defective degranulation (Paper VII).

2 AIMS

The work presented here aims at improving the current and developing new functional assays used for the diagnosis of hemophagocytic lymphohistiocytosis (HLH). This would lead to better patient management and improved prognosis. Studying how specific PID genes affect overall health is also important in broadening our general understanding of human biology. More specifically, our study objectives were:

Paper I:

- To provide an up-to-date standard operating procedure for performing NK cell phenotypic assessment and NK cell degranulation assay.

Paper II:

- To standardize NK degranulation assay protocols across the major HLH referral centers in Europe.
- In a multi-center approach, to evaluate the K562-induced NK degranulation assay as a diagnostic assay for PIDs affecting NK degranulation.

Paper III:

- To evaluate all infant HLH cases in Sweden with defective NK cell degranulation.
- To identify genetic defects in infant HLH cases that have yet to receive a molecular diagnosis.

Paper IV:

- To explain a rare case of HLH in a non-consanguineous family of Chinese descent.
- To illustrate the effects of prolonged sample transport times on various cytotoxic lymphocyte functional assays.

Paper V:

- To understand the role of store-operated calcium entry in NK cell responses.
- To determine the activation stage where ORAI1 function is crucial in NK cells.

Paper VI:

- To define a T cell subset containing cytolytic proteins that could exert cytotoxic effects.
- To develop a robust CTL degranulation assay using resting, unstimulated lymphocytes and compare it to NK cell degranulation levels.
- To study resting state and IL-2 activated CTL degranulation in patients with known NK cell degranulation defects.

Paper VII:

- To document the variability in responses to target cell stimulation found in various NK cell and $CD8^+$ T cell subtypes.
- To compare NK and CTL degranulation levels in various PID patients that present clinical HLH or hyperinflammation.
- To assess the sensitivity and specificity of various degranulation assays for the diagnosis of primary defects affecting cytotoxic lymphocyte degranulation.

3 PATIENTS AND METHODS

An overview of the patient groups studied and the methods employed in this thesis is outlined here. For specific details, please refer to the methods section of the various manuscripts.

3.1 Patient recruitment

Patients from Sweden and around the world were recruited for the studies done here. Through word of mouth and various channels, we have built up a network of clinician collaborators who send blood samples of their patients with suspected HLH or other abnormal presentations to us for diagnosis. The blood shipment included a signed consent as well as a short clinical report briefly outlining the patient's current condition. Patients having confirmed functional and molecular diagnosis have been used for the papers presented here. The Regional Ethical Review Board in Stockholm has approved all studies. Healthy donors were obtained from the local blood bank.

Specifically, in Paper I, II, VI, and VII, we have included patients bearing confirmed mutations leading to defective CL killing. For Paper III, we compiled all Swedish infants born between December 2005 and January 2011 referred to our laboratory for suspected HLH. Those with a family history of HLH and/or a defect in NK cell degranulation were included in the manuscript. Also used were healthy local infant controls, confirmed *PRF1* patients, and European patients with mutations in *UNC13D*. Paper IV studied a specific family from Singapore diagnosed with a novel *UNC13D* mutation. Paper V analyzed a patient previously reported bearing an *ORAI1* mutation (194) as well as a patient with *STIM1* mutation. Paper VII included patients bearing mutations in PID genes leading to normal CL degranulation, patients being treated for MAS/SoJIA, and patients with at least 5 of 8 HLH criteria fulfilled but with no mutations in any HLH-associated genes.

3.2 PBMC preparation

In all papers, peripheral blood from patients, family members, and unrelated controls were collected in heparin tubes and delivered at room temperature within 48-hours to the laboratory. Whole blood was diluted 1:1 in phosphate buffered saline (PBS). The diluted blood was carefully layered on top of Ficoll in a 50 ml tube and spun at 800 *g* for 20 minutes with brakes off. The PBMC at the Ficoll interphase was then extracted, washed twice in PBS, and live cells counted, either with trypan blue on a haemocytometer, or using an automated cell counter (Muse, Millipore). Cells in complete media (RPMI supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine) were then rested overnight or stimulated with 500U/ml IL-2 (Proleukin, Novartis) for 36-48 hours before assays were run. All unused PBMC were frozen down in fetal calf serum (FCS) with 10% DMSO and stored in liquid nitrogen for future work. Buffy coats were processed in a similar manner.

3.3 Cell isolation

In Paper III, V, and VI where specific subsets of PBMC were evaluated, magnetic bead based isolation was used (Miltenyi Biotec). Paper III used positive selection magnetic beads for the isolation of $CD4^+$, $CD8^+$, $CD14^+$, and $CD56^+$ populations from PBMC. In Paper V and VI cells were purified using negative isolation kits. NK cells were isolated with a standard kit while $CD8^+$ T cell subsets were purified using the standard $CD8^+$ negative isolation kit supplemented with anti-CD45RO and anti-CD57 magnetic beads for naïve $CD8^+$ T cells, with anti-CD45RA and anti-CD57 for memory $CD8^+$ T cells, and with anti-CD28 beads for effector $CD8^+$ T cells. Isolated fractions were tested for purity via flow cytometery before performing further work.

3.4 Evaluation of NK cell cytotoxicity

In Paper III, V, and VI, we evaluated NK cytotoxicity via the established ^{51}Cr release assay. This sensitive and highly reproducible method (195) is still widely used for the determination of NK and T cell cytotoxicity although faced with problems involving radioactivity, waste disposal, a high cell number requirement, and the inability to be multiplexed. In this assay, cells (PBMC or purified subpopulations of NK or T cells) were incubated in medium or mixed with target cells (K562 cells, P815 cells, P815 cells added anti-CD3 mAb, L1210 cells alone, or L1210 cells added anti-CD16 mAb) pre-labeled with ^{51}Cr (Na_2CrO_4, sodium chromate in normal saline, Perkin Elmer) at specific effector to target ratios. Effector cells were first serially diluted to a range between 100 and 0.3, before the addition of ^{51}Cr-labeled target cells. To quantify spontaneous chromium release, pre-labeled target cells were incubated in media without any effector cells, while for maximum chromium release, pre-labeled target cells were incubated with 2M hydrochloric acid. Assays were run in duplicates or triplicates for four hours in a humidified 37°C incubator. The cells were then spun down and supernatant collected for gamma irradiation quantification with a gamma counter (Packard, Cobra or Wizard2, Perkin Elmer).

The specific percent lysis was calculated:

$$\%\ specific\ lysis = \frac{mean\ experimental\ cpm - mean\ spontaneous\ cpm}{mean\ maximium\ cpm - mean\ spontaneous\ cpm} \times 100$$

cpm: counts per minute.

The effector to target cell ratio at 25% specific lysis was estimated from the percent specific lysis curve, and converted to lytic units (LU) by the function below as described by Bryant et al. (196). A value of less than 10 LU_{25} was considered pathologic.

$$\text{Number of lytic units per } 10^7 \text{ effector cells} = \frac{10^7}{no.of\ target\ cells \times E{:}T\ ratio\ at\ 25\%\ lysis}$$

For detailed protocol, please refer to references 197 and 198.

3.5 Evaluation of cytotoxic lymphocyte degranulation

In all papers, the evaluation of NK and T cell degranulation was performed via the detection of CD107a on CL cell surface after stimulation with various target cells (169,199). A complete and detailed protocol can be found in Paper I. Briefly, resting or cytokine stimulated PBMC were mixed 1:1 with target cells or media alone as control for up to 6 hours. The target cells were K562, P815 with anti-CD16 antibody (clone 3G8), P815 with anti-CD3 (S4.1), or L1210 with anti-CD16 antibody in the presence of Brefeldin A. In Paper V, the SOCE blocker DPB162-AE was also added in certain experiments. The cells were then surface stained with a live/dead discriminating dye and fluorescence conjugated antibodies against CD107a (H4A3), CD3, CD4, CD8, CD56, and CD57. In certain experiments, LFA-1 open conformation (mAb24) was also evaluated. Cells were then fixed with formaldehyde (Cytofix, BD Bioscience), and stained for intracellular MIP-1β, CD69, TNF-α, and IFN-γ, in the presence of saponin (Cytoperm, BD Bioscience). Finally cells were acquired on a BD Calibur, Dako Cyan ADP, or BD Fortessa cell analyzer and analyzed on FlowJo (v7 or v9, TreeStar). The percentage NK/CTL degranulation or cytokine production was calculated by comparing effector cells stimulated with various conditions against effector cells in media alone, and termed ΔCD107a%.

3.6 Evaluation of cell granule contents

The detection of perforin levels in NK cells was performed with flow cytometry following the step-by-step procedure in Paper I. Resting PBMC were stained with a live/dead discriminating dye, and fluorescence conjugated antibodies against CD3 and CD56. Cells were then fixed with formaldehyde (Cytofix, BD Bioscience), and then stained for intracellular perforin (δG9), or isotype-matched control. Cells were acquired on a BD Calibur or BD Fortessa cell analyzer (BD Bioscience) and analyzed on FlowJo (v7 or v9, TreeStar). The median fluorescence intensity of perforin was then compared to isotype control.

Advanced phenotyping of NK and T cells in Paper VI was performed as above but with additional surface and cytoplasmic markers including CD3, CD4, CD8, CD27, CD28, CD45RA, CD45RO, CD62L, perforin, and granzyme B. Data was evaluated using Spice v5.2b 21265010 and the probability state modeling performed by Gemstone (v1.0.63, Verity Software). Details on the models are found in the supplementary data of Paper VI.

3.7 Calcium flux

The detection of intracellular calcium levels in Paper V was done with indo-1-AM. Isolated NK cells were first preincubated with thapsigargin, a calcium inhibitor, or anti-CD16 antibody before being spiked with $CaCl_2$. Cells were acquired on an LSRII (BD Bioscience).

3.8 DNA sequencing

Mutation detection for most of the patients analyzed was performed via PCR followed by Sanger sequencing. Genomic DNA was isolated from peripheral blood, fibroblasts, or PBMC according to standard procedures. Forward and reverse primers were designed to amplify exons and exon-intron boundaries of the known FHL genes by PCR. In Paper III and IV, primers targeting evolutionary conserved intronic regions of *UNC13D* were in addition designed. PCR products were then directly sequenced (BigDye v3.1, Applied Biosystems) and reactions analyzed by capillary electrophoresis (ABI3730, Applied Biosystems). Variants found were assessed *in silico* for pathogenicity using the program Alamut (Interactive Biosoftware). For a small number of patients in Paper VII, whole exome sequencing was performed. Sequences were processed through a bioinformatics pipeline including quality control, mapping, coverage analysis, variant calling, variant annotation, and filtering. Reads were mapped to genome build *hg19*. Potential candidate variants were inspected visually and thereafter confirmed with Sanger sequencing.

3.9 RNA isolation and cDNA amplification

In Paper III, total RNA was extracted from PBMC (RNeasy, Qiagen) and first strand cDNA synthesized with oligo$(dT)_{20}$ primed reverse transcription (SuperScript III, Invitrogen). Nine primer pairs were designed to amplify overlapping cDNA fragments of the *UNC13D* cDNA. The amplified products were run on an agarose gel, extracted (QIAquick, Qiagen) and cloned (TOPO-TA, Invitrogen). The resulting plasmid DNA was then extracted and sequenced.

The 3' RACE-PCR (Rapid Amplification of cDNA Ends) used primers marked with an M13R-tag for the first strand cDNA synthesis. Then the 3'-end amplification was carried out with a gene specific forward primer and an M13R reverse primer. The amplified products were then similarly ran on an agarose gel, extracted, cloned and sequenced as described above.

3.10 Allele specific RT-PCR

For Paper III, trizol extracted RNA from various isolated subpopulations of lymphocytes was used for cDNA synthesis as described above. Quantitative real-time PCR was then run to quantify allele specific transcription levels. Two different reverse primers that could differentiate a single nucleotide polymorphism in exon 11 (c.888G > C) were used. cDNA was mixed with SYBR Green PCR mix (Applied Biosystems) together with forward and reverse primers. Reactions were ran on a real-time PCR (ABI7900 HT, Applied Biosystems) in triplicates.

3.11 Western blot

PBMC from various patients in Paper III and IV were blotted for Munc13-4. PBMC were lysed with buffer containing Triton X-100 and then spun down to collect the supernatant. Protein content was first determined using Bradford assay (Bio-Rad) and equal protein amounts then loaded onto an SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane and blotted for Munc13-4 (Protein Technologies Group) and ERK1 (Santa Cruz Biotechnology).

3.12 Granule polarization assay

In Paper V, isolated NK cells were incubated with K562 cells for 20 minutes on a microscope slide and then fixed with paraformaldehyde. Phalloidin, perforin, and α-tubulin were stained for and conjugates imaged with a confocal microscope. The degree of actin, perforin, and MTOC polarization was then scored.

4 RESULTS AND DISCUSSIONS

The following chapter gives an overview of the patient samples analyzed over the years in our laboratory. Besides collating the data from papers included in the appendix of this thesis, select observations from our other published and yet unpublished works have been included here to so as to present a fuller picture of the diagnostic efforts.

4.1 Patient census

Over the past seven years, we have built up a strong network of clinician collaborators from around the world. This has led to a large number of HLH diagnostic requests. The geographical origin of patients received between the period of January 2013 and July 2015 is shown in Figure 2. During this period, 421 unique patients were received in 31 months or an average of 13.6 patients a month. This number does not include repeat samples or those from family and healthy controls shipped at the same time.

Local Swedish patients represent the highest proportion. Most of these patients do not fulfill 5 of 8 HLH criteria but with high awareness amongst local clinicians, samples have been sent in due to reoccurring infections, hyperinflammation, and abnormal clinical symptoms. This is important in light of reports of PID patients with atypical presentation (S.IV, S.IX, S.X, S.XIII). Substantial patient numbers have also been sent from our Scandinavian neighbors. Consanguinity is uncommon in this region (200-202) but small initial founder populations combined with genetic isolation or large immigrant populations with limited integration to indigenous populations have led to enrichment of certain monogenic diseases (92,203,204). Turkey and Poland also contributed a significant number of patients. These are two populous countries (78 and 38 million inhabitants respectively, compared to 25 million in Denmark, Finland, Norway, and Sweden combined) and have naturally higher number of cases. Additionally, up to a third of marriages in Turkey are consanguineous (205). With ease in travel between Schengen states and large migration from places of unrest, European countries have become more heterogeneous. Such populations tend to produce offspring within the same race group that could lead to higher rates of consanguinity, and in the case of Norway, 40% of children born to Pakistani migrants have related parents (200).

