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UNDERSTANDING CHRONIC IMMUNODEFICIENCY DISORDERS THROUGH ASSESSMENT OF CYTOTOXIC LYMPHOCYTE FUNCTION

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Front page figure: Text in latin is a translation of the first maxim (see foreword) kindly performed by professor Denis Searby, Stockholm University. The outer circle is made up of humans, among which certain are ill. The inner circle is made up of investigated cells from these humans. Some of these cells show common traits explaining the illness of the humans in the outer circle. In the center, the serpent of Asklepios entwined around a lit candle symbolizes medical discovery.

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UNDERSTANDING CHRONIC IMMUNODEFICIENCY SYNDROMES THROUGH ASSESSMENT OF CYTOTOXIC LYMPHOCYTE FUNCTION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Representing an evolving and diverse group of syndromes, immunodeficiencies can currently be viewed as a spectrum of disorders where a dysfunction of the immune system plays a significant etiologic role. Cytotoxic lymphocytes are immune cells that are pivotal for the eradication of infected and malignant cells. They also contribute to immune-regulation. Congruently, patients with defects in cytotoxic lymphocyte function show increased susceptibility to infections, malignancies and inflammation.

This work focuses on the role of cytotoxic lymphocytes in syndromes where their pathophysiological involvement is suspected, but not established. The first study investigates the variability in cytotoxic effector functions introduced by commonly used pharmacological substances, knowledge that is important for the interpretation of patient data. The second study establishes a robust and sensitive addition to the arsenal of diagnostic assays for cytotoxic lymphocyte dysfunctions. The third study shows that myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), linked to viral infections and hypothesized to be caused by immune dysregulation, is not a syndrome where a substantial fraction of the patients has any obvious primary defect in lymphocyte cytotoxicity. The fourth and the fifth studies identify and functionally dissect the impact of autoantibodies directed against an inhibitory cytotoxic lymphocyte receptor, NKG2A, in patients with systemic lupus erythematosus (SLE), an autoimmune disorder. In summary, the studies expand our knowledge and toolbox for assessing lymphocyte cytotoxicity and highlight the complexity of interpreting experiments investigating cytotoxic lymphocyte function in the context of chronic immunodeficiency disorders. Furthermore, they provide clinically relevant insights about the role of cytotoxic lymphocytes in both ME/CFS and SLE.

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TABLE OF CONTENTS

FOREWORD AND PRESENTATION OF MAXIMS

In this doctoral thesis, I have tried to condense a substantial portion of the scientific work that I (and all my collaborators) have performed over the last years. To add some of the historical weight that a doctoral thesis merits and to hopefully make the reading more enjoyable, I have tried to sum up the principles that have guided my work in the following five maxims:

Salutogenetic insight can be gained by finding
the traits defining each group of ill

For each uncommon, severe illness
there is a common, subtle counterpart

A step towards controlled constancy lies in
understanding the grounds of variability

The subtle in the macrocosm may be discerned by
finding the robust in the microcosm

The regular nature of an illness may be understood
by exploring its rare causes

The following introductory section is meant for someone not immediately working in this subfield of immunology. It provides a frame in which the studies, underpinned by these maxims, have been performed. It is succeeded by a section where the studies will be summarized and discussed, which is followed by conclusions.

I sincerely hope that you, dear reader, during and after getting acquainted with cytotoxic lymphocytes in this context, will feel as enthusiastic about them as I have been during the nine years in which I have had the opportunity to investigate them and their multifaceted functions.

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
CD	Cluster of differentiation (taxonomic term for classification of immunologic surface proteins)
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DNAM-1	DNAX accessory molecule 1
EAT-2	Ewing sarcoma/FLI1 Activated Transcript 2
EBV	Epstein-Barr Virus
Fc	Fragment crystallizable (the non-variable part of an antibody)
HLA	Human leucocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
IFN	Interferon
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITAM	Immunoreceptor tyrosine-based activating motif
ICAM	Intercellular adhesion molecule
KIR	Killer-cell immunoglobulin-like receptor
LFA-1	Leucocyte functional antigen 1
ME/CFS	Myalgic encephalomyelitis/chronic fatigue syndrome
MHC	Major histocompatibility complex
NK	Natural killer
NKG2	Natural Killer Group 2
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PLZF	Promyelocytic leukemia Zink finger protein
RNA-IC	RNA-immune complex
SLAM	Signaling lymphocytic activation molecule
SLE	Systemic lupus erythematosus
T-lymphocyte/cell	Thymus-derived lymphocyte/cell

1 INTRODUCTION

Salutogenetic insight can be gained by finding
the traits defining each group of ill

This, the first and most general maxim of this work summarizes one central motivation to conduct human medical research. The family of immunological research approaches following from this maxim have together been termed *Immunology in Natura* (1). With these approaches, the immune systems of relevant patients, patient groups or populations are studied to gain insights of the immunologic causes for their disease. When such a cause is identified, it does not only help to explain the etiology of the disease (*pathogenesis*), but also sheds light on the very components of the immune system that are necessary to keep the rest of humanity from succumbing to the same disease – in other words, points to the etiology of our healthiness (*salutogenesis*[i]). The proponents of *Immunology in Natura* claim that the large variability in genetic backgrounds and environmental exposures in the human population provides the optimal framework for identifying such immunological defects, as disease-correlated immune aberrances are likely to be ecologically relevant, *i.e.* non-redundant in a natural setting (1). That such genetic variability is present within any wild population is due to fierce competition between hosts and pathogens; a genetic variant that proves to be a selective advantage towards a certain pathogen might prove deleterious in the interaction with another. Thus, genetic variants tend not to spread to the whole population or species, but rather be confined to a subgroup, the size of which fluctuates depending on the current pathogenic pressure.

Traditionally, basic immunological research has primarily utilized experimental animal models, such as inbred mice with aseptic living conditions. Such systems have provided numerous insights and are essential in answering questions of causality, as they allow control over genetic and environmental variation and arguably make *in vivo* experiments less ethically inacceptable than they would be in humans. Yet, experimental animal models almost by definition lack the aforementioned genetic variability and complex environmental exposure patterns typical to a wild population such as humanity. Furthermore, many immune genes are among the most rapidly evolving in our genome, again due to environmental adaptations following pathogen-host competition and coevolution. Accordingly, while large

[i] Here, the term *salutogenesis* is used in its most general meaning, deviating away from the original, psychological sense-of-coherence concept introduced together with the term by Aaron Antonovsky (2).

parts of the genetic composition dictating the shape of the immune system are conserved among most vertebrates, certain important discrepancies exist even between mammal species. Thus, it has been argued that previous approaches, where phenomena have been identified in model systems and then confirmed or denied in studies on humans should be complemented with *Immunology in Natura*-research followed by verifications of causality in model systems (1).

One approach under the *Immunology in Natura* umbrella is epidemiological. This is motivated by the observation that the variability in genetic background and environmental exposures among humans combined with our sheer numbers provides an extraordinary opportunity to find small groups that, due to similar genetic aberrances and environmental triggers, end up with specific disease. Practically, patient groups with similar clinical characteristics are investigated, in search for immunological abnormalities that distinguish these patients from healthy populations. When such an abnormality is identified, this knowledge might generate a clue about the cause of the disorder. Consequently, it might also suggest that the abnormal immunological parameter is likely to be directly or indirectly involved in counteracting development of the disorder under normal conditions. Thus, this approach, when successful, provides both clinically and biologically relevant results. In this work, all studies are performed with this *Immunology in Natura* approach in mind. Now, to be able to further describe and discuss details, a general introduction of the immune system is necessary.

1.1 THE IMMUNE SYSTEM: A BASIC INTRODUCTION TO AN EVOLVING WEB OF DEFENSE SYSTEMS

Organisms on all levels of complexity have developed strategies to cope with infectious challenges and maintain homeostasis, enabling growth and reproduction. An integral part of keeping homeostasis is counteracting occupation by pathological entities. Representing the simplest cellular organisms, bacteria have numerous strategies to avoid being turned into factories of viral proteins by bacteriophages (3,4). In more complex organisms, all cells, regardless of their specialized role, have intrinsic mechanisms to sustain homeostasis and sense infection. In addition, multicellular organisms tend to dedicate a complex system of functions to the prevention of invasion by a variety of potential pathological entities, such as bacteria, viruses, parasites and malignant cells. This complex system of functions is commonly denoted the *immune system* and congruently, the cells carrying out specialized immune functions are entitled *immune cells*.

A central feature of all arms of immune systems is the ability to differentiate between normal cells or molecules within or closely associated to the organism, referred to as "self", and other cells or molecules, referred to as "non-self". One widely disseminated solution for this is maintaining a set of intra- and extracellular receptors that sense conserved pathogen-associated molecular patterns (5). In addition, the immune system identifies aberrant or stressed endogenous cells. This is in some cases done by detecting "induced-self" (6), *i.e.* molecules that are up-regulated on cells upon stress, and in other cases by identifying damage-associated molecular patterns, such as nuclear proteins or DNA in the extracellular space (5). These pattern recognition receptors are germ line encoded, and as such evolve along with the hosts. As they are constant during the life of an individual host, they are considered innate immune receptors. The pattern recognition concept is central to the function of all innate immune cells, such as granulocytes, monocytes, macrophages, dendritic cells and innate lymphoid cells. Each innate immune cell expresses a multitude of receptors with different specificities, which enables it to respond immediately upon numerous different challenges.

