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CELL-MEDIATED IMMUNITY TO FLAVIVIRUS INFECTIONS

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Cell-Mediated Immunity to Flavivirus Infections

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"An expert is a person who has found out by his own painful experience all the mistakes that one can make in a very narrow field."

Niels Bohr

ABSTRACT

NK cells and T cells constitute a major part of the cell-mediated immune system and play important roles in the host defense against viral infections. Most current knowledge of cell-mediated immune responses to primary acute viral infections comes from murine models, while relatively less is known about human responses to, in particular, acute viral infections. In this thesis, the aim was to explore the early cell-mediated immune responses to acute viral infections in humans. The live attenuated yellow fever virus vaccine (YFV 17D) was used as a human *in vivo* model to study the dynamics and regulation of NK cell and T cell responses to the infection. In parallel, the NK cell and T cell responses to a natural acute infection caused by tick-borne encephalitis virus (TBEV) were studied.

Infection with YFV 17D induced activation of NK cells and T cells. An early activation in NK cell function, around one week post-vaccination, could be observed along with increased functions in response to target cells. A specific effector CD8 T cell response occurred that reached a peak of activation around two weeks post-vaccination, while CD4 T cell activation peaked earlier, around day 10, preceding that of CD8 T cells. The functional profile of the YFV 17D-specific CD8 T cell response changed in composition as it matured from an effector- to a memory-type response, and tended to become less polyfunctional during the course of this transition. In TBEV infected patients, NK cells were activated at the time of hospitalization. The activation subsequently decreased to healthy control levels. Simultaneous activation of CD8 and CD4 T cells occurred at one week following hospitalization and the TBEV-specific CD8 T cells showed a mono-functional profile that persisted over time. Furthermore, TBEV-specific CD8 T cells showed a distinct transcriptional profile in the effector phase of the T cell response.

Results presented in this thesis contribute to the understanding of the early T cell and NK cell responses in two human acute viral infections. In addition, they demonstrate that these two flaviviruses induce immune responses with different characteristics in infected humans.

LIST OF SCIENTIFIC PAPERS

I. Nicole Marquardt, Martin A. Ivarsson, **Kim Blom**, Veronica D. Gonzalez, Monika Braun, Karolin Falconer, Rasmus Gustafsson, Anna Fogdell-Hahn, Johan K. Sandberg and Jakob Michaëlsson, The Human NK Cell Response to Yellow Fever Virus 17D Is Primarily Governed by NK Cell Differentiation Independently of NK Cell Education. *Journal of Immunology*. 2015;195: 3262–3272.

II. **Kim Blom** and Monika Braun, Jolita Pakalniene, Sebastian Lunemann, Monika Enqvist, Laura Dailidyte, Marie Schaffer, Lars Lindquist, Aukse Mickiene, Jakob Michaëlsson, Hans-Gustaf Ljunggren, Sara Gredmark-Russ. Vivid Natural Killer Cell Response to Human Acute Tick-borne Encephalitis Virus Infection, *Manuscript.*

III. **Kim Blom**, Monika Braun, Martin A. Ivarsson, Veronica D. Gonzalez, Karolin Falconer, Markus Moll, Hans-Gustaf Ljunggren, Jakob Michaëlsson and Johan K. Sandberg. Temporal Dynamics of the Primary Human T Cell Response to Yellow Fever Virus 17D As It Matures from an Effector- to a Memory-Type Response. *Journal of Immunology*, 2013, 190:2150-2158.

IV. **Kim Blom**, Monika Braun, Jolita Pakalniene, Laura Dailidyte, Vivien Béziat, Margit H. Lampen, Jonas Klingström, Nina Lagerqvist, Torbjörn Kjerstadius, Jakob Michaëlsson, Lars Lindquist, Hans-Gustaf Ljunggren, Johan K. Sandberg, Aukse Mickiene, Sara Gredmark-Russ. Specificity and Dynamics of Effector and Memory CD8 T Cell Responses in Human Tick-Borne Encephalitis Virus Infection. *Plos Pathogens*, 2015,11: e1004622–20.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
EBV	Epstein-Barr virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HIV-1	Human Immunodeficiency virus type 1
HLA	Human leukocyte antigen
HSV-2	Herpes simplex virus 2
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
KIR	Killer-cell immunoglobulin-like receptors
LCMV	Lymphocytic choriomeningitis virus
MHC	Major histocompatibility complex
MPEC	Memory precursor effector T cell
PBMC	Peripheral blood mononuclear cells
SLEC	Short lived effector T cell
TBE	Tick-Borne Encephalitis
TBEV	Tick-Borne Encephalitis virus
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell