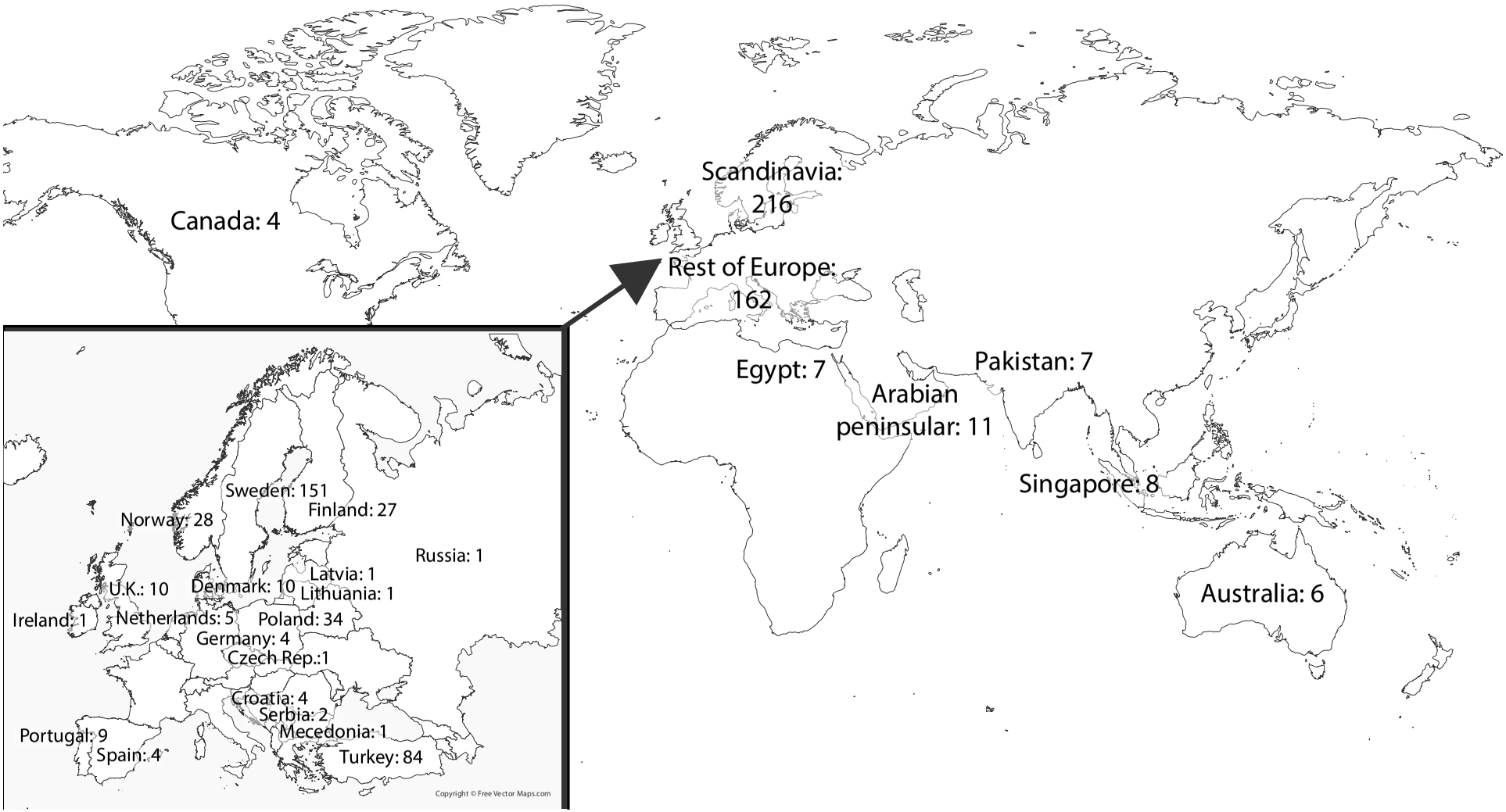

Figure 2. Patient numbers sent for HLH testing from around the world between January 2013 and July 2015. Numbers are shown for various countries and regions and are specifically broken down for European countries (inset).

4.2 Assay advancement for rapid assessment of genetic degranulation defects

CD107a is a protein usually found associated with lytic granules and late endosomes. Upon degranulation, where the membrane of lytic granules fuse with the plasma membrane, CD107a can then be found on the outer cell surface and detected easily. Should there be a damaging mutation in one of the genes implicated in lytic granule trafficking or membrane fusion, CD107a surface expression would be absent or greatly reduced after target cell stimulation. This is the biological basis behind the functional assay to diagnose for genetic degranulation defects.

When diagnostic assays were first run in our laboratory, CD107a levels on NK cell surface was studied using a 4-colour cytometer (Paper II and III). The European consortium in Paper II met to standardize protocols between four collaborating laboratories and agreed upon a basic degranulation assay consisting of two conditions, first a negative control of PBMC with no stimulation, and the second having PBMC with K562 cells as stimulus. Patients were analyzed at the various laboratories for 2½ years before the data was collated. The 4 laboratories contributed in total 120 confirmed PID patients of which 90 carried a genetic degranulation defect. Comparing these against a group of patients with secondary HLH, XLP, or *PRF1*, Receiver operating characteristic (ROC) curves returned 5.4% $CD107a^+$ NK cells as the optimal threshold giving 96% sensitivity and 88% specificity for the discrimination of genetic degranulation defects. Degranulation results from patients with secondary HLH, XLP, and *PRF1* showed no difference when stratified into groups not on medication, receiving immunosuppression, or treated with the HLH-2004 protocol. This showed that the degranulation readout was robust and not affected by treatment.

Acquisition of a 4-laser BD Fortessa with 20 channels allowed for an improved assay with the additional quantification of cytokine production (MIP-1β, TNF-α and IFN-γ) as well as cell activation status (LFA-1 and CD69) in both NK and T cell subsets (Paper I, V, VI, and VII). Thus, multiparametric flow cytometry opened the door to multiple readouts, allowing us to obtain more data from the same number of cells. These cytokine markers encompass a wide activation time-range, with the inside-out LFA1 being a very early activation marker found on NK and CTL surface after target detection (S.III) followed by CD69 and MIP-1β upregulation. Lastly, between 2 to 3 hours, TNF-α and IFN-γ will be detectable in CL (Paper I, V, and VI).

Having an assay with multiple readouts representing various periods of cell activation and response is important for the thorough characterization of novel primary deficiencies. ORAI1 and STIM1 have been identified as the molecular constituents of the calcium release-activated calcium (CRAC) channel in T cells (157). This channel regulates Ca^{2+} influx into the cell, which is essential for activation and cytokine expression. As the role of ORAI1 in cytotoxic lymphocyte degranulation and killing in general, and NK cell development and function more specifically had not been studied, we ran an expanded degranulation panel on PBMC from an ORAI1 deficient patient and found a defect in both NK cell cytokine

production and degranulation when challenged with K562 (Paper V). Patients with genetic defective degranulation normally do not show reduced cytokine production (Paper VII). The inside-out LFA-1 activation (S.III) was however intact in the ORAI1 deficient NK cells as this very early signaling is calcium independent. Besides highlighting the importance of calcium in NK cell function, our results thus suggest ORAI1, and not other Ca^{2+} channels, is the predominant CRAC and crucial for both cytotoxicity and cytokine production.

While compiling data for Paper II, the lack of a robust CTL degranulation assay became apparent. A T cell degranulation protocol relying on PBMC to be stimulated for 2-3 days with IL-2 and PHA followed by a Ficoll to remove dead cells and a further 2-3 days of low level IL-2 stimulation before being tested for degranulation using CD3/28 coated beads had obvious shortcomings as a diagnostic assay. Besides being tedious and very labor intensive, it was neither rapid nor sensitive for the detection of degranulation deficiencies. We have shown that stimulation with IL-2 leads to functional recovery in certain *UNC13D* (Paper III), *STX11* (S.VII,66), and *STXBP2* (S.II, S.V). Thus, normal levels of degranulation found in activated T cells could simply be a recovered degranulation deficit, and this assay would fail to diagnose those patients. This led to a desire to create a T cell degranulation assay that is easy to perform and utilized fresh unstimulated cells.

In Paper VI, CD57 was found to be a cell surface marker that positively correlated with internal levels of perforin and granzyme B in T cells, corresponding to a highly differentiated $CD27^{-}CD28^{-}CD45RA^{+}CD45RO^{-}$ effector T cell population. More importantly, freshly isolated $CD3^{+}CD8^{+}CD57^{+}$ cells responded to anti-CD3 antibody stimulation by degranulating as well as producing high levels of cytokines. This 'resting state' cytotoxic T cell response (degranulation and cytokine production) was more rapid and vigorous compared to $CD56^{dim}$ NK cells stimulated with anti-CD16 antibody, with more multifunctional $CD8^{+}CD57^{+}$ cells compared to NK cells. Isolated naïve, effector, and memory subsets of $CD8^{+}$ T cells were tested for specific cytotoxicity and only the effector $CD57^{+}$ subset displayed substantial anti-CD3 antibody mediated killing of target cells. When tested on patients with known molecular defects in *UNC13D*, *STX11*, and *STXBP2*, both NK cell and $CD8^{+}CD57^{+}$ T cell degranulation were abolished in resting unstimulated PBMC (Paper IV, VI, VII). Probed further, patients whose NK cell degranulation recovered with IL-2 stimulation likewise regained function to a similar extent in the $CD8^{+}CD57^{+}$ T cell compartment (Paper VI). We concluded that there are similar requirements for Munc13-4, Syntaxin-11, and Munc18-2 for both NK cell and $CD8^{+}CD57^{+}$ T cell function.

It was then reasoned that this new cytotoxic T cell degranulation assay could be useful for the diagnosis of deficiencies leading to degranulation defects. Hence, all molecularly confirmed PID patients analyzed between November 2011 (when the CTL degranulation assay was started) and December 2014 were collected and presented in Paper VII. The patients were divided to two groups; those carrying genetic defects that led to a CL degranulation deficiency, and patients with molecular defects not affecting CL degranulation. As further controls, three other groups were selected. First a cohort of 200 healthy donors from the local

blood bank, second a cohort of patients being treated for MAS/SoJIA as well as patients fulfilling HLH criteria but with no mutations found in any PID gene, and thirdly all healthy unrelated controls that were shipped together with patient samples.

Three different degranulation assays were performed on all samples, NK cell stimulation with the K562 cell line (natural cytotoxicity), NK cell stimulation with the P815 cell line together with anti-CD16 antibody (ADCC), and $CD8^{+}CD57^{+}$ T cell stimulation with the P815 together with anti-CD3 antibody (TCR stimulation). Surface CD107a expression as well as internal cytokine production was measured after 3 hours. Sensitivity and specificity of each degranulation assay were obtained by comparing patients with genetic degranulation defects against patients with other PIDs not affecting degranulation, as similarly done in Paper II. The ROC results are summarized in Table 3.

Table 3. ROC analysis on the various assays assessed. Data shows results from cohorts analyzed in Paper II and VII.

Study	Cell type evaluated	Stimulation	Cutoff (ΔCD107a%)	Sensitivity (%)	Specificity (%)
Paper II	NK	K562	5.4	96	88
Paper VII	NK	K562	7.9	86	97
Paper VII	NK	P815 with anti-CD16	12.5	97	92
Paper VII	CTL	P815 with anti-CD3	23.5	97	95

All assays ran gave good sensitivity and specificity. In Paper VII, the CTL degranulation stood out as the best overall while K562 lagged in sensitivity. Anti-CD3 antibody stimulation led to a stronger CTL degranulation response as seen with the higher cutoff compared to NK stimulation while anti-CD16 gave higher signal compared to K562 on NK cells. The higher signal to noise is appreciated when running assays on samples shipped from distant countries and have undergone transport stress. CL under stress did not degranulate well especially for the K562-induced degranulation (Paper IV, VII). This also led to poor chromium release assay killing values as it is also based on NK cell response to K562. Not only do more $CD8^{+}CD57^{+}$ T cells degranulate compared to NK cells when stimulated, they also degranulated more, displaying higher CD107a median fluorescence intensities (Figure 3). This would aid in the detection of hypothetical deficiencies that would lead to partial loss in CL degranulation.

A noticeable difference appears between the results from the K562 degranulation in Paper II and Paper VII. In Paper II, the assay was incubated for 2 hours compared to 3 hours in Paper VII to allow for cytokine detection. Secondly, the results in Paper II represents pooled data from 4 different laboratories with slightly different protocols, equipment, analysis method, and staff. Samples arriving at our lab were previously ran on the same day it arrives during the Paper II study period but are now rested overnight before running. Lastly, in Paper II, it was found that the K562 line from Stockholm gave lower levels of degranulation as compared to lines from other centers. As such, we have switched to using the K562 line that gave the highest level of degranulation.

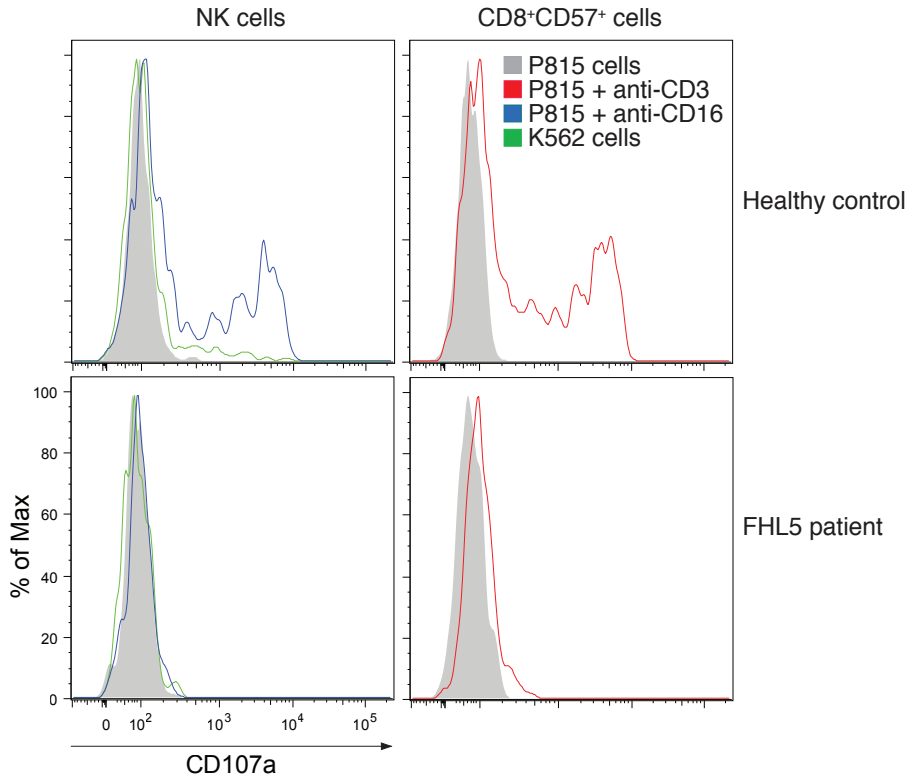

Figure 3. Defective degranulation in a *STXBP2* patient. Degranulation plots from a healthy person and a patient with compound heterozygous mutations in *STXBP2* (c.1463C>T, c.37+2T>C). More cytotoxic T lymphocytes degranulate, and to a higher median fluorescence intensity (MFI) as compared to NK cells when stimulated with K562 or P815 with anti-CD16 antibody.

Many infants between 0 – 3 mo have low $CD8^+CD57^+$ T cell counts as consequence of immunological immaturity. A census of all patients received in 2014 returned 16 patients analyzed at an age of 0 - 3 mo. Of this, 2 have low NK numbers while 7 have low $CD8^+CD57^+$ T cell number. A low count is defined as lesser than 100 gated NK/CTL per stimulation, out of 200,000 PBMC at the start of the assay. Scanning through 152 unique patients in 2014, regardless of age, found 17 patients had both low NK and CTL numbers, 23 had low CTL only, while 6 patients had poor NK numbers only. Thus, if only one of the three

different degranulation assays were run and insufficient cytotoxic cell numbers were acquired, a resampling would have to be arranged, leading to loss in time and waste of reagents besides additional patient stress. We hence suggest that at least two degranulation assays be run simultaneously to act as a safeguard against the failure of one of the assays due to poor cell numbers or human error, as well as for confirmation. The two assays suggested would be NK cell degranulation by anti-CD16 antibody stimulation and $CD8^+CD57^+$ T cell degranulation by anti-CD3 antibody stimulation. From Paper VII, should these two assays be run concurrently, a 94% sensitivity and 100% specificity is noted.

Although the NK cell CD107a assay has been validated for diagnosis of genetic degranulation defects (Paper II), it has been known that large variability exists for the level of NK cell response to K562 cell stimulation (Paper VII). We have recently published data showing NK cells displaying functional heterogeneity in a subset of CMV positive healthy individuals (S.XI). Specifically, NK cells with epigenetically downregulated EAT-2 or FcεRγ showed normal cytokine production but reduced CD107a degranulation upon stimulation with K562 cells. This effect was less pronounced with anti-CD16 stimulation. In Paper VII where 200 random healthy blood bank donors were screened, two persons with the highest percentage of FcεRγ downregulation in NK cells had K562 induced degranulation below the 7.9% diagnostic cutoff for genetic defective degranulation. Both individuals had normal anti-CD16 NK and anti-CD3 $CD8^+CD57^+$ T cell response This implies that approximately 1% of the healthy adult population could be falsely diagnosed with a degranulation defect should a large-scale K562-only screening be attempted. Once again, caution should be taken when relying on one sole degranulation assay for diagnosis.

Although the basis of the routine assays performed has not changed throughout these years, many small steps had been taken to improve the standard operating procedure. Compounded small changes can sometimes lead to huge differences. PBMC isolated from blood samples were previously immediately run on the same day but now left to rest overnight. This led to better degranulation and killing responses from healthy persons. Instead of using PBS for blood dilution and PBMC washes, we have started using whole RPMI media, giving up to 2 fold higher viable cell counts. Cell counting has also transitioned from the conventional haemocytometer to automated flow-based counting. This is more accurate and reproducible, removes human error, and allows us to confidently enumerate exact live PBMC numbers as the machine ignores RBCs, debris, and dead cells. These issues are especially important in samples from very ill patients or poor transportation that sometimes showed RBC contamination in the ficoll layer. A complete blood count including specific lymphocyte subsets is done on every sample to precisely ascertain the percentage of NK and $CD8^+CD57^+$ cells. Samples with exceptionally low CL numbers would require the use of more PBMC for the assays in order for there to be enough cells for analysis, reducing assay failure rates.