About 500 million years ago, a second arm of the immune system likely first emerged in the ancestors of the simplest present-day vertebrate animals, such as hag fish or lampreys (7). The common feature of the cells in this arm is their ability to confer memory of previous pathogen exposures in the individual host, thus mounting potent recall responses upon secondary challenge. This part of the immune system is congruently called "adaptive". Soon after the first emergence of adaptive immunity in general, recombination activating genes (8) were spread and enabled somatic recombination (9), a process that is central to adaptive immune function in all higher vertebrates (7). Somatic recombination is a cellular process in which (two or) three gene segments, called variable (V), diversity (D) and joining (J), are pasted together by gene editing systems which create a new, combined V(D)J gene product (9). In a cell that has undergone somatic recombination, the V(D)J gene product codes a key receptor unique to that specific cell and all its clonal relatives. As multiple different variants of the V, D and J segments are present in any individuals genome, and as these are stochastically selected during somatic recombination, this process, in conjunction with a few additional gene editing systems, gives rise to the presence of at least tenths of millions of adaptive immune cell clones with unique receptors in a single human, capable of recognizing a wide diversity of antigens (10). As adaptive immune cells are subject to complex selection processes, a vast majority of the circulating cells recognize non-self-molecules and can, upon recognition of their antigen, respond by dividing rapidly and form a large force of cells targeted to the present pathogen. Thus, the adaptive immune cells provide a defense system

that dynamically change depending on the environment that the individual animal lives in. There are two major classes of adaptive immune cells. Bone marrow-derived (B) cells have receptors that directly recognize antigen in the extracellular space. Subsets of B cells can produce and release soluble forms of their receptors, called antibodies. Thymus-derived (T) cells instead recognize short preprocessed signal peptides presented on *major histocompatibility complex* (MHC) proteins (in humans, MHC is called *human leucocyte antigen* or HLA). Further, the two main, classical T-cell subsets are defined by their expression of the CD4 and CD8 co-receptors. These receptors define what source of antigen that the T-cell in question detects. CD4+ on T-cells enables TCR recognition of MHC class II (MHC-II, HLA-II in humans) presented peptides, which is expressed on immune cells with the special capacity to detect and break down pathological extracellular molecules. CD8+ T-cells, on the other hand, can recognize MHC-I (HLA-I in humans) with their TCR. MHC-I is expressed on virtually all vertebrate nucleated cells and present peptides derived from the intracellular space. A random fraction of all proteins is processed and displayed on the MHC-I molecules. Thus, T cells expressing CD8 mainly assess endogenous protein production. Taken together, the adaptive lymphocyte subsets can identify specific aberrant molecules present extracellularly or intracellularly from endogenous or exogenous cells.

In summary, the innate and the adaptive arms of the immune system are complimentary in nature. The innate arm can, by always probing for a variety of the most prototypic pathological signals, respond rapidly to challenge by various pathogens. It compensates for its lower specificity and affinity for a certain pathogen-associated molecule with expressing diverse and numerous receptors on individual cells, giving rise to a high avidity for most pathogens. The adaptive cells, conversely, can after a few days of pathogenic challenge mount a tailored and strong response towards any molecule with high specificity and affinity.

Instead of structuring the immune system based on means of recognition, one can view it from the angle of hematopoietic origins. In this division, there are also two main classes of cells: the myeloid and the lymphoid cells. The myeloid progenitor cells are arguably more pluripotent, not only giving rise multiple innate immune cell types such as granulocytes, monocytes, macrophages and dendritic cells, but also to erythrocytes and thrombocytes. Lymphoid progenitors, on the other hand, form the lymphocyte populations, both the adaptive B- and T cells and the innate lymphoid cells.

A third way of dividing the immune system is based on the effector functions, which are broadly categorized as humoral or cellular. Cells active in humoral immunity produce soluble molecules (humoral coming from latin *humor* meaning ‘fluid’) such as complement factors, antimicrobial peptides and antibodies, that convey the effector functions in bodily fluids.

These molecules are primarily active in extracellular compartments and on cell surfaces. On the other hand, immune cells conferring cellular immunity directly identify and rid the body of aberrant cells, exogenous or endogenous, that are potentially pathological. In this work, the focus lies on a specific part of the cellular immune system; the cytotoxic lymphocytes.

1.1.1 Cytotoxic lymphocytes – the terminators of the cellular immune system

Cytotoxic lymphocytes are effector cells with the ability to identify and eradicate cells showing signs of pathological alteration, mainly due to either infections with intracellular pathogens or due to neoplastic transformation (11). They also play pivotal roles in controlling immune responses, *i.e*. by creating negative feedback loops through killing of activated immune cells that promote inflammation (12,13). In humans (and many animals), the two main subsets are the innate natural killer (NK) cells and the adaptive cytotoxic $CD8^+$ T cells (11).

1.1.2 Natural killer cells

Natural killer cells are considered innate lymphoid cells, as their responses are regulated by a multitude of germ-line encoded receptors (14). Typically, during interaction with an individual target cell, multiple activating and inhibitory receptors are engaged. Hence, the functional outcome of a specific NK-target cell interaction is determined by receptor expression patterns and density on the NK cells (15) and, conversely, ligand expression patterns and density on the target cell (16). Further complicating the matter, a single ligand may bind both inhibitory and activating receptors (17,18). The following section is devoted to introducing a few of the NK cell receptors that play prominent roles in this work.

One receptor that inhibits NK cell function is the natural killer group 2 A (NKG2A)/CD94 heterodimer (19,20). NKG2A is a type-2 transmembrane protein with an extracellular C-type lectin domain (21). Its intracellular domain contains an *immunoreceptor tyrosine-based motif* (ITIM) (22,23), that upon activation becomes phosphorylated and recruits phosphatases, that efficiently counteract an array of activating receptor signaling pathways (24). NKG2A/CD94 recognizes HLA-E, a relatively conserved, non-canonical HLA-I molecule that presents signal peptides from other HLA-I molecules (25–28). More specifically, only certain HLA-E/signal peptide combinations are stable on the cell surface (29) and recognized by NKG2A/CD94 (25,26). Accordingly, NKG2A-expressing NK cells are strongly inhibited by target cells that produce HLA proteins presented by HLA-E (16). This inhibition can only partially be overcome by the strongest activating stimuli (16).

However, in situations where endogenous cells evade T cell recognition by decreasing classical HLA-I expression, the HLA-E-signal peptide complexes will decrease consequently and the NK cell will no longer be inhibited by NKG2A/CD94. If the target cell expresses ligands for activating NK cell receptors in this situation, it will become susceptible to NK cell-mediated killing (16). In combination with the large group of inhibitory killer immunoglobulin receptors (KIRs), this function, called missing-self recognition, grant NK cells with a complimentary function to T cell-mediated, HLA-I-dependent surveillance and eradication of aberrant cells.

The lectin-like receptor family encoded on human chromosome 12, to which NKG2A belongs, has multiple NKG2 receptor members. The closest homologue to NKG2A is NKG2C, sharing 94% of amino acid sequence in the external domain. The NKG2C/CD94 complex is activating (22,23,30), as NKG2C does not have any intracellular signaling domain, but rather associates with the activating adaptor protein DAP12 (31). The NKG2C/CD94 complex also binds HLA-E, but with lower affinity than NKG2A/CD94 (32). This is due to differences in the contact area between CD94 and the NKG2 proteins and not directly in the HLA-E contact site (33). Still, different signal peptides give rise to different affinities (32). As NKG2A is mainly expressed on "undifferentiated" NK cells (34), and NKG2C preferentially is expressed upon pathogen challenge, especially *cytomegalovirus* (CMV) (35), these receptors are seldom co-expressed and generally have complimentary functions. Yet, in cases where they are co-expressed, the NKG2A signal overrides the NKG2C signal (36).

This duality in the NK cell response to HLA-E recognition has been proposed to be linked to the defense against human CMV infection. One of many immune evasion mechanisms by CMV is the down-regulation of HLA-I expression (37). Potentially compensating for lack of HLA-I expression, CMV also expresses the protein UL40, which contains multiple sequences similar to those of classical HLA-I signal peptides. These peptides are presented by HLA-E and induce HLA-E up-regulation, inhibiting NK cell-mediated killing via NKG2A/CD94 (38). NKG2C/CD94, on the other hand, can be engaged by HLA-E presenting UL40-derived peptides and kill the target cell anyway. However, this is dependent on the specific sequence of the UL40 protein as some UL40 peptides only elicit inhibition by NKG2A/CD94 without activating NKG2C-positive cells (39).