T_{EMRA}	Effector memory RA T cell
T_N	Naive T cell
T_{reg}	Regulatory T cell
T_{RM}	Tissue resident T cell
YFV	Yellow fever virus
YFV 17D	Yellow fever virus vaccine (Stamaril-17D 204)
VL	Viral load
WNV	West Nile virus

1 INTRODUCTION

One of the first references to immunity in humans stems from the plague of Athens in 430 BC. The historian Thucydides (who survived the disease) noted that people who recovered from the plague could nurse the sick without contracting the illness a second time [1]. The earliest forms of vaccination likely occurred in China during the 10th century, where it was known that smallpox infection could be prevented by deliberate inoculation of the smallpox virus to uninfected individuals [2]. The basis for this rather harmful vaccination was further improved in the 18th century, when Edward Jenner noted that dairy workers never contracted smallpox and speculated that this was because they had already been infected with cowpox, a less virulent virus in humans. Jenner took pus from the hand of a milkmaid with cowpox and scratched it into the arm of an 8-year-old boy. Six weeks later, Jenner inoculated the boy with smallpox and observed that he did not contract the disease [3]. In the 19th century, Louis Pasteur made observations of acquired immunity and created the first vaccines against rabies and anthrax. His discoveries supported the theory that some diseases are caused by microorganisms (the germ theory) [4], which was in direct opposition to the contemporary theories of diseases, in which "bad air" were thought to cause disease (the miasma theory) [5]. It was not until 1891 Robert Koch established the causative relationship between microbes and disease. For this he was awarded with the Nobel Prize in physiology or medicine in 1905. Yellow fever was the first identified human disease to be caused by a virus. In 1901, Walter Reed verified the existing theory that yellow fever is transmitted by mosquitoes and not by human to human contact, thereby connecting arthropods to disease [6].

1.1 FLAVIVIRUSES

The *Flaviviridae* family of viruses are divided into hepacivirus, flavivirus, pegivirus and pestivirus genera and they include many human pathogens. The family name originates from the yellow fever virus itself; *flavus* meaning yellow in latin. *Flaviviridae* viruses are enveloped and have a positive-sense single stranded RNA genome, which upon entry is recognized as messenger RNA in the host cell. This allows the virus to replicate and produce a polyprotein that is processed into ten proteins. The flaviviruses have three structural proteins; capsid (C), precursor membrane (prM) and envelope glycoproteins (E) that constitute the core of the virion (Figure 1A). There are seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1B), that associate to form a replicase complex that catalyses RNA accumulation in association with modified cytoplasmic membranes [7]. NS1 is a protein unique to flaviviruses, and intracellular expression of NS1 within the lumen of the ER is required for viral replication and viral RNA synthesis [8]. NS1 is synthesized as a monomer, after posttranslational modification in the lumen of the ER NS1 dimerizes, and is secreted into the extracellular space as a hexameric lipoprotein particle [9]. Dimeric NS1 is expressed directly on the

plasma membrane of infected cells [10]. The two hydrophobic proteins NS2A and NS2B are encoded upstream of NS3 in the polyprotein. One of these, NS2B, is the NS3 protease cofactor whereas the other, NS2A, is an essential component of the replicase [11]. NS3 is a multifunctional enzyme that together with the NS2B cofactor mediates cleavage of the flavivirus C protein from its membrane anchor, and is therefore essential for maturation. NS4A and NS2B are cofactors that contribute to the structure of NS3 anchoring the enzyme to the membrane, they are required for most NS3-mediated processing events and can modulate NS3 helicase activity. NS5 protein has RNA-dependent RNA polymerase (RdRp) and methyltransferase activities that are important for RNA synthesis and genome capping, respectively. NS2A, NS4A and NS4B proteins have so far undefined, but essential, roles in RNA accumulation [12].