4.3 Cytotoxic lymphocyte function in primary HLH

Patients with biallelic defects in one of the FHL associated genes (*PRF1*, *UNC13D*, *STX11*, and *STXBP2*) or genes linked to partial albinism (*LYST*, *AP3B2*, and *RAB27A*) show defective NK cytotoxicity. These patients commonly present with fulfilled clinical HLH criteria. Here we highlight our contribution to characterize and understand some of these deficiencies.

When initially identified, FHL was thought to be a severe disease that manifested only in the first years of life (65). Later, persons with defective CL function were found as late as the fourth to sixth decade of life (S.XIII,206). Data from patients analyzed for Paper VII showed approximately 2/3 of degranulation deficiency cases were ≤ 2 years old at time of sampling while 2/3 of other PID with no degranulation deficiency were ≥ 2 years old (Figure 4). Degranulation deficiency syndromes are thus more common in infants and adult cases of PID are less common but age cannot be used as an exclusion criteria.

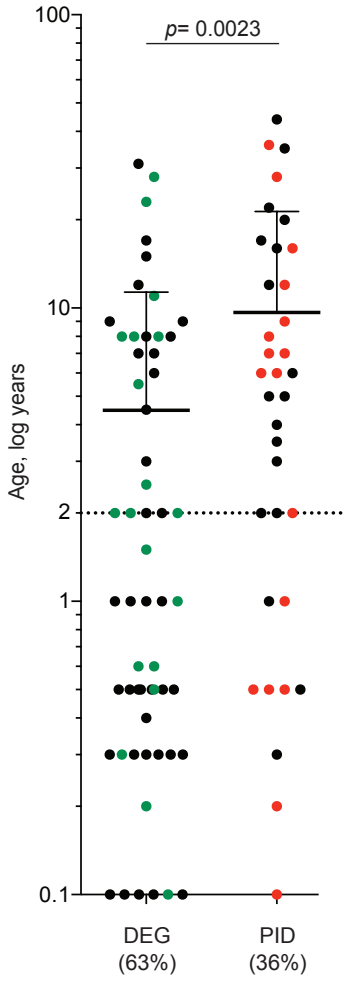

Figure 4. Age of patients and controls analyzed in Paper VII. Brackets indicate percentage of patients in each group with an age of ≤ 2 years old at time of analysis. *p* value calculated using nonparametric Mann-Whitney t-test. Green points represent patients with hypopigmentation (*LYST*, *AP3B2*, and *RAB27A*) while red points represent perforin deficient patients (*PRF1*). DEG: patients with a genetic degranulation defect, PID: immunodeficiencies not presenting with defective degranulation.

4.3.1 Perforin, *PRF1*

The lack of perforin, a syndrome termed FHL2, leads to defective cytotoxicity but unaffected levels of CL degranulation. It is thus used in Paper II, III, VI, and VII as a control group presenting HLH and having defective cytotoxicity but with normal degranulation. Perforin can be directly stained for in NK and T cells and is accurate for the diagnosis of FHL2 (207). Staining with the dG9 antibody clone was sensitive for the detection of various perforin deficiencies, rare variants, and certain heterozygous carriers (Figure 5). The relative perforin expression in these patients' NK cells correlated to cytotoxicity against K562, as demonstrated by the lytic units at 25% specific lysis (LU_{25}).

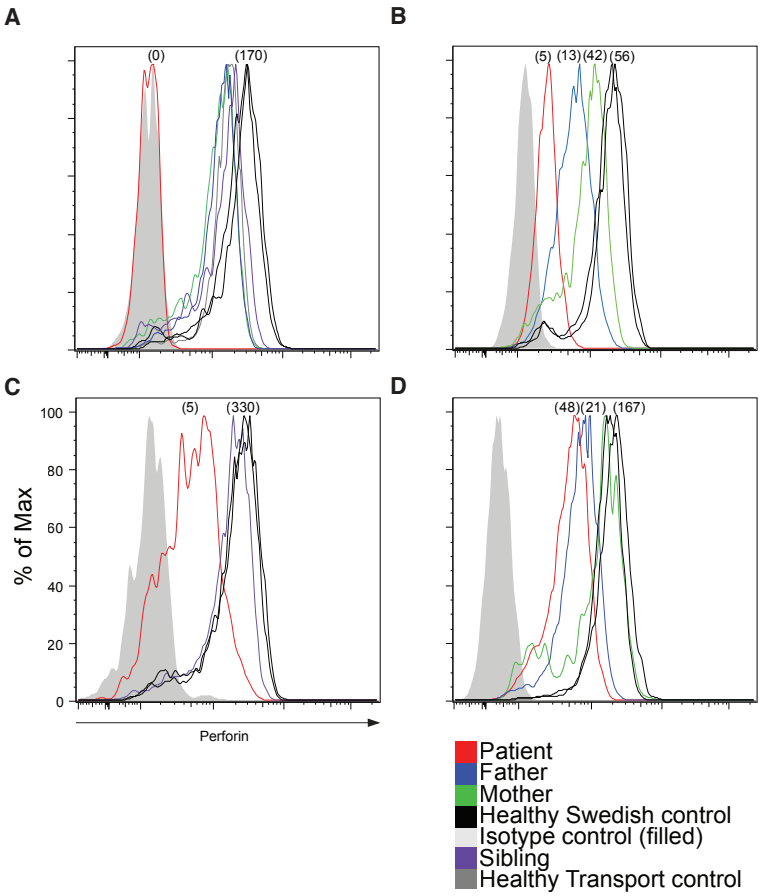

Figure 5: Perforin staining in NK cells from families with various *PRF1* mutations. A) A 3 mo patient with homozygous c. 445G>A mutation leading to total loss of perforin but normal levels in the heterozygous parents B) A 6 yo patient with homozygous c.1349C>T, with parents being heterozygous carriers C) 38 yo Patient with homozygous A91V with a heterozygous sibling, D) A family with both 7 mo patient and father heterozygous for the rare variant c.706 C>T and displaying reduced perforin levels. Brackets display LU_{25} values for representative plots.

There is a large variation in the age at presentation as well as clinical spectrum of patients with perforin mutations. Typically, patients with nonsense mutations would present HLH earlier in life while it is possible for persons carrying biallelic missense *PRF1* mutations to be completely healthy. Three sibling pairs were found in our records where each pair carried similar *PRF1* mutations but with different clinical manifestations. One sibling in each pair was affected by HLH. For the other sibling, two of three developed Hodgkin's lymphoma while the third remained healthy (S.XIII). Another unrelated 19 yo patient analyzed was found carrying homozygous c.394G>A and interestingly did not have HLH but presented with reoccurring fevers and headaches, leading to refractory seizures, blurring of vision in the right eye, and right-side hand weakness. A brain MRI detected multiple lesions as well as abnormal leptomeningeal thickening in the interfolial spaces. These neurological symptoms were cured after a matched related allogeneic hematopoietic stem cell transplantation (S.IX).

Comparing a cohort of perforin patients with biallelic nonsense mutations against perforin patients with biallelic missense mutations, the nonsense cases had a disease onset of < 1 yo while the missense cases had a later age of presentation (22 mo – 38 yo) (S.XIII). More importantly, NK cell function assessed by chromium release assay showed recovery after IL-2 stimulation in the cohort with missense mutations. Examining CL after IL-2 stimulation found a noticeable recovery in perforin in a patient where cytotoxicity improved (Figure 6). Evaluation of NK degranulation by CD107a is not informative as *PRF1* patients have normal degranulation. All together, this fits well with the hypothesis that missense mutations in *PRF1* are in fact a protein-misfolding disease (208). It remains to be understood how IL-2 stimulation corrects for misfolded perforin in CL in certain patients.

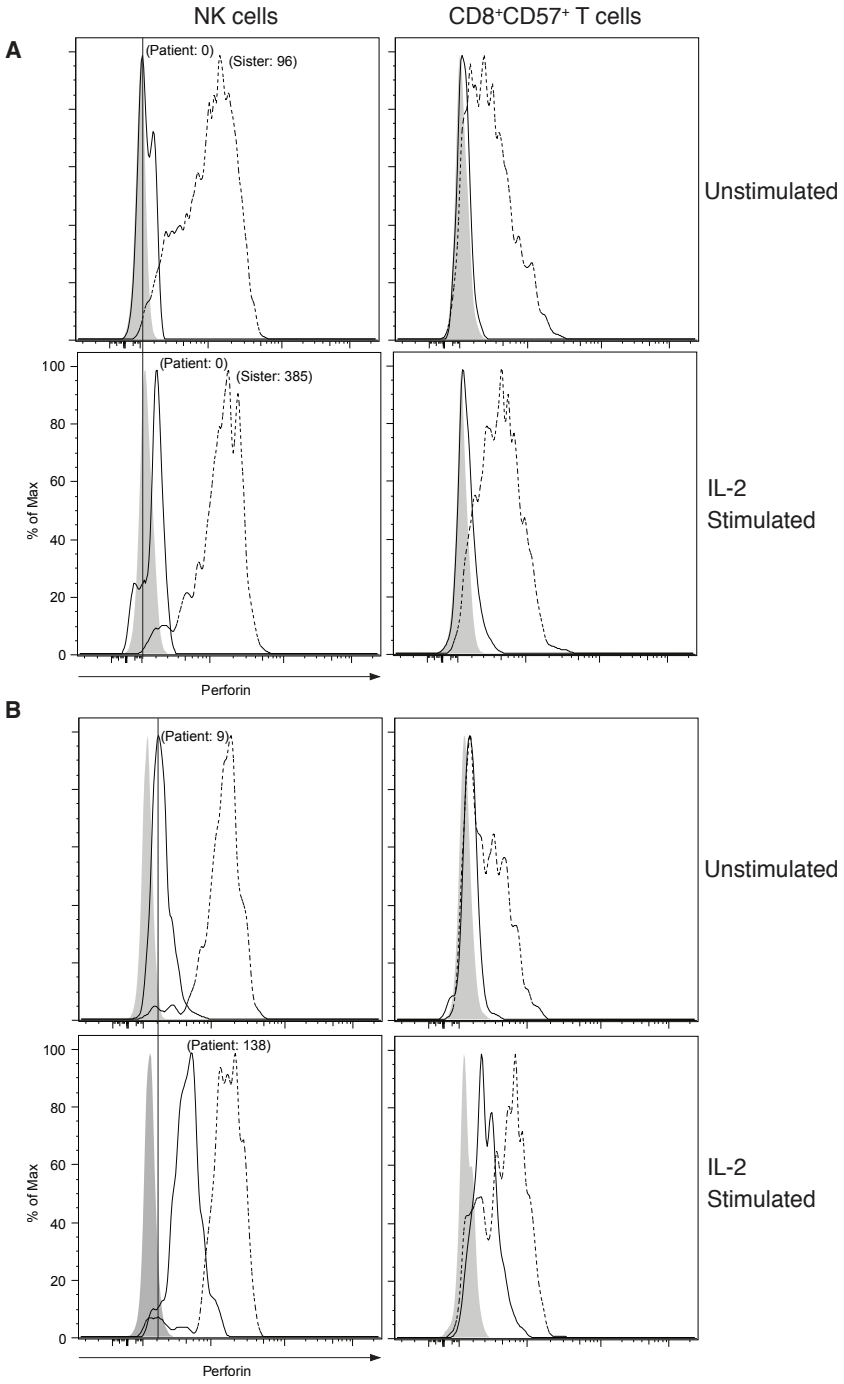

Figure 6: IL-2 mediated recovery of perforin expression leads to regained cytotoxicity. A) A patient bearing homozygous. c.673C>T with minimal perforin recovery in CLs after IL-2 stimulation shows no improvement of cytotoxicity as shown in brackets (LU_{25}), while in B) a patient with c.148G>A, c.673C>T displays substantial perforin upregulation after IL-2 stimulation and likewise improved LU_{25} with a healthy control as reference. Filled graphs represent isotype control.

4.3.2 Munc13-4, *UNC13D*

Biallelic mutations in *UNC13D, STX11, STXBP2, LYST, RAB27A,* and *AP3B1* can be functionally diagnosed by a standard NK or $CD8^{+}CD57^{+}$ degranulation assay. A patient showing lowered NK or CTL degranulation or poor cytotoxicity would trigger targeted sequencing of these genes. To save resources and effort, only the exons and intron-exon boundaries were usually sequenced.

Two Swedish infants (<2 yo) fulfilling HLH criteria with defective NK cell degranulation and cytotoxicity were sequenced for all known HLH genes but only monoallelic *UNC13D* mutations were found (Paper III). Western blot showed these patients did not express any detectable Munc13-4 protein and so we asked if the gene was transcribed normally. Specific overlapping segments of the *UNC13D* mRNA were amplified and in one of these two patients, the last two segments failed to amplify. To solve this, a 3' RACE PCR was ran and the sequence mapped to reveal a 253kb inversion. To the best of our knowledge, this represents the first description of an autosomal recessive human disease caused by an inversion. The second monoallelic patient had normal mRNA transcription and so all highly conserved intronic regions of *UNC13D* were next mapped and sequenced. Surprisingly, an intronic point mutation c.118-308C>T was found in a highly conserved region of intron 1.

Re-sequencing unsolved HLH cases revealed six infants carrying homozygous 253-kb *UNC13D* inversion. As such, it is now the most common mutation found in primary HLH cases in Swedish infants (13/26 alleles). Screening through 190 healthy adult Swedes found 1 heterozygous person or approximately half a percent of the population. Microsatelite mapping revealed a common haplotype in patients of Scandinavian ancestry suggesting a founder effect from northern Scandinavia. Following this, a proof of principle Guthrie card based screening for the inversion mutation has been designed and tested (209). This is in line with the unusually high UNC13D inversion incidence in Sweden that was close to that of SCID (210). The screen was found to be very accurate and may be implemented in the Swedish neonatal screening program.

Re-sequencing patients for the intronic mutation found hits from Denmark, Slovenia, and Croatia. Microsatelite analysis again found similar signatures suggesting a common heritage. A Korean national study then reported the c.118-308C>T intronic point mutation as the most common mutation found in Korean *UNC13D* patients and SNP analysis likewise suggested an independent Korean founder effect (211). The same group postulated earlier that conventional sequencing likely missed mutations in the second allele (212).

Later, we found an 8 yo ethnic Chinese patient carrying a point mutation one base pair downstream at position c.118-307G>A (Paper IV). The patient rapidly digressed and died. An elder brother was found to carry the same mutations but is currently asymptomatic at 15 yo. He was recommended for HSCT as the underlying molecular defect predisposed him to rapid-onset HLH as well as malignancy (S.V). However, the family declined. Following this, the Cincinnati Children's hospital published genetic data on a large cohort of 1709 suspected

HLH cases and reports that the two intronic mutation and the inversion mutation together was found in 31.6% of all *UNC13D* patients (213). In addition, a patient with a novel point mutation in intron 28 was reported, suggesting that non-coding mutations are in fact more common than thought, at least in *UNC13D*. It also draws attention to the possibility of missed mutations in reported monoallelic or hypomorphic cases (44,95,214).

The pathogenesis of c.118-307G>A and c.118-308C>T mutations was next studied (S.VIII). *UNC13D* gene transcript could be amplified indicating the mutations did not interfere with splicing while real-time PCR on sorted lymphocyte subtypes found transcription severely reduced specifically in CD4, CD8, and CD56 populations but not in CD14 cells. Next, a transcription factor binding prediction software found the mutated region to be a predicted consensus ELF1 binding site. ELF1 facilitated STAT4 binding and together recruited BRG1, leading to the acetylation of surrounding chromatin. The relaxed DNA allowed gene transcription to proceed. Both STAT4 and BRG1 were highly expressed in T and NK cells but not monocytes, supporting the real time PCR finding. In addition, a novel second transcription start site was found in intron 1 and transcription from this start site was abrogated by the point mutations. The function of alternative Munc13-4 isoforms are currently being investigated in the laboratory.