A third member of the lectin-like receptor family is NKG2D. NKG2D is expressed on all human NK cells (40) and acts through intracellular association with the activating adaptor protein DAP10 (41). The signal resulting from ligand binding is not strong enough to trigger activation by itself, but only when other activating receptors are engaged simultaneously (42).

Therefore, NKG2D is considered a co-activating receptor (42). It only has a 21% amino acid homology to the other NKG2 receptors (21) and is expressed as a homodimer (41). Multiple ligands have been identified for human NKG2D (43), all of which are structurally related to HLA-I molecules, but lack peptide presenting capacity (43). NKG2D ligands can be up-regulated by cellular stress, and NKG2D is, accordingly, one of the "induced-self" receptors (6). In an evolutionary perspective, the multitude of ligands for NKG2D may reflect an immunological "arms race" between hosts and viruses (44): as a viral infection gives rise to cellular stress and hence NKG2D ligand up-regulation, some viruses, such as CMV, produce proteins that interfere with up-regulation of NKG2D-ligands (44). This has in turn led to NKG2D ligand gene multiplication and mutation events, circumventing this interference, leading the viruses to further adapt, and so on. As a result, animal species differ in the number of NKG2D ligands and extent of their diversity (44).

Examples of other receptors that sense "induced-self" are *DNAX accessory molecule 1* (DNAM-1) and the *signaling lymphocytic activation molecule* (SLAM) family of receptors.

DNAM-1 is an adhesion molecule and a member of the Ig-like family of glycoproteins (45). It is expressed on a majority of human NK cells. It is activating (45) and, like NKG2D, works as a co-activating receptor in humans (42). It signals through the adaptor molecule Grb2 (46) and needs to be physically associated to the leucocyte functional antigen 1 (LFA-1, see section 1.1.5) to be functional (47). DNAM-1 binds CD112 and CD155, which both serve as viral receptors (48) and are up-regulated upon cellular stress and on neoplastic cells (48,49). Of note, a number of inhibitory receptors with similar ligand binding profiles as DNAM-1 have been identified (18).

The SLAM family is a group of CD2-related receptors. In human NK cells, they generally signal through both the activating adaptors *SLAM Associated Protein* and *Ewing Sarcoma/FLI1 Activated Transcript 2* (EAT-2) (50). In cell types where these adaptors are not present, including immature NK cells, the receptors are instead generally inhibitory in nature, due to intracellular recruitment of phosphatases (50). Of the receptors in the family, CD48, CD84, CD244 (2B4), CD229 (LY9), CD319 (CRACC) and CD352 (NTB-A) are expressed by NK cells (VI). Apart from CD244 which binds CD48, the SLAM family of receptors interact homotypically (50). They are upregulated upon cellular activation (VI, 51) and accordingly contribute to recognition of "induced self".

NK cells also express the low-affinity Fc-receptor CD16 (FcγRIIIA) (52). This receptor is activating, through intracellular association with the *immunoreceptor tyrosine-based activating motif* (ITAM)-containing adaptor proteins CD3ζ and FceRγ (53). CD16 differs from all other NK cell receptors in that engagement of this receptor alone is sufficient to

induce release of cytotoxic granules (42). Thus, NK cells are likely to kill antibody-opsonized target cells. This process is called *antibody-dependent cellular cytotoxicity* (ADCC) and it provides a link between NK cells and the adaptive humoral immune system and to antibody-based immunotherapies (54,55).

1.1.3 Epigenetical reprogramming of NK cells

In the last decade, evidence has mounted that NK cells in certain situations can take on features of adaptive immunity. Early evidence for this was that certain CMV positive individuals had an NK cell pool that was strongly skewed towards subsets expressing NKG2C (35). Similarly, mouse NK-cells with a certain, antigen specific mouse CMV receptor, Ly49H, could mount a rapid and specific response upon a second challenge with the same virus (56). Moreover, different haptens and vaccines can induce long-lived liver NK-cell populations with a degree of antigen specificity and an ability to respond quickly upon re-challenge, without any specific receptor having been identified for these antigens (57).

In humans, many infections as well as malignancies may induce a further expansion of reprogrammed NK cells than CMV does by itself (58–62). In the case of $NKG2C^+$ reprogrammed NK cells, this effect is noted almost exclusively in CMV^+ individuals (58–62), suggesting that prior priming with CMV might be necessary for this specific reprogrammed NK cell population. Other herpes viruses do not seem to have the same effect as CMV and the data for herpes viruses is generally somewhat contradictory. Herpes simplex virus type 2 infections do not seem to skew the NK cell pool (63). In a study of NK cells from children being seropositive for one or both of *Epstein-Barr Virus* (EBV) and CMV, EBV seemed to further pronounce the $NKG2C^+CD57^+$ NK cell profile in CMV^+ individuals (VII). This result has been opposed by another study investigating CMV positive and negative young adults undergoing acute EBV infections (64). In this study, EBV infection did not alter the size of the CMV-induced NKG2C+CD57+ NK cell populations. This apparent discrepancy might be due to the different study setups. In the first study, the order of infections was not known. Although unlikely, it is not impossible that NK cells are instead primed by EBV, so that a secondary challenge with CMV results in more pronounced NK cell skewing. Furthermore, in the first study, coinfection with CMV and EBV might represent a proxy-marker for a generally higher infectious load. This could possibly be due to different living conditions, *e.g.* different number of siblings, a factor that is not controlled in this study. In that case, other infectious agents than EBV could potentially explain the difference between the groups. Contrarily, immune systems of different individuals become increasingly diverse with age

(65). One could thus speculate that the additional effect of EBV infection on skewing of the NK cell population in the second study is so small that it, with its total of 15 subjects is underpowered to reject the null hypothesis.

The phenotype and function of the expanded NK cell populations has been further characterized since the initial recognition of the CMV-correlated NKG2C^{+} NK cell populations. In patients undergoing hematopoietic stem cell transplantation where CMV reactivates, the NK cell pool becomes strongly skewed (66, VIII). Expanded NK cells from patients with CMV infection may express distinct KIR repertoires, including DAP12-signaling activating KIRs (67). Importantly, the NK cell expansions are not always NKG2C positive, and can be more widely defined by the down-regulation of different signaling adaptors, including FcERγ, EAT-2 and SYK (VIII). This phenotypical and functional switch is due to epigenetical reprogramming of the cells (VIII). The DNA methylation patterns of the expanded NK cells more closely resemble those of CTL than conventional NK cells (VIII). A key transcription factor involved is *promyelocytic leukemia Zink finger protein* (PLZF) (VIII). Notably, small populations of cells with a reprogrammed phenotype are also detected in a fraction of CMV negative individuals (VIII), possibly indicating that other infections can expand reprogrammed NK cell populations that might be phenotypically different from the populations induced by CMV.

The reprogrammed NK cells are less efficient killers of activated autologous CD4 T-cells than conventional cells but more potent cytokine producers upon Fc receptor engagement (VIII). Furthermore, they divide rapidly when co-cultured with HCMV-infected monocytes together with anti-HCMV antibodies (VIII). The reprogrammed NK cells are also less dependent on hematopoietic stem cell repletion than their conventional counterparts, pointing to a substantial capacity for self-renewal or a longer life span. Individuals with heterozygous mutations in *GATA2*, that due to haploinsufficiency display age-related progressive loss of both myeloid and lymphoid progenitor cells, retain reprogrammed NK cells also when all other NK cell compartments are depleted (IX). Furthermore, studies on paroxysmal nocturnal hematuria patients with hematopoietic, acquired somatic mutations in the *PIGA* gene, that lead to loss of glycosylphosphatidylinositol (GPI)-anchors in stem- and progenitor cells and all cells derived from these, show that reprogrammed NK cells, also long after the mutations have occurred, retain their GPI anchors, pointing to an important non-stem cell dependent survival mechanism (X). These observations defy the general notions that these reprogrammed NK cells are short-lived and/or that they have reached a stage of “terminal differentiation”, where they cannot divide.

1.1.4 $CD8^+$ T cells in general and cytotoxic $CD8^+$ T cells in particular

The primary role of $CD8^+$ T cells is to control that endogenous cells produce normal proteins. This is achieved through TCR recognition of MHC-I (in humans, HLA-I) molecules that express signal peptides from proteins generally produced in the same cell. The TCR complex is comprised of one TCRα/β, one CD3δ/ε and one CD3γ/ε heterodimer as well as one ζ/ζ homodimer (68). Upon ligand binding, the activating signal is transmitted through a number of ITAM motifs on the CD3 subunits (68), as the TCR chains do not possess any cytoplasmic signaling motifs (68).

The $CD8^+$ T-cells are often stratified into naïve, central memory, effector memory and cytotoxic subgroups based on their expression of CD45RA, CD28, CCR7 and effector molecules amongst others (69,70). Even though such models are based on the phenotypic subsets in blood and are not always congruent with findings associated in well-controlled infections, such as infections with live vaccines (70), an attempt to delineate a typical $CD8^+$ T cell activation and differentiation will follow.