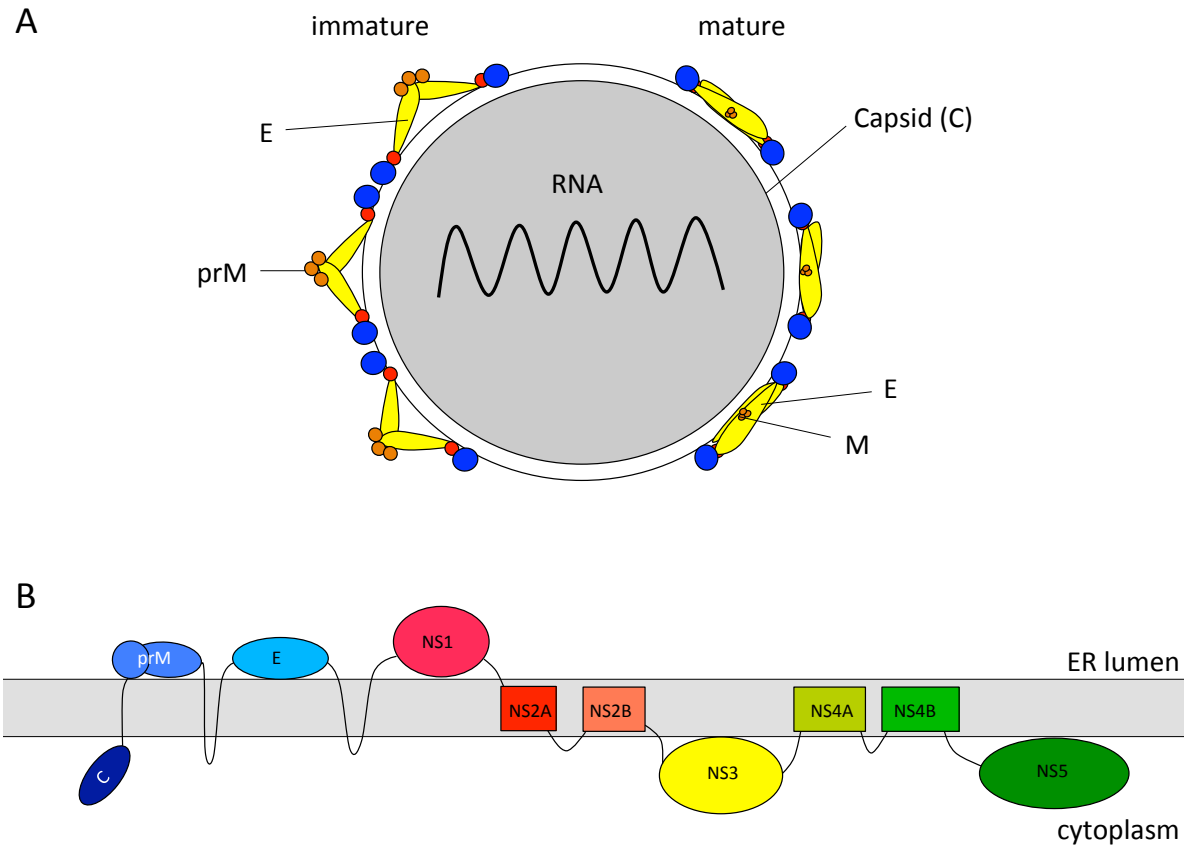

Figure 1. Overview of the flavivirus structure and polyprotein. (A) Schematic representation of a flavivirus particle. Left: immature virion; right: mature virion. (B) The polyprotein is composed of three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

Flaviviruses enters the cell through clathrin-dependent endocytosis upon attachment of the E protein to a cellular receptor [13]. The receptor or receptors that mediate flavivirus entry are, at present, poorly known. A large number of molecules have been described as candidate receptors in different cell types, but their role in virus endocytosis remains unknown. After cell entry, the virus is delivered to the endosomes [14]. The low pH within the endosome triggers the E protein to fuse with the endosomal membrane and the nucleocapsid is released into the cytosol. The assembly of immature

virions occurs in the ER, and the viral particles are transported to the Golgi apparatus [15, 16]. Formation of a deficient capsid and RNA particles is a hallmark of flavivirus infections. The virion particles are immature until the envelope protein is rearranged and prM are cleaved to M by furin (Figure 1A) in the acidic environment in the Golgi apparatus [17, 18]. Immature particles are noninfectious [17] and whereas proteolytic cleavage of prM is a prerequisite for viral infectivity, studies have shown that complete cleavage is not necessary for infectivity [19].