One curious observation was the partial recovery of NK cell cytotoxicity in patients bearing the 253-kb inversion mutation (Paper III). This recovery was not seen in patients with the c.118-308C>T intronic point mutation or other *UNC13D* coding mutations. This small increase in degranulation led to a large increase in cytotoxicity, which remains unexplained.

In conclusion, the establishment of sensitive assays for the detection of NK degranulation (Paper I, II) led us to discover novel non-coding mutations in the *UNC13D* gene (Paper III, IV). The high prevalence of these mutations in the Scandinavian population argues for compulsory neonatal screening in Sweden in the hope of early intervention. European, Asian, and North American diagnostic centers had similarly found substantial numbers of patients bearing these non-coding mutations in their cohort, improving their diagnostic capabilities. Biochemical studies have found STAT4 recruitment abrogated by the intronic point mutations leading to poor DNA uncoiling and transcription at the *UNC13D* locus in CL. The study of rare diseases utilizing powerful functional assays are thus not just diagnostic, they also shed light on basic cell function.

4.3.3 Syntaxin 11, *STX11*

Patients with defects in *STX11* are labeled FHL4 and these mutations lead to a range of reduction in CL degranulation, depending on mutation severity (Paper VI,215). All *STX11* patients analyzed in our laboratory showed partial recovery of NK cell degranulation after IL-2 stimulation (S.VII,66). As seen, there is a trend for better K562-induced NK degranulation recovery compared to other stimulations (Figure 7).

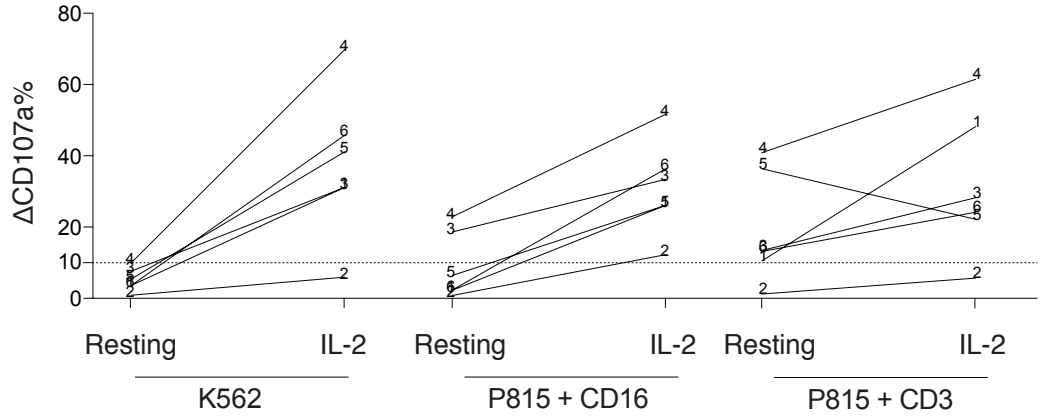

Figure 7. Degranulation of resting and IL-2 stimulated CL from *STX11* patients. PBMC from six patients with *STX11* biallelic mutations were stimulated with K562, P815 with anti-CD16, or P815 with anti-CD3. Results show the CD107a percentage after subtracting basal level degranulation. Patient numbers arranged to indicate increasing age from 0 to 7 years old.

The precise role of various FHL-associated proteins and their domains crucial for cytotoxic function is still largely unknown. Studying point mutations found in various domains is one way of deciphering protein function. *STX11* patients with N-terminus missense mutations were found lacking Munc18-2 interaction. Without this stabilizing interaction, we suspected Stx11 would be degraded and hence not detected by Western blot (S.VII). Similar to deficiencies in *UNC13D* and *STXBP2*, it is still unclear how IL-2 stimulation led to functional recovery.

4.3.4 Munc18-2, *STXBP2*

The *STXBP2* gene codes for Munc18-2 and is the latest member of the FHL syndrome group, called FHL5. A census of 11 *STXBP2* patients found variable age of onset (2 mo to 17 yo) and clinical presentation (S.II). Besides immunological changes, various neurologic symptoms were noted in half the patient population and gastrointestinal symptoms including chronic diarrhea, vomiting, and abdominal pain in 6 of 11 cases. Interestingly, the gastrointestinal symptoms resolved after commencing HLH treatment with no reoccurrences in one patient 8 years post transplantation. These chronic symptoms predate HLH manifestation and clinicians should be aware that such could point to possible immunodeficiencies as described in S.IX. These proteins traditionally linked to cytotoxic function may play unknown roles in the brain and gastrointestinal system. However, the fact that HSCT led to the resolution of most symptoms in these patients suggested that the pathology was driven by hematopoietic cells with defective Munc18-2 function.

The oldest patient enrolled in the above study developed a nearly fatal EBV triggered HLH but recovered with HLH-94 treatment. She was not transplanted due to her late onset and

relatively stable health after the initial attack (S.V). As seen in Figure 8, this patient showed very robust IL-2 mediated NK and T cell degranulation recovery with IL-2 stimulated values comparable to healthy donors. This is not the general trend seen in other *STXBP2* patients (Figure 9). Almost four years later, a rapidly developing classical Hodgkin's lymphoma was detected in the cervical lymph nodes. After treating the lymphoma, she was transplanted and today, 2 years post-transplant, she is doing well. It is postulated that robust recovery of function might confer partial protection leading to severe disease only later in life. Patients bearing milder mutations could escape severe HLH during infancy but have a higher chance of developing malignancies, neurological, gastrointestinal, and other diseases with age.

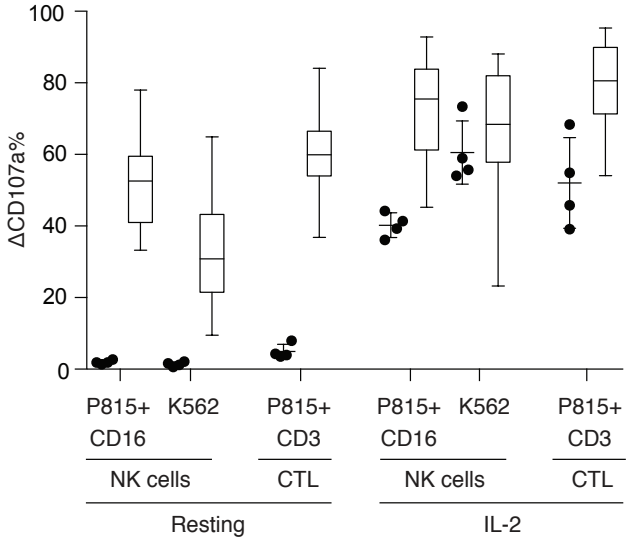

Figure 8. Repeated evaluation of an adolescent *STXBP2* patient. Resting and IL-2 stimulated NK cell and T cell degranulation are plotted next to 30 healthy controls ran during the same period. Activated cells from the patient showed strong functional recovery. Bar graphs depict data from healthy controls while dots represent repeated patient samples taken over a 2-month period.

Similar to *STX11* patients, some *STXBP2* patients recover function with IL-2 stimulation. The induced recovery of K562 degranulation seemed better than anti-CD16 or in T cells (Figure 9). It is however difficult to correlate age of onset with IL-2 recovery or genotype.

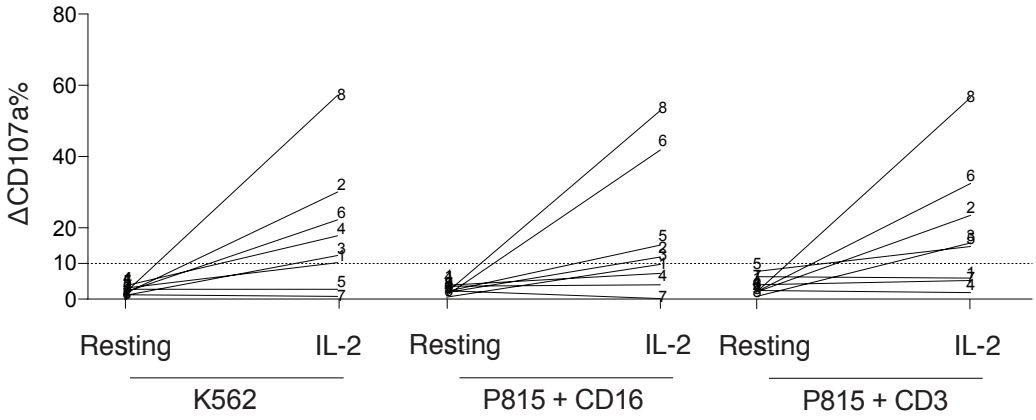

Figure 9. Degranulation of resting and IL-2 stimulated CL from *STXBP2* patients. PBMC from eight patients with *STXBP2* biallelic mutations were stimulated with K562, P815 with anti-CD16, or P815 with anti-CD3. Results show the CD107a percentage after subtracting basal level degranulation. Patient numbers arranged to indicate increasing age. Patient 8 was evaluated at 17 yo while all others were at 6 mo or below.

4.4 General discussion

The data provided demonstrated that certain patients in all the various FHL subgroups showed recovery of function after IL-2 stimulation. It was however difficult to predict which would recover, and the degree of recovery. Correlating the mutation, age, clinical symptoms, or resting functional results did not clearly predict the level of functional recovery. The collection of more patient data would hopefully facilitate better prediction.

Searching our database for siblings having similar mutations in genes involved in cytotoxic cell degranulation (not perforin), we found three families. The functional results for these pairs are shown in Table 4. In most cases, one sibling was found manifesting HLH while the other sibling was either asymptomatic or presented atypical symptoms. We expected the siblings to show roughly similar levels of NK and T cell degranulation but found variable activity especially in IL-2 stimulated cells. Additionally, the sibling that came down with HLH not necessarily had poorer function. Again, there was a disparity between clinical and functional data. The interplay of other genes, diet, and epigenetic factors most likely led to this finding. In a recent study, a team of researchers took 7 years to create 1400 RNAi worm knock-outs on two different strains (216). Significant differences in the phenotype severity was found in 20% of gene mutations between the two strains of worms. This was partly due to varying gene expression levels between the two strains. As humans have heterogeneous genetic backgrounds with large variations in gene expression, a large disparity can also be expected between siblings with one similar gene mutation.

Other PID could manifest HLH without showing reduced cytotoxic cell degranulation. We have reported HLH patients with biotinidase deficiency (S.IV), IFN-γ receptor deficiency (S.X), and *MAGT1* (S.XII). As such, patients with peculiar or chronic clinical presentation and family history should be followed up with sequencing even if CL function is normal. Research in the past years had provided answers to some patients but many unsolved cases remain. We have seen cases of clearly deficient degranulation or family history that have yet to be solved even after performing whole exome sequencing (not published) while apparently unrelated cases with neurological or gastrointestinal symptoms were found bearing primary defects (S.II, S.IX). The once clear domains of primary and secondary HLH are now becoming blurred with the discovery of various associated genes and novel mutations. Some previously monoallelic cases linked to late onset or atypical HLH have been found to carry non-coding mutations (Paper III,212,213).

Cytotoxic lymphocytes play a crucial role in protecting the body. Failure of these cells to function normally due to an overwhelming intrusion on top of a primary defect leads to severe repercussions and even death. We have studied patients bearing such defects and found the NK degranulation assay sensitive in distinguishing patients bearing certain genetic defects of the degranulation pathway. This assay was further refined and was instrumental in detecting novel mutations, allowing us to diagnose more patients and better understand the biology of granule exocytosis. We have also established a parallel T cell degranulation assay and found it more sensitive when evaluated alongside the established NK assay. With continual development and expansion, we hope to expand the various assays to diagnose more primary diseases and improve assay stability.

Table 4. Functional evaluation of three sibling pairs bearing similar biallelic mutations. Shown are CD107a degranulation levels in NK and T cells as well as NK cytotoxicity analyzed on both resting and IL-2 activated PBMC. LU: Lytic units.

				Resting degranulation				**IL2 stimulated degranulation**			
Pat no.	Diagnosis	Referral age (mo)	HLH criteria fulfiled	NK K562 degran (%)	NK CD16 degran (%)	CTL degran (%)	NK LU	NK K562 degran (%)	NK CD16 degran (%)	CTL degran (%)	NK LU
1a	*RAB27A*	18	6	2	7	3	19	14	13	9	560
1b	*RAB27A*	24	0	2	8	2	34	30	27	13	446
2a	*LYST*	36	6	8	24	18	9	n/a	n/a	n/a	n/a
2b	*LYST*	24	0	8	17	8	3	n/a	n/a	n/a	n/a
3a	*UNC13D*	96	5	0	0	1	n/a	32	20	9	n/a
3b	*UNC13D*	144	0	0	1	1	n/a	6	7	10	n/a

5 CONCLUSIONS

NK cells and CTL are together referred to as cytotoxic lymphocytes and play an important role in clearing infections and immunoregulation. This includes cytokine release and targeted cell killing via perforin granule exocytosis. The release of perforin granules is accompanied by the presence of surface CD107a, and termed degranulation. This assay can be used to evaluate both NK and T cells and represents a diagnostic assay to detect primary defects affecting cytotoxic lymphocyte function. The assay results can often be correlated to clinical data and has been reproduced in various diagnostic centers worldwide.

Specific conclusions:

Paper I

- A detailed updated CD107a degranulation assay protocol is provided.
- The intracellular staining of perforin is a reliable method of detecting defects and polymorphisms in the *PRF1* gene.

Paper II

- A consensus K562-induced NK cell degranulation protocol was found to have an overall 96% sensitivity and 88% specificity for the diagnosis of genetic degranulation defects, namely in *UNC13D*, *STX11*, *STXBP2, LYST,* and *RAB27A*.
- Results from the degranulation assay were not affected by immunosuppressive treatment and could still be performed after the initiation of the HLH-2004 protocol.
- CTL blasts show low-level of degranulation after stimulation with CD3/CD28 beads and could possibly be used for evaluation of CTL function in patients where initial cell numbers are low.

Paper III

- The K562 induced NK cell degranulation assay was sensitive for the detection of defective degranulation in infants.
- Two new non-coding mutations in *UNC13D* were described. The first is the c.118-308C>T point mutation in a highly conserved region of intron 1, and the second a 253kb inversion at the 3' end of the gene. The inversion mutation was the first known description of an autosomal recessive human disease caused by an inversion.
- Mutations in non-coding regions of *UNC13D* were found in 8 of 13 Swedish HLH infants between December 2005 and January 2011. Six of these eight were homozygous for the inversion mutation.
- The inversion mutation was the most common *UNC13D* mutation as well as the most common genetic degranulation defect mutation in Sweden, with a possible founder effect originating from northern Scandinavia.
- Patients bearing the c.118-308C>T intron 1 point mutation could be found throughout Europe with a similar haplotype and have spread over a large geographical area.

Paper IV

- We described a novel point mutation in a highly conserved region of intron 1 of *UNC13D*, c.118-307G>A.
- While the index patient quickly succumbed to illness, the older brother carrying a similar genotype and poorer cytotoxic cell function remaied asymptomatic, highlighting the large variability in clinical presentation and the challenges in drawing genotype-phenotype correlations.
- Long transportation times led to lowered K562 induced NK degranulation in the samples shipped. The anti-CD16 and anti-CD3 induced degranulation assays were more robust and could still differentiate persons with genetic degranulation defects.

Paper V

- The loss of ORAI1 led to abrogated NK cell degranulation, cytotoxicity, as well as cytokine production.
- Calcium mobilization is thus crucial for functional NK responses and ORAI1 is the SOCE mediator in NK cells.
- Early LFA-1 signaling and granule polarization remained intact. Calcium influx is thus not required for initial signaling leading to adhesion and granule polarization.

Paper VI

- The $CD3^+CD8^+CD57^+$ T cell compartment was shown to have high perforin and granzyme B content, and can be thus regarded as a *bona fide* cytotoxic T lymphocyte.
- Stimulation with anti-CD3 antibody coated P815 cells induced rapid $CD3^+CD8^+CD57^+$ T cell degranulation, cytokine production, and target cell killing.
- This simple and rapid assay measured $CD3^+CD8^+CD57^+$ T cell *ex vivo* activity without the need for any prior cell stimulation.
- Patients having mutations in *UNC13D*, *STX11*, and *STXBP2* were found to have a comparable defect in NK and $CD3^+CD8^+CD57^+$ cell degranulation. IL-2 stimulation led to comparable levels of degranulation recovery in NK and $CD3^+CD8^+CD57^+$ cells.
- The T cell degranulation assay could possibly be used as a diagnostic assay for genetic degranulation defects.