Naïve $CD8^+$ T cells are cells that have undergone rigorous selection to be non-self-reactive, but have not so far been exposed to their antigen. They express the chemokine receptor CCR7. As the chemokines for this receptor is abundantly expressed in secondary lymphoid organs, naïve $CD8^+$ T cells in circulation always re-enter, or home, to these. In the secondary lymphoid organs, naïve $CD8^+$ T cells interact with many professional antigen presenting cells. Subgroups of professional antigen presenting cells can namely ingest non-endogenously produced proteins and present these on their HLA-I molecules, in a process called cross-presentation. When a naïve $CD8^+$ T cell interacts with a professional antigen presenting cell that displays a peptide that it recognizes and gets co-stimulation through CD28, it becomes properly activated and starts dividing, mainly creating effector cells. The effector cells have high levels of cytotoxic proteins and lack CCR7 expression, and therefore no longer home to secondary lymphoid organs, but can migrate to the site of inflammation and perform their functions. After the initial infection has ceased, a number of memory T cell subsets are retained, among which a subgroup in the blood has remaining cytotoxic potential, and are called effector memory cells. Upon a second stimulus with antigen, these memory populations rapidly expand and give rise to effector cells, creating a robust response, generally diminishing the clinical effect of the second infection.

In any individual, multiple developments from naïve cells to memory cells have taken, and are taking place, due to recently passed as well as chronic infections. In healthy individuals, there is for this reason a pool of cytotoxic $CD8^+$ T cells with different phenotypes present at any given time. In models of $CD8^+$ T cell differentiation, these are generally pooled

and denoted the effector-memory population. As a subpopulation of these cells re-express the naïve T cell marker CD45RA, they are often referred to as *effector memory T cells with CD45RA* or T_{EMRA}, which are considered the most differentiated. Yet, composition of the effector-memory population varies greatly between individuals (see Supplementary figure 1, study 2 for example). For this reason, we will herein not attempt to separate the different $CD8^+$ T cell populations with a cytotoxic potential, but instead denote them all *cytotoxic $CD8^+$ T lymphocytes*, or CTL. Further characterization of these cells for comparative and diagnostic purposes is provided in **Study 2**.

1.1.5 Shared traits among cytotoxic lymphocyte subsets

Even though cytotoxic lymphocytes use distinct receptors for their activation, their effector machinery, from integration of the activating signals to the release of the cytotoxic proteins, is largely similar.

Adhesion by integrins represent one of the first crucial steps in productive function of cytotoxic lymphocytes. Integrins are required for extravasation into tissues upon responses to infections, but also play important roles in establishing the contact site between the target cell and the cytotoxic lymphocyte, often denoted the "immune synapse". Integrins are heterodimers consisting of one alpha and one beta subunit. An integrin central to cytotoxic lymphocyte function is *leucocyte functional antigen 1* (LFA-1) (71). In LFA-1, both subunits have one knee, but the binding head is present in the alpha subunit (71). Integrins have three prototypic conformations. When the cell is not activated, the two subunits are bent and closely associated, generating a conformation with low ligand affinity. The molecule can also extend its knees, but retain low affinity, as the head is still bent down (71). However, if activating receptors are engaged, inside-out signals to the integrin induce a spatial separation of the cytoplasmic domains of the subunits that result in extension of the head, which increases the affinity for the ligand tremendously, in the case of LFA-1 10,000-fold (71). Interaction with multiple different activating receptors can generate this effect, and it occurs even if the receptor stimulus is not strong enough to induce release of cytotoxic granules (16). While strong stimuli can induce release of cytotoxic granules from cytotoxic lymphocytes also in the absence of LFA-1-ligand interactions, such release is poorly directed. LFA-1 is needed to recruit cytotoxic granules to the immune synapse, concentrating the release of their contents towards target cells, both increasing efficiency and decreasing bystander killing (16). Thus, LFA-1 plays a pivotal role in the effector functionality of cytotoxic lymphocytes.

Many other activating and inhibitory receptors associated with NK cells are also expressed on CTL, *e.g*. NKG2A, NKG2C and DNAM-1. NKG2A can partially inhibit, whereas NKG2C can potentiate TCR activation of T cell subsets expressing these receptors (72). DNAM-1 is widely expressed on CTL and functions as a co-activating receptor (45).

Cytotoxic lymphocytes also all express a set of proteins that confer cytotoxicity. These are retained in so-called cytotoxic granules, a type of secretory lysosome. Three of these proteins are *perforin*, *granzyme A* and *granzyme B*. Perforin acts to create pores in the membrane of the target cell (73), allowing entry of other apoptosis-inducing enzymes, such as the granzymes (73) and that in addition gives rise to cellular stress by itself. Upon entering the target cell, granzyme A mainly cleaves enzymes involved in DNA repair while granzyme B activates the caspase cascade (74).

In addition to having similar capabilities of responding with cytotoxicity upon activation, these cells also share the ability to rapidly produce and release large amounts of pro-inflammatory cytokines, such as TNF and IFN-γ when activated (75). They also recruit other cells by quickly releasing chemokines, such as CCL3 (MIP-1a) and -4 (MIP-1b) and -5 (RANTES) (76,77).

In summary, albeit typically defined as innate or adaptive lymphocytes, respectively, NK cells and CTL share intracellular mechanisms of signal integration and activation, adhesion, cytotoxic effector function and produce similar cyto- and chemokines.

1.2 IMMUNODEFICIENCIES AND THE EVOLUTION OF THEIR DEFINITIONS

For each uncommon, severe illness
there is a common, subtle counterpart

Traditionally, immunodeficiencies have been viewed as a group of rare, monogenetic defects with severe and early-onset clinical manifestations, resulting from the complete loss of a cell type or an immune function. Over time, mounting genetic evidence has challenged this view. Some investigators now argue that any disorder where the immune system plays a pathogenic role could be classified as an immunodeficiency (78). It has also been argued that, in the broadest sense, anyone being threatened by death by infection may harbor some form of immunodeficiency (78). Given the considerable variability in the human genes involved in immunity and especially as at least 100 genes are estimated to be deleteriously mutated at one allele in any healthy individual (79), it is hypothetically possible that for each human, there

might be a pathogen strain with the exactly right combination of virulence factors that during an infection would cause a gap between the otherwise overlapping immune functions, generating a situation-specific primary immunodeficiency.

In the case of lymphocyte cytotoxicity, the prototypic associated immunodeficiency syndrome is termed *hemophagocytic lymphohistiocytosis* (HLH). This syndrome is, in its familial forms, caused by a complete loss of cytotoxic function (80). Typically, when presented with a viral infection early in life, the familial HLH patients' inability to clear the virally infected cells and control the ensuing immune response leads to a state of hyper-inflammation. In healthy individuals, such a hyper-inflammatory state would be terminated by cytotoxic killing of hyper-activated immune cells, but due to the general lack of cytotoxicity, the immune system spirals out of control, leading to multi-organ failure and ultimately death if treatment with cytostatic drugs is not initiated (81). Presently, the only cure for familial HLH is allogeneic hematopoietic stem cell transplantation (82). There are multiple monogenetic causes for familial HLH. Whereas familial HLH type 2 is caused by mutations in *PRF1*, encoding perforin, the other known mutations affect genes of proteins required for trafficking and release of cytotoxic granules (80).

In line with the rest of the immunodeficiency field, the notion of possible clinical presentations caused by defects in lymphocyte cytotoxicity is also changing. Although mutations in the genes causing familial HLH leading to complete loss of protein function generally have complete, early disease penetrance, patients with mutations in *STXBP2* can show gastrointestinal problems and mild bleeding disorders in childhood but not present with HLH until adolescence (83). Moreover, patients with missense mutations in *PRF1* leading to reduction of function have been shown to have variable presentations, including hematological malignancies, cytopenias, systemic autoimmunity and gastro-intestinal symptoms (84). Some patients with *PRF1* missense mutations might not develop HLH or not have any symptoms at all (84). These studies and others have led to the establishment of a spectral view of mutations affecting lymphocyte cytotoxicity, where complete loss of function results in HLH, whereas reduction of function can cause variable phenotypes (85). This, in turn, has led to an increasing interest in studying disorders where a partial defect in cytotoxic lymphocyte function might be suspected.

1.3 THE DILEMMA OF THE OPTIMAL EXPERIMENT

Reproducible experiments are the cornerstone of all empirical research. When attempting to study common, and potentially subtle, counterparts to the uncommon severe

immunoregulatory disorders, there is an additional need for highly sensitive experiments. Still, weighing these two factors is not always straight-forward; assays that are sensitive are not per definition also reproducible. This is exemplified by the establishment of functional cytotoxic lymphocyte assays utilizing antibodies specific to the open conformation of the integrin LFA-1 (86, XI). The conformational change of integrins to their open, high affinity state is a read-out of very early activation of cytotoxic lymphocytes. As LFA-1 is expressed at high levels on all cytotoxic lymphocytes, this conformational change is readily detectable, rendering the assay highly sensitive to cytotoxic lymphocyte activation. Yet, this sensitivity causes low robustness: as the conformational change of LFA-1 can occur even after very weak activating signals, subtle experimental variations can lead to large differences in the amount of activation that is seen. This means that the signal-to-noise ratio, indicated by the z-factor (87), varies greatly even between batches in the same experiment (Supplementary table 1, **Study 1**).