Diseases caused by flaviviruses are divided into hemorrhagic e.g., yellow fever, dengue fever and West Nile fever and encephalitic e.g., tick-borne encephalitis (TBE), japanese encephalitis (JE). Flaviviruses are further divided into two clades: vector borne and viruses with unknown vectors [20]. The vector borne clade can in turn be divided into a mosquito-borne clade and a tick-borne clade [21]. The yellow fever virus (YFV) is transmitted to humans by the *Aedes aegypti* mosquito. The virus causes an estimated 200,000 illnesses and 30,000 deaths yearly, and 90% of these infections occur in Africa. Yellow fever can be associated with acute immunopathology, including life-threatening viral hemorrhagic fever [22, 23]. The vast majority of yellow fever cases are preventable, because of an effective vaccine that was developed in the 1930s after successful attenuation of the YFV Asibi strain [24]. The yellow fever vaccine has in recent years received renewed attention due to its high efficacy and safety, and since it provides a model for studies of human immune responses to a mild, but nevertheless replication-competent, viral infection [25-28].

The first tick borne encephalitis-like disease was described in Scandinavian church records from the 18th century [29]. Tick-borne encephalitis virus (TBEV) is transmitted to humans by infected *Ixodes* tics, and it is estimated that one third of infected individuals develop clinical disease (Figure 2). TBE is a biphasic disease where more than a third of the TBE patients will suffer from life-long sequelae after the acute phase of the infection, including neuropsychiatric symptoms, severe headaches and a decreased quality of life [30]. Numbers of reported cases have increased rapidly over the last decades in both Europe and Asia [31, 32]. Three subtypes of TBEV have been identified: European (TBEV-Eu); Siberian (TBEV-Sib); and Far Eastern (TBEV-FE). The mortality rates differ between the strains, and infection with the far eastern strain have a mortality of 5-35 % whereas the other two have reported mortalities of 1-3% [32]. The pathogenesis of TBEV infection is far from fully understood. TBEV viral proteins and immune cell infiltrates have been detected in neuronal tissues from cases of fatal TBE, which indicates that the pathology could be mediated by direct viral effects as well as by immune-mediated mechanisms [33, 34].

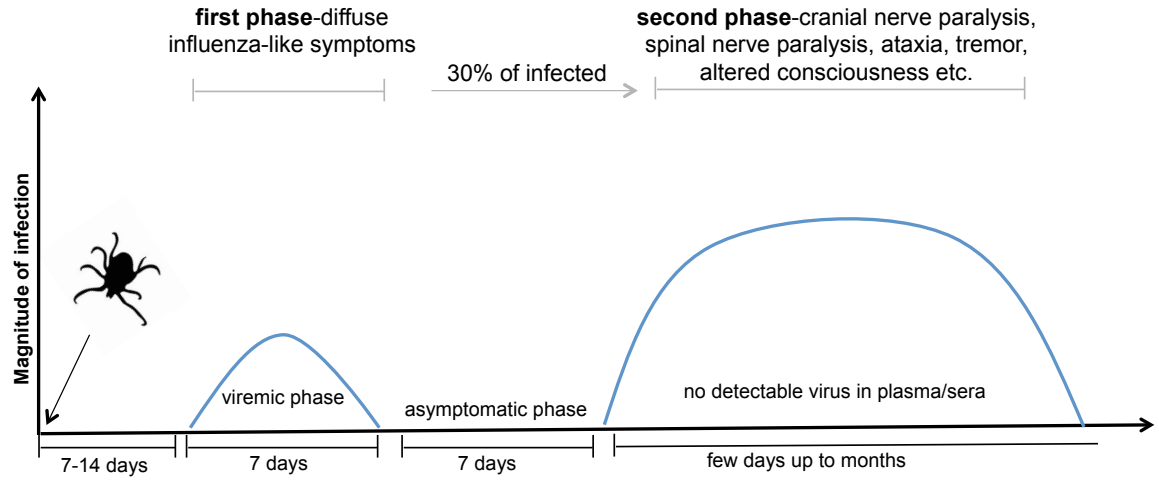

Figure 2. Classical overview of the biphasic disease in TBEV infection. The first phase includes influenza-like symptoms and occurs 7-14 days after transmission. About 65-70% of infected individuals clear the virus, but for one third of the patients a second disease stage follows. The virus reaches the CNS and cause severe symptoms of meningitis or encephalitis e.g., fever, severe headache, tremor, nystagmus, altered state of consciousness, cranial nerve paralysis and spinal nerve paralysis.