Paper VII

- K562 stimulated NK cells displayed lower CD107a degranulation and a higher variance when compared to anti-CD16 NK cell and anti-CD3 T cell stimulation.
- The downregulation of epigenetic factors FcεRγ and EAT-2 in NK cells corresponded to lowered K562 degranulation.
- The anti-CD16 NK and anti-CD3 $CD8^+CD57^+$ degranulation assays were more accurate for the rapid functional diagnosis of genetic degranulation defects compared to the previous K562 induced NK degranulation standard.
- We proposed running two degranulation assays simultaneously for confirming results and better overall specificity.

6 FUTURE DIRECTIONS

The true impact of a genetic abnormality can only be determined using assays that reflect the function of that specific protein or pathway. Mutations can then be differentiated from polymorphisms. We have shown that direct staining of perforin as well as the magnitude of CD107a degranulation are assays that can separate out persons having defects in genes important for CL function. And while this thesis has focused on the clear black and white differences in resting state CL degranulation found in most primary HLH patients, the assay distinguishes shades of grey that we are currently exploring.

While the current CD107a assay is good, CL degranulation still does not equate to actual target cell killing, which is the 'gold standard' for a cytotoxicity test. We hope to be able to incorporate a cell death marker into the degranulation assay as a cytotoxicity readout. Merging degranulation and cytotoxicity assays would allow for simultaneous detection of both effector cell and target cell response, strengthening the assay and saving on patient material. Specific effector to target ratios can be calculated for per cell killing levels similar to the chromium release assay. It could also be possible to directly evaluate $CD57^+$ T cell killing. At the moment, because this population of cells constitutes a very small percentage of total lymphocytes, its killing effect is not apparent when performed on bulk PBMC.

Using cell lines as targets produces significant variability when ran over prolonged periods (Paper VII). A possible solution would be to switch to a bead-based assay where specific antibodies are first captured on microbeads before added into PBMC giving more consistent results over time and improving assay accuracy.

Concerning the mechanism by which CL gain function after cytokine stimulation, we would like to evaluate NK cells at resting and IL-2 stimulated states for loci with open DNA conformations. It has been reported that the overexpression of Munc13-4, Rab27a, and Slp3 in T cells from patients bearing *LYST* mutations led to recovery of function (217). Could similar compensatory mechanisms operate following cytokine stimulation, and why do only some defects show recovery and not others?

Only one large population based functional evaluation of CL has been reported (218). NK cell cytotoxic function was examined in 3625 persons >40 yo with the aim of predicting cancer occurrences. Interestingly, the report did not mention any finding of defective NK cell cytotoxicity in the sample population. While the incidence of primary HLH in children has been studied in Sweden and estimated to be 1.2:1,000,000 (92), the many reports of late onset and atypical clinical presentation of primary HLH cases suggests that there could be a sizeable number of asymptomatic persons with defective CL function in the healthy population. As such, we would like to analyze and follow longitudinally a cross section of the Swedish population to examine how CL function and phenotype could be correlated to various disease occurrences in the long run. It is difficult to provide a global estimate of primary HLH rates as some mutations are stratified by ethnicity and geography. Confound that with increasing human mobility, number of interracial families, and the complexity of

posttranslational modifications that are not gene encoded, one quickly realizes that such a study could raise more questions than answers. Still, this important work should be performed, and by including clinical, genetic, epigenetic, and functional results, we hope the data would allow us to better understand the importance of CL function in human disease.

A registry study found an increased rate of malignancy in first-degree female relatives of primary Swedish HLH patients who are genetic carriers (S.XIV). However, evaluation of CL activity did not reveal any differences between carriers and healthy controls. This could be due to the small sample size, a lack of sensitivity in assays ran, or an organ specific NK functional deficiency, as cervical carcinoma was the most frequent cancer reported. NK cells in the periphery vary from those in organs and we are lacking specific knowledge on how NK cells with primary defects in degranulation are affected in their function in the various organs. Clearly, more work is required to explain this observation of increased cancer rates.

At the beginning of my PhD, I have hoped that my work would make a difference in the lives of the many very ill patients we evaluate. We have progressed steadily and have now a very rapid and robust group of assays for the diagnosis of genetic diseases predisposing to defective CL degranulation. While this has been evaluated for a few different genes, the booming PID field mandates tremendous effort to be placed in the development of new and better diagnostic assays. As a case in point, one survey found 19 new PID genes published in a year (130). Lately, we have also found with more regularity primary HLH patients with atypical presentations and more PID patients presenting with clinical HLH. Because it is impossible to run every single assay on every patient, we look forward to the near future where rapid whole exome/genome sequencing will play a major role. An overnight sequencer will generate a list of possible candidate genes, directing us to perform specific validation assays when we come in to the lab the next morning.

7 ACKNOWLEDGEMENTS

I realized, very early on, the acknowledgements was, for many, the most/only read part of the whole thesis. Thus I want to point out that there is another section immediately after this, which was more thoughtfully designed, and can be thoroughly enjoyed by the average layman. But I digress.

It has been a long marathon. A lot of failures, nights and weekends, sweat, brain cells, repetitive work, and vials of my own blood has been poured into this work.

> Throwing yourself into worthwhile, fruitful hard work that you believe in, as much as you can handle, and more, is a kind of luxury not everyone gets to experience. It is also exhausting.
>
> -Benjamin Mee, 'We Bought a Zoo'

Thank you:

God, without whom, I would not be.

Yenan Bryceson, my supervisor for showing me that the *fear of missing out is greater than the fear of messing up*. I remember while sending in a grant application you said 'if you send it in, your chances are exponentially higher.' **Hans-Gustaf Ljunggren** for believing in me from the first interview. Thanks for giving me a chance in CIM. **Jan-Inge Henter**, for your constant support and efforts in patient recruitment.

Jakob Theorell, I will remember the late night labs and the dozens of samples that we thawed, froze, and analysed. **Heinrich Schlums**, for working my part while I was on holiday (before Jelve came). **Jelve Zendegani**, you are an amazing help. I am very thankful you are here. **Stephanie Wood** for keeping me in check, and the Facs-plot cake! **Martha Muller**, for company in the small office and Westerns. **Cyril Fauriat** for introducing me to multi-colour flow. **Misty Marshall** for the bantering. **Hongya** for help with the defense party. Attachment students who delayed experiments and didn't understand protocols, but were hardworking and generated good data in the end: **Felicia Er** and **Ngian Ruo Han**. The other past/present YTB lab people: **Tim Holmes**, **Frank C, Arne H**, **Lamberto R**, **Matthias V, Stephanie D**.

Bianca Tesi for slogging through all the sequencing, putting up with my corny emails, and proofreading. **Marie M**, **Miriam E**, **Wang Yini**, and the rest of the genetics people at CMM for, erm, genetics work. The rest of the Solna group: **Alexandra L**, **Tatiana G**, and **Désirée G**. **Karina M and AnnaCarin H**. **Magnus N**. Great working with all of you.

The many many many clinicians and collaborators around Sweden, Europe, and the world who have tirelessly sent us samples, reagents, and emails.

CIM: Eliisa K I go to you when my kids fall sick. **Venkat M** friendly downstairs neighbour, **Sofia A** horror mum stories, **Julius J** cord blood bag, **Annette S.** keeping the Yellow Lab working, **Sandra A, David M,** and **Martin I** taking care of *Daniel*, **Vivien B** profiling my NK cells, **Kim B** helping me open a bank account, **Erika H** durian sweets, **Margit L** my compass in Japan. **Hernan H** teaching me the Calibur, **Steve A** help with equipment purchase, **Susanna B** Form 9 helpline. **Monika E** ideas on CD57, **Magda L** ideas on neutrophils, **Egle K** company in Toronto. **Jakob M** bicycle pump, **Nicole M** curious discussions while FACS-ing. **Niklas B** the corner room advisor, **Senait A** lunchroom talks, **Aline P** the friendly small office neighbor. The CIM secretaries **Margit E** and **Carina L** who navigated me through lots of red tape. The techs **Lena, Annette,** and **Elisabeth** keeping CIM intact. **Anna NT** for leading this unit. Those in CIM who helped me in some way, did not steal my FACS time, and spoke in English.

The A* gals: **Michelle Wong**, **Jamie Mong**, **Selina Poon**, **Kai Er**, **XiaoHui**. Thanks for all the wonderful times we shared. **Siti Mariam** my Marmite supplier.

Sarene Koh the laughter and tears we shared. Hey sis, I made it!

Terry Huang watching from above. Missing you dearly.

The Flavi lab people in NUS for good grounding in basic techniques. **Prof Mary**, **Boon**, **Bhuvana R**, **TTTT**, **Justin Chu**, **Josephine H**.

Mum and **Dad** allowing me to wander away.

Papa and **Mummy** trusting their daughter to a crazy science guy.

Jin Ming, my soulmate. **Sanna** and **Erika** my beloved daughters. You inspire me to finish work early. Where would I be without you?

In the lab of course.

8 LAYMAN'S SUMMARY

"I'm not trying to be mysterious about it,
it's just that what I do is tough to explain."

MacGyver (Season 6, Jerico Games)

This section aims to distill in simple English the gist of this thesis, what this is all about, and what all the results mean.

All living humans (should) have blood which is mainly made up of red blood cells that transport oxygen, platelets that help wound clotting, and white blood cells (WBC), the soldier cell that keeps us healthy by killing off 'bad' cells. 'Bad' cells could mean our own cells that have been compromized by bacteria/viruses, cells growing out of control: tumors, or other white blood cells that have done their job and should be removed. So WBC clear infections and regulate the body's defense system.

This thesis looks at a subset of WBC termed cytotoxic cells. Cytotoxic means being able to directly kill (toxic) other cells (cyto). There are two major types of cytotoxic cells: natural killer (NK) cells and cytotoxic T lymphocytes (CTL).

NK cells make and store little packets of poison, then roam around the body looking for a bad cell. When it finds one, the NK cell grabs on to the bad cell and releases poison at the precise area where the link is formed, killing it. The NK cell then breaks the link and goes away looking for another target. The mechanism of exactly how these NK cells work is only roughly known. Doubtless, should this army of specialized killer cells fail, one will fall sick quite easily.

A person's DNA originates from his or her biological parents, i.e. one gets two copies (alleles) of the same gene, a copy from each parent, for every gene (except those on the X and Y chromosomes, girls have XX, boys have XY). If the father passed down a defective gene, but the maternal one is normal, most times the child would be normal because it's enough to have one functional copy. However, if both copies are defective, the defect will be noticeable.

Some unfortunate persons are born with two defective copies of genes required in the NK cell killing process and thus carry malfunctioning killer cells. These defective cells lead to an increased susceptibility to infections and cancer. The current treatment for this is bone marrow transplantation (removing all blood cells and receiving a transplant from a healthy donor). This is a serious decision and is the same treatment for leukemia. As such, there is a need to quickly and accurately diagnose patients to ensure that proper treatment is given.

The laboratory receives blood samples from suspected patients around the world. Functional tests are run on them which entails mixing in certain cancer cells together with white blood

cells to determine if natural killer (NK) cells are able to kill those cancer cells. **Paper I** is a protocol on how to run this test.

If the patient's cytotoxic cells did not respond by killing the added cancer cells, there is a high chance the poor killing is caused by an error at the DNA/gene level; this is termed 'primary' disease. If the cells look and respond normally, it might be a one-off overreaction of the body, and termed 'secondary' disease. Or, the patient might have a different primary disease and the assay does not detect it. DNA from patients with poor killing potential were then sequenced, which basically means to read their DNA at particular locations to see if it reads the same as in healthy persons. If there is a significant mistake, it could be the cause of disease.

Compiling the sequencing and cancer killing test results from four European laboratories, we collected 120 primary cases. From that, it was found that poor natural killer (NK) cancer-response killing result can be used as a predictor of patients having a DNA error in one of the genes important for cytotoxic cell function (**Paper II**). This test has a 96% sensitivity and 88% specificity. That means if we test 100 patients that truly have defects in genes linked to cytotoxic-cell tumor-responding potential, our test would pick out 96 of them (true positives). Conversely, if we tested 100 healthy persons with no genetic defect, our test will say 88 of them are normal (true negatives).

What puzzled us was some patients having poor tumor killing did not show any errors in the regular suspect genes. Instead, after much testing, errors were found in the 'junk' (intronic) parts of the gene (**Paper III**). A gene is a region of DNA that usually contains codes for one product (protein). Some DNA sections are code for parts that end up in the product (exon) while some are code that don't get into the final product (intron). Thus, some regard intronic regions as 'junk' DNA. However we found that in some patients, errors in a 'junk' region of the gene *UNC13D* led to defective NK function. We did not find this error initially as regular DNA sequencing does not include 'junk' regions (like reading a magazine and skipping all the ads in between). In **Paper IV**, we found another intronic mutation neighboring the one found in Paper III, highlighting the importance of this particular section of 'junk' DNA. We follow up on this in supplementary paper S.VIII and found this particular 'junk' region to be important in unwinding DNA, without which, the DNA remained tightly coiled and cannot be employed in protein production.

Paper V. Metal ions (in salt form) play important roles in cell function. We studied a patient having a defective protein (ORAI1) crucial for calcium transport into the cell. The movement of salt into cells leads to concentration changes, which triggers cells to perform certain actions. When ORAI1 is defective, we found that calcium cannot get into NK cells when it recognizes a cancer cell, which should normally happen. This leads to the NK cell not being able to mount a killing response or release signals to warn other nearby immune cells. This proves that NK cells require a flux of salt to function and ORAI1 is the channel specifically mediating NK cell activation.

All this while, only NK cells were studied. The test for CTLs at that point had shortcomings and so we decided to develop a new assay. NK cells have poison packets already in them from the start but CTLs are traditionally thought to be 'slower', having to be activated a few days before they can kill. A test that could be run immediately with fresh blood cells is preferred because sometimes the patients/doctors can't wait a few days.

CTLs are a mixed bunch; so those that had ready poison packets in them were singled out. By chance, these CTLs had the marker CD57 on the outside surface. When a compound to activate CTLs was added in, CTLs expressing CD57 were found to kill cancer cells (**Paper VI)**. Better yet, patients with a defect in NK tumor killing were tested and found to be also defective in CTL cell killing, i.e. the underlying genetic defect affects both NK and CTL.

To evaluate this CTL test, we compared it with the original NK test (**Paper VII)**. This meant repeating Paper II with an additional CTL degranulation test. Collecting approximately a hundred patients over three years, the CTL assay was found to be better than the NK test. The sensitivity and specificity for the CTL assay is 97% and 95% respectively. So the CTL test is more accurate compared to the first NK assay in predicting which persons have a true genetic defect. However, we suggested that both the NK and CTL test be run simultaneously on all patients just in case one fails as well as a confirmatory test.