CD107a expression on NK-cells after stimuli, which is a central assessment in the cellular diagnostics of cytotoxic immunodeficiency syndromes, often shows a suboptimal signal-to-noise ratio (Supplementary table 1, **Study 1**), the reasons for which are not thoroughly established. In the cases where complete defects in exocytotic capacity is sought after, this does not present a problem, as the signal is always strong enough to provide a highly specific and sensitive difference between patients and controls (88). Still, in some instances, when NK cells are scarce, or if the aim is finding more subtle, partial defects in cytotoxicity, this variability becomes problematic. One potential source of variability in this context is the intake of pharmacological substances, many of which act on widely expressed receptors. Investigating the effect of pharmacological substances in clinical use on NK cell functions might thus contribute to understanding the variability. This represented the rationale behind **study 1**. In addition, identifying other primary human cytotoxic lymphocyte populations with a more robust CD107a expression upon stimulation might also prove useful in a diagnostic setting, which is the aim of **study 2**.

1.4 MYALGIC ENCEPHALOMYELITIS/CHRONIC FATIGUE SYNDROME AND THE CONNECTION TO DEFECTS IN LYMPHOCYTE CYTOTOXICITY

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a syndrome based on a number of clinical criteria. The main characteristic is post-exertional neuro-immune exhaustion (89)*, i.e.* that ME/CFS patients after physical or psychological stress experience

flares. These flares are commonly characterized by influenza-like symptoms and neuro-hormonal disturbances in addition to severe fatigue and typically go on for days to weeks (89). In a majority of ME/CFS cases, there is a connection between the onset and infections. In a prospective study in 196 primary care centers in Australia, all patients presenting either with infections by EBV, Ross River Virus or the intracellular parasite *Coxiella burnetti*, or having typical infectious symptoms, but without an identified agent, were followed with diagnostic ME/CFS questionnaires over time. After 6 month, 11% of either group of patients fulfilled the criteria for ME/CFS. There was no correlation to any specific agent, but rather a correlation to the severity of the initial disease episode (90). There are also numerous reports on epidemics of ME/CFS, both in direct association with known epidemics and where no associated pathogen has been identified (91–93). As cytotoxic lymphocytes are implicated in the defense against intracellular infections, they were hypothesized to be involved already when the syndrome was defined (94,95). Multiple studies have shown that cytotoxic lymphocytes are indeed abnormal as measured by lower killing of target cells or lower perforin expression, or that they are less abundant than in healthy individuals (94–96). Yet, negative results have conflicted the field (97,98), and critical reviews have even argued that there is a negative correlation between the overall quality of the studies and the number of findings (99), suggesting a publication bias. Still, as patients with mutations in *STXB2*, if not previously exposed to EBV, can get severely ill by mononucleosis in adolescence (83) and other, more subtle symptoms may arise in patients with missense mutations in familial HLH genes (84), it seems possible that a subgroup of ME/CFS patients indeed have a mild defect in lymphocyte cytotoxicity that may explain their symptoms. Moreover, as infections in general and CMV in particular are known to trigger epigenetically altered expansions of NK cells, and these changes in turn render the cells less efficient immune modulators and more targeted towards combatting infection (VIII), certain cases of ME/CFS could hypothetically be explained by excessive expansions of such reprogrammed NK cells. Finally, as patients with ME/CFS show imbalances in the hypothalamus-pituitary-adrenal axis (100) and altered catecholaminergic responses (101,102), their hypofunction in NK cells could also be due to a pathological interplay between the humoral stress response and the cellular immune responses.

In summary, thorough investigations of cytotoxic lymphocyte functions in ME/CFS are warranted. This is the focus of **study 3**.

1.5 ROLES FOR NK CELLS IN SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder. Amongst the more well-known symptoms of SLE are sun sensitivity and a typical butterfly-shaped rash of the face, that occurs in 30-60% of the cases. Other organ systems often affected are the kidneys, joints and the nervous system, but most organs can be affected in this heterogeneous disorder. Typical for SLE is a development of a wide range of autoantibodies, amongst which the most clinically screened for are so called anti-nuclear antibodies directed towards multiple disparate nuclear antigens. Albeit not specific to SLE patients (103), ANA are in the context of SLE hypothesized to arise as a response to an imbalance between apoptosis and clearance of apoptotic cells (104). The importance of this imbalance is underscored by the identification of monogenetic defects in the complement system leading to early-onset SLE (104). Amongst the different ANA, antibodies binding RNA are typical for SLE (103). These give rise to *RNA-antibody immune complexes* (RNA-IC), which contribute to the SLE pathogenesis in various ways, including aggregating in the kidneys, causing damage (105). In total, more than 180 different autoantibody specificities have so far been described, which is more than in any other autoimmune disorder (106).

A phenomenon that is associated with the pathology of SLE is increased levels of IFN-α. While most human cells can express small amounts of IFN-α in response to IFN-α stimuli, the main *a priori* IFN-α producers are plasmacytoid dendritic cells (pDC) (107). A strong stimulus for pDC is RNA-IC, which is detected mainly via TLR7 (104). Intriguingly, if pDC can interact with NK cells *in vitro*, their production of IFN-α upon stimulation with RNA-IC is potentiated a 1,000-fold (108). This interaction is in part mediated by soluble factors, such as CCL4, but is also LFA-1 dependent (108). The SLAM family members CD229 and CD319 are possibly also involved in the pDC-NK cell interaction as they are up-regulated on pDC and NK cells after *in vitro* co-incubation with RNA-IC (VI). The activating adaptor molecules SLAM Associated Protein and EAT-2 are not expressed in pDC, likely rendering these receptors inhibitory (VI). The possible interaction between NK cells and pDC through SLAM family receptors might be dysregulated in SLE, shown by lower baseline SLAM family receptor expression in SLE patients (VI) and the genome-wide association studies linking the 1q23 locus, where the SLAM genes are present to SLE (109). Analogous findings have also been made in mice (110).

Patients with SLE generally have fewer NK cells with lower cytotoxic capacity, and an activated NK cell phenotype (111). They also express higher levels of NKG2A and NKG2C as well as certain KIR receptors and react with stronger pro-inflammatory cytokine production upon stimulation (111). This, in combination with their capacity in potentiating

IFN-α production from pDC, has led to the hypothesis that NK cells are involved in the pathogenesis of SLE. Underscoring this, cases with very early-onset SLE have been reported in patients with biallelic missense mutations in perforin (84). Furthermore, amongst the few monogenetic causes of SLE-like disorders, mutations in *FASLG*, encoding Fas ligand, and *fas*, encoding the Fas receptor, are present in SLE patients and mouse models, respectively (104). Fas ligand is in resting state primarily expressed in cytotoxic lymphocytes, which can induce apoptosis by engaging Fas on target cells (112).

Further building on the hypothesis of an NK cell involvement in SLE pathogenesis, study **4** and **5** aims at investigating the functional impact of autoantibodies to NKG2A and -C receptors in SLE patients.

2 ETHICAL CONSIDERATIONS

All studies in this work have been performed with human cells. For this reason, ethical approval has been obtained before the start of each study. In the cases where blood from healthy human donors has been used, informed, oral consent has been received. As these individuals were de-identified, no results have been reported back to them. For all experiments involving patient material, written informed consent was obtained.

In the case of **Study 3**, there was an interest from a few patients in retrieving their personal study results. This led to thorough discussions due to an ethical dilemma: in accordance with the patient information, the individual patients had the right to retrieve the personal results if these could improve the clinical outcome of the individual. On the other side, the results of an individual are generally hard to interpret, both as normal intervals are lacking and as a seemingly "pathological" phenotypes or functional responses are, in most cases, very hard to directly associate to clinical symptoms. In the end, the patients urging to know their results have gotten an interpretation of these, coupled with information regarding the uncertainty of the results. The interpretation was performed by Dr Yenan Bryceson and myself together in each instance to leave minimal room for over-interpretation. In one case, where the p.A91V variant of perforin was present in a homozygous form, the patient was actively contacted and referred to clinical genetics, to further explore the possible influence of this perforin variant in the clinical course of the disease for this specific patient.