In a nutshell, if you skipped all the way to this paragraph, what I accomplished during my doctoral studies was to create, improve, and evaluate diagnostic tests for a rare inherited syndrome (familial hemophagocytic lymphohistiocytosis), which led me (together with a whole team of scientists and doctors) to a better understanding of the importance and the function of cytotoxic white blood cells: what makes them tick and why they sometimes fail to function

9 REFERENCES

1. Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992:13(1):11-16.
2. Fan S, Fehr HG, Adams D. Activation of macrophages for ADCC in vitro: effects of IL-4, TNF, interferons-alpha/beta, interferon-gamma, and GM-CSF. Cell Immunol 1991:135(1):78-87.
3. Cavaillon JM. Cytokines and macrophages. Biomed Pharmacother 1994:48(10):445-453.
4. Artis D, Spits H. The biology of innate lymphoid cells. Nature 2015:517(7534):293-301.
5. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol 2001:1(3):220-228.
6. Crome SQ, Lang PA, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses. Trends Immunol 2013:34(7):342-349.
7. Rosenberg EB, Herberman RB, Levine PH, Halterman RH, McCoy JL, Wunderlich JR. Lymphocyte cytotoxicity reactions to leukemia-associated antigens in identical twins. Int J Cancer 1972:9(3):648-658.
8. Brandstadter JD, Yang Y. Natural killer cell responses to viral infection. J Innate Immun 2011:3(3):274-279.
9. Fauci AS, Mavilio D, Kottilil S. NK cells in HIV infection: paradigm for protection or targets for ambush. Nat Rev Immunol 2005:5(11):835-843.
10. Stewart CA, Vivier E, Colonna M. Strategies of natural killer cell recognition and signaling. Curr Top Microbiol Immunol 2006:298:1-21.
11. Wu J, Lanier LL. Natural killer cells and cancer. Adv Cancer Res 2003:90:127-156.
12. Bryceson YT, Chiang SC, Darmanin S, Fauriat C, Schlums H, Theorell J, Wood SM. Molecular mechanisms of natural killer cell activation. J Innate Immun 2011:3(3):216-226.
13. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 1986:319(6055):675-678.
14. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 1990:11(7):237-244.
15. Wagtmann N, Biassoni R, Cantoni C, Verdiani S, Malnati MS, Vitale M, Bottino C, Moretta L, Moretta A, Long EO. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. Immunity 1995:2(5):439-449.
16. Yokoyama WM. The search for the missing 'missing-self' receptor on natural killer cells. Scand J Immunol 2002:55(3):233-237.
17. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 1998:391(6669):795-799.
18. Sepulveda FE, Maschalidi S, Vosshenrich CA, Garrigue A, Kurowska M, Menasche G, Fischer A, Di Santo JP, de Saint Basile G. A novel immunoregulatory role for NK-cell cytotoxicity in protection from HLH-like immunopathology in mice. Blood 2015:125(9):1427-1434.
19. Brittenden J, Heys SD, Ross J, Eremin O. Natural killer cells and cancer. Cancer 1996:77(7):1226-1243.
20. Berrien-Elliott MM, Romee R, Fehniger TA. Improving natural killer cell cancer immunotherapy. Curr Opin Organ Transplant 2015.

21. Strowig T, Brilot F, Munz C. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. J Immunol 2008:180(12):7785-7791.
22. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood 2006:107(1):159-166.
23. Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. Philadelphia: Elsevier/Saunders; 2012. 6ed, 545 p. p.
24. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. Innate or adaptive immunity? The example of natural killer cells. Science 2011:331(6013):44-49.
25. Uhrberg M. The KIR gene family: life in the fast lane of evolution. Eur J Immunol 2005:35(1):10-15.
26. Beziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, Hervier B, Theodorou I, Martinot M, Debre P, Bjorkstrom NK, Malmberg KJ, Marcellin P, Vieillard V. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. Eur J Immunol 2012:42(2):447-457.
27. Reeves RK, Li H, Jost S, Blass E, Li H, Schafer JL, Varner V, Manickam C, Eslamizar L, Altfeld M, von Andrian UH, Barouch DH. Antigen-specific NK cell memory in rhesus macaques. Nat Immunol 2015:16(9):927-932.
28. Andrews DM, Estcourt MJ, Andoniou CE, Wikstrom ME, Khong A, Voigt V, Fleming P, Tabarias H, Hill GR, van der Most RG, Scalzo AA, Smyth MJ, Degli-Esposti MA. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J Exp Med 2010:207(6):1333-1343.
29. Moretta A, Marcenaro E, Sivori S, Della Chiesa M, Vitale M, Moretta L. Early liaisons between cells of the innate immune system in inflamed peripheral tissues. Trends Immunol 2005:26(12):668-675.
30. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, Scott JM, Kamimura Y, Lanier LL, Kim S. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. Immunity 2015:42(3):431-442.
31. Tscharke DC, Croft NP, Doherty PC, La Gruta NL. Sizing up the key determinants of the CD8 T cell response. Nat Rev Immunol 2015.
32. Haworth KB, Leddon JL, Chen CY, Horwitz EM, Mackall CL, Cripe TP. Going back to class I: MHC and immunotherapies for childhood cancer. Pediatr Blood Cancer 2015:62(4):571-576.
33. Ochs HD, Hitzig WH. History of primary immunodeficiency diseases. Curr Opin Allergy Clin Immunol 2012:12(6):577-587.
34. Bruton OC. Agammaglobulinemia. Pediatrics 1952:9(6):722-728.
35. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Franco JL, Gaspar HB, Holland SM, Klein C, Nonoyama S, Ochs HD, Oksenhendler E, Picard C, Puck JM, Sullivan K, Tang ML. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol 2014:5:162.
36. de Vries E, European Society for Immunodeficiencies m. Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol 2012:167(1):108-119.
37. Ishii E, Ohga S, Tanimura M, Imashuku S, Sako M, Mizutani S, Miyazaki S. Clinical and epidemiologic studies of familial hemophagocytic

lymphohistiocytosis in Japan. Japan LCH Study Group. Med Pediatr Oncol 1998:30(5):276-283.
38. Bousfiha AA, Jeddane L, Ailal F, Benhsaien I, Mahlaoui N, Casanova JL, Abel L. Primary immunodeficiency diseases worldwide: more common than generally thought. J Clin Immunol 2013:33(1):1-7.
39. Boyle JM, Buckley RH. Population prevalence of diagnosed primary immunodeficiency diseases in the United States. J Clin Immunol 2007:27(5):497-502.
40. Ludviksson BR, Sigurdardottir ST, Johannsson JH, Haraldsson A, Hardarson TO. Epidemiology of Primary Immunodeficiency in Iceland. J Clin Immunol 2014.
41. Kirkpatrick P, Riminton S. Primary immunodeficiency diseases in Australia and New Zealand. J Clin Immunol 2007:27(5):517-524.
42. Ishimura M, Takada H, Doi T, Imai K, Sasahara Y, Kanegane H, Nishikomori R, Morio T, Heike T, Kobayashi M, Ariga T, Tsuchiya S, Nonoyama S, Miyawaki T, Hara T. Nationwide survey of patients with primary immunodeficiency diseases in Japan. J Clin Immunol 2011:31(6):968-976.
43. Gathmann B, Goldacker S, Klima M, Belohradsky BH, Notheis G, Ehl S, Ritterbusch H, Baumann U, Meyer-Bahlburg A, Witte T, Schmidt R, Borte M, Borte S, Linde R, Schubert R, Bienemann K, Laws HJ, Dueckers G, Roesler J, Rothoeft T, Kruger R, Scharbatke EC, Masjosthusmann K, Wasmuth JC, Moser O, Kaiser P, Gross-Wieltsch U, Classen CF, Horneff G, Reiser V, Binder N, El-Helou SM, Klein C, Grimbacher B, Kindle G. The German national registry for primary immunodeficiencies (PID). Clin Exp Immunol 2013:173(2):372-380.
44. Spessott WA, Sanmillan ML, McCormick ME, Patel N, Villanueva J, Zhang K, Nichols KE, Giraudo CG. Hemophagocytic lymphohistiocytosis caused by dominant-negative mutations in STXBP2 that inhibit SNARE-mediated membrane fusion. Blood 2015:125(10):1566-1577.
45. Latour S, Aguilar C. XIAP deficiency syndrome in humans. Semin Cell Dev Biol 2015:39:115-123.
46. Fusco F, Pescatore A, Conte MI, Mirabelli P, Paciolla M, Esposito E, Lioi MB, Ursini MV. EDA-ID and IP, Two Faces of the Same Coin: How the Same IKBKG/NEMO Mutation Affecting the NF-kappaB Pathway Can Cause Immunodeficiency and/or Inflammation. Int Rev Immunol 2015.
47. Haapaniemi EM, Kaustio M, Rajala HL, van Adrichem AJ, Kainulainen L, Glumoff V, Doffinger R, Kuusanmaki H, Heiskanen-Kosma T, Trotta L, Chiang S, Kulmala P, Eldfors S, Katainen R, Siitonen S, Karjalainen-Lindsberg ML, Kovanen PE, Otonkoski T, Porkka K, Heiskanen K, Hanninen A, Bryceson YT, Uusitalo-Seppala R, Saarela J, Seppanen M, Mustjoki S, Kere J. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood 2015:125(4):639-648.
48. Wood SM, Ljunggren HG, Bryceson YT. Insights into NK cell biology from human genetics and disease associations. Cell Mol Life Sci 2011:68(21):3479-3493.
49. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, Ladisch S, McClain K, Webb D, Winiarski J, Janka G. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007:48(2):124-131.
50. Henter JI, Elinder G, Ost A. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. Semin Oncol 1991:18(1):29-33.

51. Henter JI, Arico M, Egeler RM, Elinder G, Favara BE, Filipovich AH, Gadner H, Imashuku S, Janka-Schaub G, Komp D, Ladisch S, Webb D. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. HLH study Group of the Histiocyte Society. Med Pediatr Oncol 1997:28(5):342-347.
52. Gupta A, Weitzman S, Abdelhaleem M. The role of hemophagocytosis in bone marrow aspirates in the diagnosis of hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2008:50(2):192-194.
53. Bien E, Balcerska A. Serum soluble interleukin 2 receptor alpha in human cancer of adults and children: a review. Biomarkers 2008:13(1):1-26.
54. Leoni S, Buonfrate D, Angheben A, Gobbi F, Bisoffi Z. The hyper-reactive malarial splenomegaly: a systematic review of the literature. Malar J 2015:14:185.
55. Wormsbecker AJ, Sweet DD, Mann SL, Wang SY, Pudek MR, Chen LY. Conditions associated with extreme hyperferritinaemia (>3000 mug/L) in adults. Intern Med J 2015:45(8):828-833.
56. Ho C, Yao X, Tian L, Li FY, Podoltsev N, Xu ML. Marrow assessment for hemophagocytic lymphohistiocytosis demonstrates poor correlation with disease probability. Am J Clin Pathol 2014:141(1):62-71.
57. Evans SS, Repasky EA, Fisher DT. Fever and the thermal regulation of immunity: the immune system feels the heat. Nat Rev Immunol 2015:15(6):335-349.
58. Schram AM, Campigotto F, Mullally A, Fogerty A, Massarotti E, Neuberg D, Berliner N. Marked hyperferritinemia does not predict for HLH in the adult population. Blood 2015:125(10):1548-1552.
59. Henter JI. HLH-2004 clinical study update. ABSTRACTS FROM THE 31stANNUAL MEETING OF THE HISTIOCYTE SOCIETY ATHENS, GREECE SEPTEMBER 28-30, 2015. Pediatric Blood & Cancer 2015:62(S3):S121-S141.
60. Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. Blood 2011:118(15):4041-4052.
61. Jenkins MR, Rudd-Schmidt JA, Lopez JA, Ramsbottom KM, Mannering SI, Andrews DM, Voskoboinik I, Trapani JA. Failed CTL/NK cell killing and cytokine hypersecretion are directly linked through prolonged synapse time. J Exp Med 2015:212(3):307-317.
62. Wigzell H. Quantitative Titrations of Mouse H-2 Antibodies Using Cr-51-Labelled Target Cells. Transplantation 1965:3:423-431.
63. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 1968:14(2):181-196.
64. Ostler T, Schamel K, Hussell T, Openshaw P, Hausmann J, Ehl S. An improved protocol for measuring cytotoxic T cell activity in anatomic compartments with low cell numbers. J Immunol Methods 2001:257(1-2):155-161.
65. Janka GE. Familial hemophagocytic lymphohistiocytosis. Eur J Pediatr 1983:140(3):221-230.
66. Bryceson YT, Rudd E, Zheng C, Edner J, Ma D, Wood SM, Bechensteen AG, Boelens JJ, Celkan T, Farah RA, Hultenby K, Winiarski J, Roche PA, Nordenskjold M, Henter JI, Long EO, Ljunggren HG. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. Blood 2007:110(6):1906-1915.
67. Rudd E, Bryceson YT, Zheng C, Edner J, Wood SM, Ramme K, Gavhed S, Gurgey A, Hellebostad M, Bechensteen AG, Ljunggren HG, Fadeel B, Nordenskjold M,

Henter JI. Spectrum, and clinical and functional implications of UNC13D mutations in familial haemophagocytic lymphohistiocytosis. J Med Genet 2008:45(3):134-141.
68. Cote M, Menager MM, Burgess A, Mahlaoui N, Picard C, Schaffner C, Al-Manjomi F, Al-Harbi M, Alangari A, Le Deist F, Gennery AR, Prince N, Cariou A, Nitschke P, Blank U, El-Ghazali G, Menasche G, Latour S, Fischer A, de Saint Basile G. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. J Clin Invest 2009:119(12):3765-3773.
69. Santoro A, Cannella S, Bossi G, Gallo F, Trizzino A, Pende D, Dieli F, Bruno G, Stinchcombe JC, Micalizzi C, De Fusco C, Danesino C, Moretta L, Notarangelo LD, Griffiths GM, Arico M. Novel Munc13-4 mutations in children and young adult patients with haemophagocytic lymphohistiocytosis. J Med Genet 2006:43(12):953-960.
70. Marcenaro S, Gallo F, Martini S, Santoro A, Griffiths GM, Arico M, Moretta L, Pende D. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. Blood 2006:108(7):2316-2323.
71. Janka GE, Lehmberg K. Hemophagocytic lymphohistiocytosis: pathogenesis and treatment. Hematology Am Soc Hematol Educ Program 2013:2013:605-611.
72. George MR. Hemophagocytic lymphohistiocytosis: review of etiologies and management. J Blood Med 2014:5:69-86.
73. Fischer A, Cerf-Bensussan N, Blanche S, Le Deist F, Bremard-Oury C, Leverger G, Schaison G, Durandy A, Griscelli C. Allogeneic bone marrow transplantation for erythrophagocytic lymphohistiocytosis. J Pediatr 1986:108(2):267-270.
74. Horne A, Trottestam H, Arico M, Egeler RM, Filipovich AH, Gadner H, Imashuku S, Ladisch S, Webb D, Janka G, Henter JI, Histiocyte S. Frequency and spectrum of central nervous system involvement in 193 children with haemophagocytic lymphohistiocytosis. Br J Haematol 2008:140(3):327-335.
75. Imashuku S, Hibi S, Ohara T, Iwai A, Sako M, Kato M, Arakawa H, Sotomatsu M, Kataoka S, Asami K, Hasegawa D, Kosaka Y, Sano K, Igarashi N, Maruhashi K, Ichimi R, Kawasaki H, Maeda N, Tanizawa A, Arai K, Abe T, Hisakawa H, Miyashita H, Henter JI. Effective control of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis with immunochemotherapy. Histiocyte Society. Blood 1999:93(6):1869-1874.
76. Arico M, Janka G, Fischer A, Henter JI, Blanche S, Elinder G, Martinetti M, Rusca MP. Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. Leukemia 1996:10(2):197-203.
77. Farquhar JW, Claireaux AE. Familial haemophagocytic reticulosis. Arch Dis Child 1952:27(136):519-525.
78. Nagle DL, Karim MA, Woolf EA, Holmgren L, Bork P, Misumi DJ, McGrail SH, Dussault BJ, Jr., Perou CM, Boissy RE, Duyk GM, Spritz RA, Moore KJ. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. Nat Genet 1996:14(3):307-311.
79. Fadeel B, Orrenius S, Henter JI. Induction of apoptosis and caspase activation in cells obtained from familial haemophagocytic lymphohistiocytosis patients. Br J Haematol 1999:106(2):406-415.
80. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene

defects in familial hemophagocytic lymphohistiocytosis. Science 1999:286(5446):1957-1959.
81. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachee-Chardin M, Chedeville G, Tamary H, Minard-Colin V, Vilmer E, Blanche S, Le Deist F, Fischer A, de Saint Basile G. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell 2003:115(4):461-473.
82. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, Kabisch H, Schneppenheim R, Nurnberg P, Janka G, Hennies HC. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum Mol Genet 2005:14(6):827-834.
83. Menasche G, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, Wulffraat N, Bianchi D, Fischer A, Le Deist F, de Saint Basile G. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat Genet 2000:25(2):173-176.
84. Badolato R, Parolini S. Novel insights from adaptor protein 3 complex deficiency. J Allergy Clin Immunol 2007:120(4):735-741; quiz 742-733.
85. Enders A, Zieger B, Schwarz K, Yoshimi A, Speckmann C, Knoepfle EM, Kontny U, Muller C, Nurden A, Rohr J, Henschen M, Pannicke U, Niemeyer C, Nurden P, Ehl S. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. Blood 2006:108(1):81-87.
86. zur Stadt U, Rohr J, Seifert W, Koch F, Grieve S, Pagel J, Strauss J, Kasper B, Nurnberg G, Becker C, Maul-Pavicic A, Beutel K, Janka G, Griffiths G, Ehl S, Hennies HC. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. Am J Hum Genet 2009:85(4):482-492.
87. Abo T, Roder JC, Abo W, Cooper MD, Balch CM. Natural killer (HNK-1+) cells in Chediak-Higashi patients are present in normal numbers but are abnormal in function and morphology. J Clin Invest 1982:70(1):193-197.
88. Arico M, Nespoli L, Maccario R, Montagna D, Bonetti F, Caselli D, Burgio GR. Natural cytotoxicity impairment in familial haemophagocytic lymphohistiocytosis. Arch Dis Child 1988:63(3):292-296.
89. Dotta L, Parolini S, Prandini A, Tabellini G, Antolini M, Kingsmore SF, Badolato R. Clinical, laboratory and molecular signs of immunodeficiency in patients with partial oculo-cutaneous albinism. Orphanet J Rare Dis 2013:8:168.
90. Cetica V, Hackmann Y, Grieve S, Sieni E, Ciambotti B, Coniglio ML, Pende D, Gilmour K, Romagnoli P, Griffiths GM, Arico M. Patients with Griscelli syndrome and normal pigmentation identify RAB27A mutations that selectively disrupt MUNC13-4 binding. J Allergy Clin Immunol 2015:135(5):1310-1318 e1311.
91. Meeths M, Bryceson YT, Rudd E, Zheng C, Wood SM, Ramme K, Beutel K, Hasle H, Heilmann C, Hultenby K, Ljunggren HG, Fadeel B, Nordenskjold M, Henter JI. Clinical presentation of Griscelli syndrome type 2 and spectrum of RAB27A mutations. Pediatr Blood Cancer 2010:54(4):563-572.
92. Meeths M, Horne A, Sabel M, Bryceson YT, Henter JI. Incidence and clinical presentation of primary hemophagocytic lymphohistiocytosis in Sweden. Pediatr Blood Cancer 2014.
93. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. Acta Paediatr Scand 1991:80(4):428-435.