3 INTRODUCTION TO METHODS AND MATERIALS

3.1 MULTICOLOR FLOW CYTOMETRY

For the non-flow cytometrist, a short introduction to this technique might be necessary to follow the experimental work in these studies. Flow cytometry is the art of investigating individual cells in suspension, by fluxing them through a thin chamber, wherein their characteristics can be assessed using electrochemical methods. Most commonly, the cells have been pretreated by incubation with fluorophore-conjugated antibodies that bind specific antigens on the cell surface or in intracellular compartments. Fluorophores are fluorescent biomolecules. When cells covered with such antibody-fluorophore conjugates flow through the detection chamber of the flow cytometer, they are illuminated with lasers that excite the fluorophores, that subsequently emit specific signals that are captured by detectors. With this method, the strength of the signal emitted by a certain fluorophore corresponds to the amount of bound antibody-fluorophore conjugates on the cell, which by itself is a proxy marker for the amount of protein. Due to the discovery of a multitude of fluorophores, up to 30 such assessments can be done simultaneously for one cell, and thousands of cells can be investigated every second. This efficient generation of complex data has rendered flow cytometry a vital tool for immunologists, but it has been made possible partially on the cost of robustness. First, the method is sensitive to day-to-day variation in the optics of the flow cytometer itself, but also to the state of the antibody-flurophore conjugates, on top of the variability caused by slight perturbations of the experimental procedures preceding the flow cytometry assessment. Secondly, standards to calculate how much protein a certain signal corresponds to are hard to obtain. Consequently, flow cytometry is best suited for extensive experiments on small sets of patients and controls (<100) analyzed together.

3.2 PERIPHERAL BLOOD MONONUCLEAR CELLS

In almost all the experimental work in this thesis, peripheral blood mononuclear cells, or PBMC, are used. PBMC are made up of lymphocyte and monocyte subsets mainly. PBMC are obtained from whole blood by density-gradient centrifugation, utilizing the lower density that PBMC show compared to red blood cells or neutrophils. The isolation is performed to enable long-term experiments, freezing of cells and to reduce the effect of the external environment but not completely exclude the physiological possibility of cell-cell interactions.

4 RESULTS AND DISCUSSION

4.1 STUDY 1

A step towards controlled constancy lies in
understanding the grounds of variability

A common, yet often underestimated source of bias in functional immunological studies is pharmacological treatments. A majority of available drugs show significant polypharmacology (113) and even recently developed specific inhibitors also exhibit off-target effects. Most patients with suspected immunodeficiencies are treated with multiple drugs. In cases where these drugs affect lymphocyte cytotoxicity, it might contribute to the pronounced inter-donor variability in cytotoxicity assays with clinical material. Lymphocyte cytotoxicity is known to be affected by multiple pharmacological treatments *in vitro* (114–116) and *in vivo* (117,118). However, systematical screening of the effect of in-use compounds on primary human cytotoxic lymphocyte functions has not previously been performed.

In an attempt to overcome this, **Study 1** was conducted. Here, the impact of 1,200 in-use or previously used drugs on NK cell functions was investigated. First, PBMC isolated from one donor the previous day and rested overnight was pre-incubated with each drug individually. After 15 minutes, an anti-CD16 antibody as added to all PBMC-drug co-cultures, and the cells were incubated for one hour, followed by staining and flow cytometry. Of the 1,200 drugs tested, 56 and 12 were identified as inhibiting and activating NK cell function, respectively.

In this study, the chosen screening concentration was 5 μM. This concentration is lower than the standard 10 μM (Thomas Lundbäck, personal communication and (119)) and it was agreed upon as a trade-off between ensuring that a number of hits would be identified and that toxicity would be limited. In hindsight, a lower screening concentration would possibly have been preferable as 5 μM is unphysiologically high for most substances, which might result in α– and β–errors. The α–errors were dealt with within the study, as a titration was performed for all hits. At 0.2 μM, 29 compounds retained more than 50% of their 5 μM activity, all of which were inhibitors (Table 1). The problem of β–errors is less well dealt with within the study. The high concentration of the substances namely resulted in significant toxicity or base line shifts for 26 substances, which were excluded from further testing (see

Fig 2, Study 1). It is plausible that a fraction of these would have shown significant inhibitory or activating capacity at lower concentrations.

Of the 29 inhibitors with retained significant activity at 0.2 μM, all but naftifine have been previously associated to inhibition of lymphocyte cytotoxicity (Table 1)(114–116,120–123). It is worth noting that of all the 29 substances with strong inhibitory activity in the sub-micromolar range, 19 had effects dependent on cAMP increase, underscoring the vital inhibitory role for cAMP in cytotoxic lymphocytes. This finding might also underscore that the selected hits in this assay are profoundly dependent on direct interference with activating signaling, which is likely a bias introduced by the short time-frame of the assay. If the drug pre-incubation time had been extended to a few hours, substances with effects on transcription, such as corticosteroid-derivatives would likely have been identified as hits (124). Of note, a majority of the substances identified in the screen, such as β-adrenergic agonists and histaminergic antagonists, influence signaling pathways that are known to be important under physiological, non-pharmacologically influenced conditions. This was all taken into consideration in later work where *understanding the grounds of variability* turned out to be important (see **Study 3**).

Table 1. Substances in **Study 1** with retained activity at 0.2 μM

Name	Pharmagological group	Likely mechanism	Effects previously reported on lymphocyte cytotoxicity
Isoetharine	ADRB2 agonist	cAMP increase	Yes
Dipivefrin	ADRB2 agonist	cAMP increase	Yes
Metaproterenol	ADRB2 agonist	cAMP increase	Yes
Terbutaline	ADRB2 agonist	cAMP increase	Yes
Racepinephrine	ADRB2 agonist	cAMP increase	Yes
(-)-Isoproterenol	ADRB2 agonist	cAMP increase	Yes
Formoterol	ADRB2 agonist	cAMP increase	Yes
Levalbuterol	ADRB2 agonist	cAMP increase	Yes
Fenoterol	ADRB2 agonist	cAMP increase	Yes
Clenbuterol	ADRB2 agonist	cAMP increase	Yes
Salmeterol	ADRB2 agonist	cAMP increase	Yes
Ibudilast	PDE4I	cAMP increase	Yes
Zardaverine	PDE4I	cAMP increase	Yes
Rolipram	PDE4I	cAMP increase	Yes
Ethaverine	PDE-inhibitor	cAMP increase	Yes
Misoprostol	prostaglandin	cAMP increase	Yes
Alprostadil	prostaglandin	cAMP increase	Yes
Desloratadine	HRH1 inhibitor	PLC inactivity (?)	Yes
Chlorprothixene	phenotiazine	Calmodulin inhibition (?)	Yes
Norcyclobenzaprine	phenotiazine	Calmodulin inhibition (?)	Yes
Zuclopenthixol	phenotiazine	Calmodulin inhibition (?)	Yes
Thiethylperazine	phenotiazine	Calmodulin inhibition (?)	Yes
Moricizine	phenotiazine derivative	Calmodulin inhibition (?)	Yes
Clomipramine	Tricyclic antidepressant	cAMP increase possible	Yes
Sertraline	Specific serotonin reuptake inhibitor	cAMP increase possible	Only indirectly
Nisoldipine	L-type Ca channel blocker	Likely Ca-dependent	Yes
Naftifine	fungal ergosterol synthesis inhibitor	Not clear	No
Tracazolate	GABAA-inhibitor	Not clear	Yes
Cyclosporin A	Calcineurin inhibitor	Calcineurin inhibitor	Yes

4.2 STUDY 2

The subtle in the macrocosm may be discerned by
finding the robust in the microcosm

As discussed in the introduction, an alternative to NK cell degranulation for diagnostic purposes of immunodeficiency syndromes involving lymphocyte cytotoxicity has been sought for. In clinical practice, stable, widely distributed flow cytometers were for long limited to four simultaneous assessable markers. As the three markers CD3, CD56 and CD107a had already been included in the diagnostic panel for evaluation of lymphocyte cytotoxicity, attempts at identifying a fourth marker that could differentiate a diagnostically useful subpopulation was made. This led to the early testing of $CD3^+CD8^+$ T cell CD107a expression as an additional marker (88). However, investigating CD107a expression in bulk, unactivated CD8 T cells did not prove to give sufficient signal-to-noise ratio, likely due to inter-donor variations in the size of the cytotoxic fraction of total $CD3^+CD8^+$ T cells. To circumvent this, strategies investigating CD107a expression in CD8 T cell blasts came into practice, but have not been widely implemented due to large day-to-day variability and insufficient blast generation in samples from immune-suppressed patients (88). Establishment of a robust and preferably simple marker setup for definition of CTL for diagnostic settings could thus potentially prove to be an example of *finding the robust in the microcosm.*

To attempt this, **Study 2** was undertaken. Here, it was shown that among a number of investigated extracellular markers, CD57 was most strongly positively correlated to expression of cytotoxic proteins, in line with previous studies (125). When comparing $CD8^+CD57^+$ T cells to NK cells, $CD8^+CD57^+$ T cells expressed cytotoxic proteins to the same extent, and upon stimulation they released cytotoxic granules, TNF and IFN-γ in higher and more reliable numbers than NK cells did. Finally, it was shown that for familial HLH patients, the same pattern of loss or reduction of cytotoxic function was seen in $CD8^+CD57^+$ T cells as in NK cells.

That CD57 is a useful marker for cytotoxic lymphocytes has been clear since its identification in 1981 (126). Some early definitions of cytotoxic lymphocytes even used CD57 as the only defining marker, thus not separating NK and CTL (127). With this in mind, it was attempted to substitute CD8 with CD57 in the diagnostic definition of CTL, which indeed was more useful than CD8 alone (see supplementary figure S4, Study 2).

After the publication of Study 2, it has been shown in clinical materials that the sensitivity and specificity of identifying primary defects in the release of cytotoxic granules

by investigating degranulation by $CD3^+CD8^+CD57^+$ T cells is 97 and 95 percent, respectively (128), making it, by itself, superior to investigating NK cell degranulation. The combination of the two leads to even further increases in diagnostic usefulness (Samuel Chiang *et al*, manuscript in preparation), providing evidence that investigating $CD8^+CD57^+$ T cells for diagnostic purposes in the contexts of immunodeficiencies involving lymphocyte cytotoxicity indeed represents something *robust in the microcosm.*

4.3 STUDY 3

For each uncommon, severe illness
there is a common, subtle counterpart

The identification of missense mutations in cytotoxicity genes giving rise to milder phenotypes (84) and the establishment of robust cytotoxicity evaluations, led to an interest in identifying syndromes that could represent an example of a common, subtle counterpart to familial HLH. As discussed in the introductory section, with its connection to viral infections and compelling but inconclusive data on NK cell aberrances, ME/CFS seemed to be a suitable candidate syndrome for this purpose. Therefore, **Study 3** investigated cytotoxic lymphocyte function and phenotype in ME/CFS patients. To study this patient group and the subtleties that were hypothesized to separate them from controls, samples from all study subjects were simultaneously assessed, to avoid batch effect. Further, to increase the external validity of the findings, a setup with two substudies was chosen, where all findings identified in samples from one cohort were validated in samples from another cohort.

In this study, no reproducible differences were noted in the number of cytotoxic cells in whole blood, the cytotoxic granule constituents, nor in the ability to release cytotoxic granules. Further, no differences were noted in the killing or the cytokine production capacity of the cells.

As a link between the acquisition of the syndrome and viral infections was established (90) and as NK cells in certain individuals had been shown to be epigenetically reprogrammed by viral infections, which dampened their immunoregulatory capacity (VIII), it was hypothesized that the noted hypofunction could be due to aberrances in the sizes and frequencies of such reprogrammed NK cell compartments among the ME/CFS patients. This was tested and turned out not to be the case.

When all tested inherent, direct functions of the cytotoxic lymphocytes had been assessed and turned out normal, a thorough review of the methodology in the previous NK

cell reports in the field was conducted (see Supplementary Table 4 of **Study 3)**. From this overview, it was clear that all previous functional NK cell experiments had been conducted in either whole blood or directly after isolation from whole blood, thus allowing for serum factors to influence the functions. One compelling group of such factors is catecholamines. Numerous reports, including **Study 1,** have shown that cytotoxic lymphocytes in general, and NK cells in particular, are inhibited by stimulation through the β2-adrenergic receptor (114). In addition, ME/CFS patients have been shown to have abnormal catecholaminergic stress responses (102). Considering this, it was investigated whether NK cells from ME/CFS individuals responded differently to a common stimulus, in this case anti-CD16 antibody stimulation, during suppression of functions by co-incubation with adrenaline. Indeed, a difference in response, where the ME/CFS patients showed less inhibition and hence stronger responses towards anti-CD16 antibodies was seen among the patients in the first substudy. Due to experimental error, this experiment could not be validated in the second substudy, but our findings substantiate those of a previous study (101). To further explore the possible effects of serum factors on cytotoxic lymphocyte function in ME/CFS patients, serum-transfer experiments could be conducted, where PBMC from healthy individuals would be co-incubated with serum from patients and from controls. If the serum from the patients indeed would dampen lymphocyte cytotoxicity, selected antagonists towards receptors likely to contribute to this inhibition could be used in the co-cultures. If such an antagonist could reverse the effects of the serum, the serum factor responsible for dampening of ME/CFS patient lymphocyte cytotoxicity could be identified.

As no differences were found on the group level, the focus was instead turned to a search for subgroups or single individuals with aberrant phenotypic or functional traits. No subgroups were visual in any single immune parameter. One individual was identified that expressed lower levels of perforin in all investigated cell subsets, which was explained by biallelic expression of a common variant of the *PRF1* gene, p.A91V. After this individual had been identified, a screen was performed for this variant in 41 of the study patients where DNA was available. This showed that the overall allele frequency of this variant was in normal range (7.3%), compared to a population study with 30,000 European individuals (4.7%) (129). In the population study, the homozygous carriership frequency was 1 per 450 individuals (129). This is the expected frequency according to the Hardy-Weinberg principle (130), minimizing the risk of a selection bias excluding sick homozygous carriers of p.A91V among the 30,000 individuals. Still, monoallelic and biallelic expression of this gene variant is associated with a risk of developing malignancies (Löfstedt *et al,* submitted) and homozygous carriers occasionally present with HLH-like symptoms (84). Notably, in a case

report, a patient homozygous for the *PRF1* p.A91V variant acquired HLH during a tuberculosis infection (131). This patient had a monozygotic twin that did not suffer from tuberculosis and did not get HLH (131). Thus, the penetrance and the clinical presentations are variable, and with only one ME/CFS patient in the study showing this variant, it cannot be confirmed that it is implicated in the etiology of the disorder for this individual patient. Yet, this finding warrants population-based studies investigating a possible correlation between biallelic expression of the *PRF1* p.A91V variant with symptoms associated with ME/CFS.

In a final attempt to detect subtle differences between the groups, all common immunological parameters to both substudies were investigated together using a supervised dimensionality reduction approach. The used method, called *sparse partial least squares discriminant analysis* (132), identifies the vector through a multidimensional data cloud that maximally discriminates the pre-defined groups (in this case patients and controls). It then uses a penalty to exclude immunological parameters that do not contribute to this discriminant vector. This analysis was done for each substudy individually. After this, the resulting discriminant vectors were reconstructed for the other substudy in a cross-over manner, and used for prediction purposes. With this approach, the optimal separation was only between 60 and 70 percent sensitive and specific for the identification of individual patients, meaning that the groups are practically inseparable with the data from this study. Notably, though, only immune parameters that were present for a majority of patients and controls from both substudies were included in this analysis. Amongst others, this excluded the NK cell killing as well as the adrenergic inhibition assays. As the latter showed a significant trend towards separating the patients from controls by themselves, it is not impossible that adding these would have improved the sensitivity and specificity.

Taken together, the results of this study did not identify reductions in cytotoxic lymphocyte function in all or a subgroup of ME/CFS patients. Nonetheless, as one patient identified by the screening was homozygous for a *PRF1* variant with connections to HLH, it is possible that a small fraction of ME/CFS patients may indeed represent a *subtle counterpart* to familial HLH, but this needs to be further studied in larger materials. Moreover, with the hints towards a difference in the response to adrenergic stimuli, integrating the knowledge of how pharmacological substances and serum factors impact lymphocyte cytotoxicity may have contributed to *understanding the grounds of variability,* in this case the variability between studies. Lastly, by utilizing *the robust in the microcosm,* in this case the set of reproducible assays, we can, with some confidence, confirm that lymphocyte cytotoxicity is not generally reduced in ME/CFS.

4.4 STUDIES 4 AND 5

The regular nature of an illness may be understood
by exploring its rare causes

Being a classical autoimmune disorder, systemic lupus erythematosus represents another angle to the connection between primary immunodeficiencies and dysfunctions in lymphocyte cytotoxicity. A curious link between SLE and cytotoxic lymphocyte function is **Study 4** that presents a patient with SLE harboring autoantibodies directed against NKG2A. This autoantibody was identified *en passant* during screening efforts to establish receptors implicated in the interaction between NK cells and plasmacytoid dendritic cells. When incubating NK cells with RNA-immune complexes (RNA-IC) with IgG obtained from this specific SLE patient, all NKG2A staining suddenly disappeared. The IgG solutions were in this case obtained from a plasmapheresis that the patient had undergone during a severe SLE flare. It was established that this effect was due to an autoantibody directed against NKG2A. This autoantibody blocked CD94/NKG2A binding of HLA-E, but did not interfere with CD94/NKG2C-HLA-E interaction. The abolishment of NKG2A-HLA-E interactions also had functional implications, as $CD56^{bright}$ and $CD56^{dim}NKG2C^{-}$ cells, that are enriched for $NKG2A^{+}$ cells, were not inhibited by HLA-E expression on target cells in the presence of IgG from the SLE patient. Furthermore, the presence of the autoantibody correlated with the SLE disease activity index (Spearman correlation 0.78). Thus, this NKG2A autoantibody seemed to underscore a potential role for NK cells in the SLE pathology.