94. Niece JA, Rogers ZR, Ahmad N, Langevin AM, McClain KL. Hemophagocytic lymphohistiocytosis in Texas: observations on ethnicity and race. Pediatr Blood Cancer 2010:54(3):424-428.
95. Cetica V, Sieni E, Pende D, Danesino C, De Fusco C, Locatelli F, Micalizzi C, Putti MC, Biondi A, Fagioli F, Moretta L, Griffiths GM, Luzzatto L, Arico M. Genetic predisposition to hemophagocytic lymphohistiocytosis: Report on 500 patients from the Italian registry. J Allergy Clin Immunol 2015.
96. Yang X, Hoshino A, Taga T, Kunitsu T, Ikeda Y, Yasumi T, Yoshida K, Wada T, Miyake K, Kubota T, Okuno Y, Muramatsu H, Adachi Y, Miyano S, Ogawa S, Kojima S, Kanegane H. A female patient with incomplete hemophagocytic lymphohistiocytosis caused by a heterozygous XIAP mutation associated with non-random X-chromosome inactivation skewed towards the wild-type XIAP allele. J Clin Immunol 2015:35(3):244-248.
97. Gao L, Zhu L, Huang L, Zhou J. Synergistic defects of UNC13D and AP3B1 leading to adult hemophagocytic lymphohistiocytosis. Int J Hematol 2015.
98. Zhang K, Chandrakasan S, Chapman H, Valencia CA, Husami A, Kissell D, Johnson JA, Filipovich AH. Synergistic defects of different molecules in the cytotoxic pathway lead to clinical familial hemophagocytic lymphohistiocytosis. Blood 2014:124(8):1331-1334.
99. Cohen JI, Niemela JE, Stoddard JL, Pittaluga S, Heslop H, Jaffe ES, Dowdell K. Late-onset severe chronic active EBV in a patient for five years with mutations in STXBP2 (MUNC18-2) and PRF1 (perforin 1). J Clin Immunol 2015:35(5):445-448.
100. Ciambotti B, Mussolin L, d'Amore ES, Pillon M, Sieni E, Coniglio ML, Ros MD, Cetica V, Arico M, Rosolen A. Monoallelic mutations of the perforin gene may represent a predisposing factor to childhood anaplastic large cell lymphoma. J Pediatr Hematol Oncol 2014:36(6):e359-365.
101. Clementi R, Locatelli F, Dupre L, Garaventa A, Emmi L, Bregni M, Cefalo G, Moretta A, Danesino C, Comis M, Pession A, Ramenghi U, Maccario R, Arico M, Roncarolo MG. A proportion of patients with lymphoma may harbor mutations of the perforin gene. Blood 2005:105(11):4424-4428.
102. Ghosh S, Bienemann K, Boztug K, Borkhardt A. Interleukin-2-inducible T-cell kinase (ITK) deficiency - clinical and molecular aspects. J Clin Immunol 2014:34(8):892-899.
103. Salzer E, Daschkey S, Choo S, Gombert M, Santos-Valente E, Ginzel S, Schwendinger M, Haas OA, Fritsch G, Pickl WF, Forster-Waldl E, Borkhardt A, Boztug K, Bienemann K, Seidel MG. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. Haematologica 2013:98(3):473-478.
104. Spinner MA, Ker JP, Stoudenmire CJ, Fadare O, Mace EM, Orange JS, Hsu AP, Holland SM. GATA2 deficiency underlying severe blastomycosis and fatal herpes simplex virus-associated hemophagocytic lymphohistiocytosis. J Allergy Clin Immunol 2015.
105. Marsh RA, Madden L, Kitchen BJ, Mody R, McClimon B, Jordan MB, Bleesing JJ, Zhang K, Filipovich AH. XIAP deficiency: a unique primary immunodeficiency best classified as X-linked familial hemophagocytic lymphohistiocytosis and not as X-linked lymphoproliferative disease. Blood 2010:116(7):1079-1082.
106. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, Soulas P, Galicier L, Le Deist F, Rieux-Laucat F, Revy P, Fischer A, de Saint Basile G, Latour S. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature 2006:444(7115):110-114.

107. Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, Cahn AP, Durham J, Heath P, Wray P, Pavitt R, Wilkinson J, Leversha M, Huckle E, Shaw-Smith CJ, Dunham A, Rhodes S, Schuster V, Porta G, Yin L, Serafini P, Sylla B, Zollo M, Franco B, Bolino A, Seri M, Lanyi A, Davis JR, Webster D, Harris A, Lenoir G, de St Basile G, Jones A, Behloradsky BH, Achatz H, Murken J, Fassler R, Sumegi J, Romeo G, Vaudin M, Ross MT, Meindl A, Bentley DR. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet 1998:20(2):129-135.
108. Marsh RA, Bleesing JJ, Filipovich AH. Using flow cytometry to screen patients for X-linked lymphoproliferative disease due to SAP deficiency and XIAP deficiency. J Immunol Methods 2010:362(1-2):1-9.
109. Ammann S, Elling R, Gyrd-Hansen M, Duckers G, Bredius R, Burns SO, Edgar JD, Worth A, Brandau H, Warnatz K, Zur Stadt U, Hasselblatt P, Schwarz K, Ehl S, Speckmann C. A new functional assay for the diagnosis of X-linked inhibitor of apoptosis (XIAP) deficiency. Clin Exp Immunol 2014:176(3):394-400.
110. Serwas NK, Cagdas D, Ban SA, Bienemann K, Salzer E, Tezcan I, Borkhardt A, Sanal O, Boztug K. Identification of ITK deficiency as a novel genetic cause of idiopathic CD4+ T-cell lymphopenia. Blood 2014:124(4):655-657.
111. Gifford CE, Weingartner E, Villanueva J, Johnson J, Zhang K, Filipovich AH, Bleesing JJ, Marsh RA. Clinical flow cytometric screening of SAP and XIAP expression accurately identifies patients with SH2D1A and XIAP/BIRC4 mutations. Cytometry B Clin Cytom 2014:86(4):263-271.
112. Egeler RM, Shapiro R, Loechelt B, Filipovich A. Characteristic immune abnormalities in hemophagocytic lymphohistiocytosis. J Pediatr Hematol Oncol 1996:18(4):340-345.
113. Waggoner SN, Cornberg M, Selin LK, Welsh RM. Natural killer cells act as rheostats modulating antiviral T cells. Nature 2012:481(7381):394-398.
114. Dandoy C, Grimley M. Secondary hemophagocytic lymphohistiocytosis (HLH) from a presumed brown recluse spider bite. J Clin Immunol 2014:34(5):544-547.
115. Gokce M, Unal O, Hismi B, Gumruk F, Coskun T, Balta G, Unal S, Cetin M, Kalkanoglu-Sivri HS, Dursun A, Tokatli A. Secondary hemophagocytosis in 3 patients with organic acidemia involving propionate metabolism. Pediatr Hematol Oncol 2012:29(1):92-98.
116. Ben Turkia H, Tebib N, Azzouz H, Abdelmoula MS, Ben Chehida A, Caillaud C, Ben Dridi MF. Phenotypic continuum of type 2 Gaucher's disease: an intermediate phenotype between perinatal-lethal and classic type 2 Gaucher's disease. J Perinatol 2009:29(2):170-172.
117. Atteritano M, David A, Bagnato G, Beninati C, Frisina A, Iaria C, Bagnato G, Cascio A. Haemophagocytic syndrome in rheumatic patients. A systematic review. Eur Rev Med Pharmacol Sci 2012:16(10):1414-1424.
118. Cron RQ, Davi S, Minoia F, Ravelli A. Clinical features and correct diagnosis of macrophage activation syndrome. Expert Rev Clin Immunol 2015:11(9):1043-1053.
119. Lehmberg K, Sprekels B, Nichols KE, Woessmann W, Muller I, Suttorp M, Bernig T, Beutel K, Bode SF, Kentouche K, Kolb R, Langler A, Minkov M, Schilling FH, Schmid I, Vieth S, Ehl S, Zur Stadt U, Janka GE. Malignancy-associated haemophagocytic lymphohistiocytosis in children and adolescents. Br J Haematol 2015:170(4):539-549.

120. Raju S, Kalyanaraman S, Swaminathan K, Nisha A, Praisid S. Hemophagocytic lymphohistiocytosis syndrome in Dengue hemorrhagic fever. Indian J Pediatr 2014:81(12):1381-1383.
121. van der Ven AJ, Netea MG, van der Meer JW, de Mast Q. Ebola Virus Disease has Features of Hemophagocytic Lymphohistiocytosis Syndrome. Front Med (Lausanne) 2015:2:4.
122. Fardet L, Lambotte O, Meynard JL, Kamouh W, Galicier L, Marzac C, de Labarthe A, Cabane J, Lebbe C, Coppo P, Molina JM, Martinez V. Reactive haemophagocytic syndrome in 58 HIV-1-infected patients: clinical features, underlying diseases and prognosis. AIDS 2010:24(9):1299-1306.
123. Blazquez-Gamero D, Dominguez-Pinilla N, Chicharro C, Negreira S, Galan P, Perez-Gorricho B, Calvo C, Prieto L, De la Parte M, Otheo E, Vivanco JL, Ruiz-Contreras J, Madrid Leishmaniasis Study G. Hemophagocytic lymphohistiocytosis in children with visceral leishmaniasis. Pediatr Infect Dis J 2015:34(6):667-669.
124. Cantero-Hinojosa J, Diez-Ruiz A, Santos-Perez JL, Aguilar-Martinez JL, Ramos-Jimenez A. Lyme disease associated with hemophagocytic syndrome. Clin Investig 1993:71(8):620.
125. Saidi W, Gammoudi R, Korbi M, Aounallah A, Boussofara L, Ghariani N, Sriha B, Braham N, Denguezli M, Belajouza C, Nouira R. Hemophagocytic lymphohistiocytosis: an unusual complication of leprosy. Int J Dermatol 2015:54(9):1054-1059.
126. Hui YM, Pillinger T, Luqmani A, Cooper N. Haemophagocytic lymphohistiocytosis associated with Mycobacterium tuberculosis infection. BMJ Case Rep 2015:2015.
127. Cascio A, Pernice LM, Barberi G, Delfino D, Biondo C, Beninati C, Mancuso G, Rodriguez-Morales AJ, Iaria C. Secondary hemophagocytic lymphohistiocytosis in zoonoses. A systematic review. Eur Rev Med Pharmacol Sci 2012:16(10):1324-1337.
128. Aguilar C, Latour S. X-linked inhibitor of apoptosis protein deficiency: more than an X-linked lymphoproliferative syndrome. J Clin Immunol 2015:35(4):331-338.
129. Sumegi J, Huang D, Lanyi A, Davis JD, Seemayer TA, Maeda A, Klein G, Seri M, Wakiguchi H, Purtilo DT, Gross TG. Correlation of mutations of the SH2D1A gene and epstein-barr virus infection with clinical phenotype and outcome in X-linked lymphoproliferative disease. Blood 2000:96(9):3118-3125.
130. Parvaneh N, Casanova JL, Notarangelo LD, Conley ME. Primary immunodeficiencies: a rapidly evolving story. J Allergy Clin Immunol 2013:131(2):314-323.
131. Quintana-Murci L, Alcais A, Abel L, Casanova JL. Immunology in natura: clinical, epidemiological and evolutionary genetics of infectious diseases. Nat Immunol 2007:8(11):1165-1171.
132. Dalal BI, Vakil AP, Khare NS, Wang SY, Richards MJ, Chen LY. Abnormalities of the lymphocyte subsets and their immunophenotype, and their prognostic significance in adult patients with hemophagocytic lymphohistiocytosis. Ann Hematol 2015:94(7):1111-1117.
133. Lehmberg K, McClain KL, Janka GE, Allen CE. Determination of an appropriate cut-off value for ferritin in the diagnosis of hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2014:61(11):2101-2103.

134. Saeed H, Woods RR, Lester J, Herzig R, Gul Z, Monohan G. Evaluating the optimal serum ferritin level to identify hemophagocytic lymphohistiocytosis in the critical care setting. Int J Hematol 2015:102(2):195-199.
135. Yasumi T, Hori M, Hiejima E, Shibata H, Izawa K, Oda H, Yoshioka K, Nakagawa K, Kawai T, Nishikomori R, Ohara O, Heike T. Laboratory parameters identify familial haemophagocytic lymphohistiocytosis from other forms of paediatric haemophagocytosis. Br J Haematol 2015:170(4):532-538.
136. Meeths M, Chiang SC, Lofstedt A, Muller ML, Tesi B, Henter JI, Bryceson YT. Pathophysiology and spectrum of diseases caused by defects in lymphocyte cytotoxicity. Exp Cell Res 2014:325(1):10-17.
137. Trottestam H, Horne A, Arico M, Egeler RM, Filipovich AH, Gadner H, Imashuku S, Ladisch S, Webb D, Janka G, Henter JI, Histiocyte S. Chemoimmunotherapy for hemophagocytic lymphohistiocytosis: long-term results of the HLH-94 treatment protocol. Blood 2011:118(17):4577-4584.
138. Mahlaoui N, Ouachee-Chardin M, de Saint Basile G, Neven B, Picard C, Blanche S, Fischer A. Immunotherapy of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins: a single-center retrospective report of 38 patients. Pediatrics 2007:120(3):e622-628.
139. Hamidieh AA, Pourpak Z, Hashemi S, Yari K, Fazlollahi MR, Movahedi M, Behfar M, Moin M, Ghavamzadeh A. Fludarabine-based reduced-intensity conditioning regimen for hematopoietic stem cell transplantation in primary hemophagocytic lymphohistiocytosis. Eur J Haematol 2014:92(4):331-336.
140. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000:288(5466):669-672.
141. Check E. A tragic setback. Nature 2002:420(6912):116-118.
142. Booth C, Carmo M, Gaspar HB. Gene therapy for haemophagocytic lymphohistiocytosis. Curr Gene Ther 2014:14(6):437-446.
143. Pachlopnik Schmid J, Ho CH, Chretien F, Lefebvre JM, Pivert G, Kosco-Vilbois M, Ferlin W, Geissmann F, Fischer A, de Saint Basile G. Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. EMBO Mol Med 2009:1(2):112-124.
144. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood 2004:104(3):735-743.
145. Kostova EB, Beuger BM, Veldthuis M, van der Werff Ten Bosch J, Kuhnle I, van den Akker E, van den Berg TK, van Zwieten R, van Bruggen R. Intrinsic defects in erythroid cells from familial hemophagocytic lymphohistiocytosis type 5 patients identify a role for STXBP2/Munc18-2 in erythropoiesis and phospholipid scrambling. Exp Hematol 2015.
146. Nakamura L, Bertling A, Brodde MF, Zur Stadt U, Schulz AS, Ammann S, Sandrock-Lang K, Beutel K, Zieger B, Kehrel BE. First characterization of platelet secretion defect in patients with familial hemophagocytic lymphohistiocytosis type 3 (FHL-3). Blood 2015:125(2):412-414.
147. Popeskou S, Gavillet M, Demartines N, Christoforidis D. Hemophagocytic Lymphohistiocytosis and Gastrointestinal Bleeding: What a Surgeon Should Know. Case Rep Surg 2015:2015:745848.
148. Sandrock K, Nakamura L, Vraetz T, Beutel K, Ehl S, Zieger B. Platelet secretion defect in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL-5). Blood 2010:116(26):6148-6150.