To further explore the presence of NKG2-autoantibodies in SLE, **Study 5** was undertaken. Here, a screen of 212 SLE patients and 90 controls was performed. In this study, six additional patients harboring autoantibodies directed against NKG2A were identified, two of which showed cross-reactivity with NKG2C. For four of the patients, blocking of HLA-E-NKG2A interactions was observed, whereas the serum from one patient enhanced HLA-E binding of both NKG2A and NKG2C. In accordance with their specificities, the different autoantibodies showed different functional profiles. When the cells from four of the patients were investigated, two showed unusually low NK cell numbers (1.9 and 2.3%, respectively), whereas two had normal numbers, but did not show any cells expressing NKG2A or NKG2C. As this could be due to either detection errors due to the presence of autoantibodies, or opsonization and subsequent deletion of NKG2A or NKG2C positive cells, the levels of NKG2A and NKG2C mRNA transcripts was measured in bulk isolated NK cells. The mRNA

levels were comparable between patients and controls, suggesting that the effect might be covering of the epitopes leading to detection error. Additional experiments however showed that cell lines expressing NKG2A or NKG2C were selectively killed by IL-2 activated PBMC when co-incubated with sera containing the specific autoantibodies, in favor of the hypothesis that the NK cells expressing NKG2A or NKG2C could be opsonized and killed. Clinically, the small subgroup of SLE patients expressing these autoantibodies had a more severe disease course than other SLE patients. The presence of autoantibodies also seemed to be more common during flares of the disorder for individual patients, but this was not statistically verified overall (Spearman correlation between the level of NKG2A antibodies and SLE disease activity index for all patients together was 0.25).

Notably, in 2008 a patent was established for the use of Monalizumab, a humanized anti-NKG2A antibody, as treatment for malignancies and autoimmune disorders (133). The rationale behind this was that blocking NKG2A would lead to dis-inhibition of NK cells and congruently to increased killing of neoplastic cells or more efficient immunoregulation, which is in line with studies of administration of NKG2A antibodies in mouse multiple sclerosis models (134). Early results indicated a good safety profile for patients with rheumatoid arthritis (unpublished data, but see (135)). Subsequently, the autoimmune indications have been dropped, but Monalizumab is currently being tested in clinical trials for a variety of malignancies (136). At a first glance, it might seem contradictory that an autoantibody with possible negative impact on the clinical outcome of a systemic autoimmune disease might be clinically useful and safe to administer in other related and unrelated syndromes. However, although the binding profile of Monalizumab and the NKG2 autoantibodies are closely related, the Ig subclass is IgG4 for Monalizumab and IgG1 and IgG3 for the autoantibodies. This means that the Fc binding profile will be very different between the antibodies; whereas IgG1 and IgG3 binds all Fcγ receptors, IgG4 is bound only by a fraction of Fcγ receptors and generally with lower affinity. For example, only certain allele variants of FcγRIIIA/CD16 binds IgG4 at all (137). This will result in a lower risk of ADCC towards NKG2A expressing cells when Monalizumab is used compared to the situation with the autoantibodies. With this in mind, it might be argued that the most important way that the autoantibodies towards NKG2A might contribute to pathology is by clearance of NKG2A positive cells, but this would need to be further proven.

Taken together, the results of Study 4 and 5 show that a fraction of SLE patients possess autoantibodies to NKG2 receptors. These autoantibodies are, at least in certain individuals, correlated to clinical symptoms and have functional impact on a cellular level, possibly representing *a rare cause* of SLE flares. These findings warrant further studies of NK cell

involvement in SLE. One current path of follow up is widening the screening efforts to detect autoantibodies directed against other NK cell receptors, especially KIRs. This would be in line with previously reports (138) and preliminary data on patients in the already screened group indicate presence of such autoantibodies (139).

5 CONCLUSION

Currently, the number of identified disease-correlated immunophenotypic aberrances is increasing rapidly. Still, a causal link between patient symptoms and immunologic aberrances is seldom identified. This inevitably leads to a growing gap between the number of possible and confirmed immunodeficiencies. To narrow this gap, methodologically sound, and carefully interpreted studies on immune cell phenotype and function on well-defined patient groups are needed.

In this work, Study 1 and 2 contribute to establishing a framework for assessing and interpreting the function of cytotoxic lymphocytes from patients with potential defects in cytotoxicity. Study 1 identifies a set of clinically used molecules with acute effects on cytotoxic lymphocyte function, and as a by-effect underscores the pivotal role of β-adrenergic stimuli in the regulation of cytotoxic lymphocyte function. Study 2 instead establishes CD57 as a robust identifier for cytotoxic T-cells in diagnostic experimental setups. The last three studies investigate cytotoxic lymphocyte function in patient groups. In Study 3, no evidence for a general primary defect in cytotoxic lymphocytes from myalgic encephalomyelitis patients is found. In Study 4 and 5, a subgroup of patients with systemic lupus erythematosus are identified that express peculiar autoantibodies directed against cytotoxic lymphocyte receptors, which are shown to have functional implications.

In conclusion, these studies attempt to narrowing the gap between the suspected and confirmed immunodeficiency syndromes. In that, they arguably all, by their own means, contribute to the *gaining of salutogenetic insights by finding the traits defining,* or not defining, *groups of ill.*

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Detta arbete söker på olika sätt belysa hur nedsatt funktion hos en grupp immunceller, kallade cytotoxiska lymfocyter, kan tänkas inverka på förloppet vid olika sjukdomstillstånd. De cytotoxiska lymfocyterna har den huvudsakliga uppgiften att identifiera och döda infekterade, cancerösa eller överaktiverade celler i kroppen, så att dessa problem inte sprids vidare. Om de cytotoxiska lymfocyterna inte fungerar som de ska, kan det följaktligen leda till att infektioner eller cancer sprids, eller att autoimmuna problem får fäste. Det är fördjupad förståelse av dessa cellers roll i olika sjukdomar som står i fokus för denna doktorsavhandling. Alla delarna i avhandlingen utgår i grunden från ett forskningsmässigt tillvägagångssätt där patienter med liknande symptom grupperas och deras immunförsvar jämförs dem emellan och med friska kontroller. När systematiska avvikelser identifieras hos patienterna i en sådan grupp, görs försök att koppla samman dessa med utvecklingen av de symptom de har gemensamt. När ett sådant samband kan säkerställas, innebär detta dels att den identifierade avvikelsen kan bli mål för behandlingar. Det bidrar dock också till att öka förståelsen för hur immunförsvaret arbetar med att skydda oss andra från att insjukna på samma sätt som patienterna med den identifierade avvikelsen. Detta sammanfattas i den första av fem maximer som kondenserar de mest väsentliga underliggande tankarna bakom detta arbete:

Salutogenetisk insikt (kunskap om vad som gör oss friska)
kan nås genom att finna drag definierande varje grupp sjuka

En kort resumé av studierna:

Den första studien handlar om hur 1500 vanligt förekommande läkemedel påverkar de cytotoxiska lymfocyternas funktioner. Flera grupper av läkemedel identifieras som har extra stor påverkan, av vilka den dominerande gruppen är adrenalinrelaterade substanser. Medvetenhet om dessa läkemedels inverkan på cellfunktionen är viktig att ha i tolkning experimentella data från såväl läkemedelsbehandlade som akut stressade patienter. I den andra studien etableras en ny diagnostisk metod för att hitta genetiska avvikelser som påverkar cytotoxisk lymfocytfunktion. Den tredje studien undersöker huruvida patienter med myalgisk encefalomyelit/kroniskt trötthetssyndrom (ME/CFS) visar tecken på nedsatt cytotoxisk lymfocytfunktion, vilket inte visar sig vara fallet, utom möjligen i en av 48 patienter. De två sista studierna handlar om autoantikroppar mot receptorer uttryckta på cytotoxiska lymfocyter; hur sådana antikroppar dels påverkar cellfunktioner, och dels om det

finns en koppling mellan sådana antikroppar och skov i systemisk lupus erytematosus (SLE), en autoimmun sjukdom.

I en tillvaro med allt bättre möjligheter att göra komplexa genetiska och proteinbaserade tester, hittas allt fler korrelationer mellan immunförsvarsavvikelser och olika sjukdomar. Antalet säkerställda kopplingar mellan immunförsvaret och sjukdomens uppkomst, där en så kallad immunbrist kan sägas föreligga, ökar dock inte i samma takt. Detta bidrar till att skapa ett växande gap mellan de misstänkta och de säkerställda immunbristproblemen. Sammantaget söker studierna i denna avhandling först skapa goda förutsättningar för att göra bra tolkningar av experimentella data och sedan undersöka såväl ME/CFS som SLE med hjälp av dessa förbättrade förutsättningar. De syftar därför till att i möjligaste mån minska gapet mellan de misstänkta och säkerställda immunbristproblemen, och att därigenom öka vår *salutogenetiska insikt*.

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