149. Zlotnikov-Klionsky Y, Nathansohn-Levi B, Shezen E, Rosen C, Kagan S, Bar-On L, Jung S, Shifrut E, Reich-Zeliger S, Friedman N, Aharoni R, Arnon R, Yifa O, Aronovich A, Reisner Y. Perforin-Positive Dendritic Cells Exhibit an Immuno-regulatory Role in Metabolic Syndrome and Autoimmunity. Immunity 2015.
150. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med 1995:181(3):1235-1238.
151. Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science 2003:301(5640):1720-1725.
152. Sims TN, Dustin ML. The immunological synapse: integrins take the stage. Immunol Rev 2002:186:100-117.
153. Hajishengallis G, Chavakis T. Endogenous modulators of inflammatory cell recruitment. Trends Immunol 2013:34(1):1-6.
154. Schulte-Mecklenbeck A, Bittner S, Ehling P, Doring F, Wischmeyer E, Breuer J, Herrmann AM, Wiendl H, Meuth SG, Gross CC. The two-pore domain K2 P channel TASK2 drives human NK-cell proliferation and cytolytic function. Eur J Immunol 2015:45(9):2602-2614.
155. Abram CL, Lowell CA. Convergence of immunoreceptor and integrin signaling. Immunol Rev 2007:218:29-44.
156. Shaw PJ, Feske S. Regulation of lymphocyte function by ORAI and STIM proteins in infection and autoimmunity. J Physiol 2012:590(Pt 17):4157-4167.
157. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 2006:441(7090):179-185.
158. Jairaman A, Yamashita M, Schleimer RP, Prakriya M. Store-Operated Ca2+ Release-Activated Ca2+ Channels Regulate PAR2-Activated Ca2+ Signaling and Cytokine Production in Airway Epithelial Cells. J Immunol 2015:195(5):2122-2133.
159. Kraft R. STIM and ORAI proteins in the nervous system. Channels (Austin) 2015:235-243.
160. Wen L, Voronina S, Javed MA, Awais M, Szatmary P, Latawiec D, Chvanov M, Collier D, Huang W, Barrett J, Begg M, Stauderman K, Roos J, Grigoryev S, Ramos S, Rogers E, Whitten J, Velicelebi G, Dunn M, Tepikin AV, Criddle DN, Sutton R. Inhibitors of ORAI1 Prevent Cytosolic Calcium-Associated Injury of Human Pancreatic Acinar Cells and Acute Pancreatitis in 3 Mouse Models. Gastroenterology 2015:149(2):481-492 e487.
161. Feske S. ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca2+ entry in the immune system and beyond. Immunol Rev 2009:231(1):189-209.
162. Gerbec ZJ, Thakar MS, Malarkannan S. The Fyn-ADAP Axis: Cytotoxicity Versus Cytokine Production in Killer Cells. Front Immunol 2015:6:472.
163. Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. Nature 2006:443(7110):462-465.
164. Liu D, Meckel T, Long EO. Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. PLoS One 2010:5(9):e12870.
165. Kurowska M, Goudin N, Nehme NT, Court M, Garin J, Fischer A, de Saint Basile G, Menasche G. Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/Slp3/Rab27a complex. Blood 2012:119(17):3879-3889.

166. Elstak ED, Neeft M, Nehme NT, Voortman J, Cheung M, Goodarzifard M, Gerritsen HC, van Bergen En Henegouwen PM, Callebaut I, de Saint Basile G, van der Sluijs P. The munc13-4-rab27 complex is specifically required for tethering secretory lysosomes at the plasma membrane. Blood 2011:118(6):1570-1578.
167. Ma C, Su L, Seven AB, Xu Y, Rizo J. Reconstitution of the vital functions of Munc18 and Munc13 in neurotransmitter release. Science 2013:339(6118):421-425.
168. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004:294(1-2):15-22.
169. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003:281(1-2):65-78.
170. Lopez JA, Jenkins MR, Rudd-Schmidt JA, Brennan AJ, Danne JC, Mannering SI, Trapani JA, Voskoboinik I. Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. J Immunol 2013:191(5):2328-2334.
171. Woodsworth DJ, Dunsing V, Coombs D. Design Parameters for Granzyme-Mediated Cytotoxic Lymphocyte Target-Cell Killing and Specificity. Biophys J 2015:109(3):477-488.
172. Hunziker W, Simmen T, Honing S. Trafficking of lysosomal membrane proteins in polarized kidney cells. Nephrologie 1996:17(7):347-350.
173. Kundra R, Kornfeld S. Asparagine-linked oligosaccharides protect Lamp-1 and Lamp-2 from intracellular proteolysis. J Biol Chem 1999:274(43):31039-31046.
174. Praper T, Besenicar MP, Istinic H, Podlesek Z, Metkar SS, Froelich CJ, Anderluh G. Human perforin permeabilizing activity, but not binding to lipid membranes, is affected by pH. Mol Immunol 2010:47(15):2492-2504.
175. Eskelinen EL. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Mol Aspects Med 2006:27(5-6):495-502.
176. Andrejewski N, Punnonen EL, Guhde G, Tanaka Y, Lullmann-Rauch R, Hartmann D, von Figura K, Saftig P. Normal lysosomal morphology and function in LAMP-1-deficient mice. J Biol Chem 1999:274(18):12692-12701.
177. Krzewski K, Gil-Krzewska A, Nguyen V, Peruzzi G, Coligan JE. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. Blood 2013:121(23):4672-4683.
178. Sarafian V, Jadot M, Foidart JM, Letesson JJ, Van den Brule F, Castronovo V, Wattiaux R, Coninck SW. Expression of Lamp-1 and Lamp-2 and their interactions with galectin-3 in human tumor cells. Int J Cancer 1998:75(1):105-111.
179. Saitoh O, Wang WC, Lotan R, Fukuda M. Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. J Biol Chem 1992:267(8):5700-5711.
180. Trapani JA, Kwon BS, Kozak CA, Chintamaneni C, Young JD, Dupont B. Genomic organization of the mouse pore-forming protein (perforin) gene and localization to chromosome 10. Similarities to and differences from C9. J Exp Med 1990:171(2):545-557.
181. Sarma JV, Ward PA. The complement system. Cell Tissue Res 2011:343(1):227-235.

182. Yagi H, Conroy PJ, Leung EW, Law RH, Trapani JA, Voskoboinik I, Whisstock JC, Norton RS. Structural Basis for Ca2+-mediated Interaction of the Perforin C2 Domain with Lipid Membranes. J Biol Chem 2015.
183. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev 2010:235(1):105-116.
184. Brodovitch A, Shenderov E, Cerundolo V, Bongrand P, Pierres A, van der Merwe PA. T lymphocytes need less than 3 min to discriminate between peptide MHCs with similar TCR-binding parameters. Eur J Immunol 2015:45(6):1635-1642.
185. Poenie M, Tsien RY, Schmitt-Verhulst AM. Sequential activation and lethal hit measured by [Ca2+]i in individual cytolytic T cells and targets. EMBO J 1987:6(8):2223-2232.
186. Vanherberghen B, Olofsson PE, Forslund E, Sternberg-Simon M, Khorshidi MA, Pacouret S, Guldevall K, Enqvist M, Malmberg KJ, Mehr R, Onfelt B. Classification of human natural killer cells based on migration behavior and cytotoxic response. Blood 2013:121(8):1326-1334.
187. Vasconcelos Z, Muller S, Guipouy D, Yu W, Christophe C, Gadat S, Valitutti S, Dupre L. Individual Human Cytotoxic T Lymphocytes Exhibit Intraclonal Heterogeneity during Sustained Killing. Cell Rep 2015:11(9):1474-1485.
188. Shafer-Weaver K, Sayers T, Strobl S, Derby E, Ulderich T, Baseler M, Malyguine A. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. J Transl Med 2003:1(1):14.
189. Karimi MA, Lee E, Bachmann MH, Salicioni AM, Behrens EM, Kambayashi T, Baldwin CL. Measuring cytotoxicity by bioluminescence imaging outperforms the standard chromium-51 release assay. PLoS One 2014:9(2):e89357.
190. Fischer K, Mackensen A. The flow cytometric PKH-26 assay for the determination of T-cell mediated cytotoxic activity. Methods 2003:31(2):135-142.
191. Nakagawa Y, Watari E, Shimizu M, Takahashi H. One-step simple assay to determine antigen-specific cytotoxic activities by single-color flow cytometry. Biomed Res 2011:32(2):159-166.
192. Crozat K, Hoebe K, Ugolini S, Hong NA, Janssen E, Rutschmann S, Mudd S, Sovath S, Vivier E, Beutler B. Jinx, an MCMV susceptibility phenotype caused by disruption of Unc13d: a mouse model of type 3 familial hemophagocytic lymphohistiocytosis. J Exp Med 2007:204(4):853-863.
193. Terrell CE, Jordan MB. Mixed hematopoietic or T-cell chimerism above a minimal threshold restores perforin-dependent immune regulation in perforin-deficient mice. Blood 2013:122(15):2618-2621.
194. Schlesier M, Niemeyer C, Duffner U, Henschen M, Tanzi-Fetta R, Wolff-Vorbeck G, Drager R, Brandis M, Peter HH. Primary severe immunodeficiency due to impaired signal transduction in T cells. Immunodeficiency 1993:4(1-4):133-136.
195. Pross HF, Baines MG. Studies of human natural killer cells. I. In vivo parameters affecting normal cytotoxic function. Int J Cancer 1982:29(4):383-390.
196. Bryant J, Day R, Whiteside TL, Herberman RB. Calculation of lytic units for the expression of cell-mediated cytotoxicity. J Immunol Methods 1992:146(1):91-103.
197. Nelson DL, Kurman CC, Serbousek DE. 51Cr release assay of antibody-dependent cell-mediated cytotoxicity (ADCC). Curr Protoc Immunol 2001:Chapter 7:Unit 7.27.

198. Whiteside TL. Measurement of NK-Cell Activity in Humans. In Detrick, Barbara, Hamilton, Robert G. and Folds, James D. (ed), Manual of Molecular and Clinical Laboratory Immunology, 7th ed. ASM Press, Washington, DC. 2006:296-300.
199. Kannan K, Stewart RM, Bounds W, Carlsson SR, Fukuda M, Betzing KW, Holcombe RF. Lysosome-associated membrane proteins h-LAMP1 (CD107a) and h-LAMP2 (CD107b) are activation-dependent cell surface glycoproteins in human peripheral blood mononuclear cells which mediate cell adhesion to vascular endothelium. Cell Immunol 1996:171(1):10-19.
200. Stoltenberg C, Magnus P, Lie RT, Daltveit AK, Irgens LM. Birth defects and parental consanguinity in Norway. Am J Epidemiol 1997:145(5):439-448.
201. Jorde LB, Pitkanen KJ. Inbreeding in Finland. Am J Phys Anthropol 1991:84(2):127-139.
202. Brodszki N, Jonsson G, Skattum L, Truedsson L. Primary immunodeficiency in infection-prone children in southern Sweden: occurrence, clinical characteristics and immunological findings. BMC Immunol 2014:15:31.
203. Sipila K, Aula P. Database for the mutations of the Finnish disease heritage. Hum Mutat 2002:19(1):16-22.
204. Hemminki K, Li X, Forsti A, Sundquist J, Sundquist K. Incidence of hereditary amyloidosis and autoinflammatory diseases in Sweden: endemic and imported diseases. BMC Med Genet 2013:14:88.
205. Akbayram S, Sari N, Akgun C, Dogan M, Tuncer O, Caksen H, Oner AF. The frequency of consanguineous marriage in eastern Turkey. Genet Couns 2009:20(3):207-214.
206. Nagafuji K, Nonami A, Kumano T, Kikushige Y, Yoshimoto G, Takenaka K, Shimoda K, Ohga S, Yasukawa M, Horiuchi H, Ishii E, Harada M. Perforin gene mutations in adult-onset hemophagocytic lymphohistiocytosis. Haematologica 2007:92(7):978-981.
207. Abdalgani M, Filipovich AH, Choo S, Zhang K, Gifford C, Villanueva J, Bleesing JJ, Marsh RA. Accuracy of flow cytometric perforin screening for detecting patients with FHL due to PRF1 mutations. Blood 2015:126(15):1858-1860.
208. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. Nat Rev Immunol 2015:15(6):388-400.
209. Borte S, Meeths M, Liebscher I, Krist K, Nordenskjold M, Hammarstrom L, von Dobeln U, Henter JI, Bryceson YT. Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. J Allergy Clin Immunol 2014:134(1):226-228.
210. van der Spek J, Groenwold RH, van der Burg M, van Montfrans JM. TREC Based Newborn Screening for Severe Combined Immunodeficiency Disease: A Systematic Review. J Clin Immunol 2015:35(4):416-430.
211. Seo JY, Song JS, Lee KO, Won HH, Kim JW, Kim SH, Lee SH, Yoo KH, Sung KW, Koo HH, Kang HJ, Shin HY, Ahn HS, Han DK, Kook H, Hwang TJ, Lyu CJ, Lee MJ, Kim JY, Park SS, Lim YT, Kim BE, Koh KN, Im HJ, Seo JJ, Kim HJ, Korea Histiocytosis Working P. Founder effects in two predominant intronic mutations of UNC13D, c.118-308C>T and c.754-1G>C underlie the unusual predominance of type 3 familial hemophagocytic lymphohistiocytosis (FHL3) in Korea. Ann Hematol 2013:92(3):357-364.
212. Yoon HS, Kim HJ, Yoo KH, Sung KW, Koo HH, Kang HJ, Shin HY, Ahn HS, Kim JY, Lim YT, Bae KW, Lee KO, Shin JS, Lee ST, Chung HS, Kim SH, Park CJ, Chi HS, Im HJ, Seo JJ. UNC13D is the predominant causative gene with recurrent splicing mutations in Korean patients with familial hemophagocytic lymphohistiocytosis. Haematologica 2010:95(4):622-626.

213. Qian Y, Johnson JA, Connor JA, Valencia CA, Barasa N, Schubert J, Husami A, Kissell D, Zhang G, Weirauch MT, Filipovich AH, Zhang K. The 253-kb inversion and deep intronic mutations in UNC13D are present in North American patients with familial hemophagocytic lymphohistiocytosis 3. Pediatr Blood Cancer 2014:61(6):1034-1040.
214. Zhang K, Jordan MB, Marsh RA, Johnson JA, Kissell D, Meller J, Villanueva J, Risma KA, Wei Q, Klein PS, Filipovich AH. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. Blood 2011:118(22):5794-5798.
215. Macartney CA, Weitzman S, Wood SM, Bansal D, Steele M, Meeths M, Abdelhaleem M, Bryceson YT. Unusual functional manifestations of a novel STX11 frameshift mutation in two infants with familial hemophagocytic lymphohistiocytosis type 4 (FHL4). Pediatr Blood Cancer 2011:56(4):654-657.
216. Vu V, Verster AJ, Schertzberg M, Chuluunbaatar T, Spensley M, Pajkic D, Hart GT, Moffat J, Fraser AG. Natural Variation in Gene Expression Modulates the Severity of Mutant Phenotypes. Cell 2015:162(2):391-402.
217. Sepulveda FE, Burgess A, Heiligenstein X, Goudin N, Menager MM, Romao M, Cote M, Mahlaoui N, Fischer A, Raposo G, Menasche G, de Saint Basile G. LYST controls the biogenesis of the endosomal compartment required for secretory lysosome function. Traffic 2015:16(2):191-203.
218. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet 2000:356(9244):1795-1799.