There is no specific treatment for TBE, but the disease can be prevented by vaccination. There are Russian vaccines based on TBEV-FE and in Europe two vaccines against TBE-Eu strains are licensed (strain Neudoerfl, FSME-IMMUN, Baxter Vaccines and Strain K23, Encepur, Novartis). The vaccines are based on formalin-inactivated virus, however over the last years, an increased number of vaccine failures have been reported. There is a possibility that more vaccine failures remain unnoticed partly due to unusual antibody-kinetics making it difficult to diagnose TBE in this group of patients [35].

1.2 NK CELLS

Natural killer (NK) cells were first identified through their ability to mediate cellular cytotoxicity against tumor cells [36]. The T cell receptor recognizes MHC class I, a necessity for the T cell to kill its target cells, and studies of NK cells were focused on finding a similar NK cell receptor, but no such receptor was found. It was observed that NK cells rather had the ability to kill cells lacking MHC class I, thus becoming activated by recognition of "missing self" (absence of self-MHC class I molecules) [37]. NK cells are identified as a component of the innate immune response against pathogens since they are activated in response to type I interferons (IFNs), do not rearrange genes to form receptors (like T and B cells do) and several other features [38]. Although NK cells are considered as a part of the innate immune system, several studies in mice suggest they mediate recall responses to several pathogens and haptens [39-41]. Human NK cells are classically defined as $CD3^-$ (T cell receptor negative), $CD56^+$ lymphocytes and represent about 15% of peripheral blood lymphocytes. One important function of NK cells, in particular NK cells of a $CD56^{dim}$ phenotype, is to induce cell lysis in malignant or virally infected cells. This cytolysis is mediated by a number of mechanisms including

(I) exocytosis of cytoplasmic granules containing perforin and granzymes, (II) fas-ligand-mediated induction of apoptosis, (III) antibody-dependent cellular cytotoxicity (ADCC) through activation of CD16 (FCγ-receptor). $CD56^{bright}$, as well as to some extent also $CD56^{dim}$ NK cells, also play an important immunomodulatory role, bridging the adaptive and innate immune responses by the production of soluble factors such as cyto- or chemokines [42]. NK cells recognize infected cells through an arsenal of different activating and inhibitory receptors, and the interactions among down-stream signals are still not entirely understood. This thesis will focus primarily on a few of these receptors that are likely important in human viral infection.

1.2.1 NK cell receptors

Killer Immunoglobulin-like Receptors

Humans and mice have two structurally distinct but functionally similar receptors that are important in regulating NK cell function. In humans, they are referred to as killer immunoglobulin like receptors (KIRs) that can activate signaling pathways that lead to inhibitory or activating outcomes in the NK cell (Figure 3). This primarily occurs through interaction with MHC class I molecules [43]. KIRs interact with classical (-A, -B and –C) and non-classical (-E and –G) HLA class I receptors. Activation or inhibition of NK cells is dependent on the strength of the binding between each KIR and its ligand, which is also partly dependent on the peptide presented by MHC class I. The proteins encoded by KIR genes have different immunoglobulin domains. KIRs that have two immunoglobulin domains are named KIR2D and KIRs with three domains are named KIR3D. Whether the protein becomes activating or inhibitory is determined by the presence or absence of specific signaling motifs in the cytoplastic domain of the cell. The receptor is inhibitory if the cytoplastic tail is long (KIR2DL, KIR3DL). If the protein is short, i.e. it lacks the inhibitory motifs, it has an activating function (KIR2DS, KIR3DS). KIR3DL1 and KIR3DS1, binds to the serological motif w4 that is carried by HLA-B molecules [44] while KIR2DL1, KIR2DL2 and KIR2DL3 all bind to HLA-C molecules. Furthermore, HLA-C alleles can carry either an aspargine at position 80 and are then classified as group 1 (HLA-C1), or a lysine and then belong to group 2 (HLA-C2). KIR2DL2/3 preferentially binds HLA-C1 and KIR2DL1 preferentially binds HLA-C2, with the binding affinity of the latter combination being stronger than the former (Table 1). The KIR gene family is highly polymorphic, and this further complicates the general NK cell response. Two alleles for each KIR gene exist and two main haplotypes have been identified. Haplotype A have inhibitory KIRs exclusively (with the exception of KIR2DS4), and haplotype B have both inhibitory and activating KIR genes. Thus, possible haplotypes are A/A, A/B and B/B [45]. Since genes encoding for KIR receptors display a great diversity in number and are expressed in a stochastic manner, unique subsets of NK cells with different activation thresholds are formed.

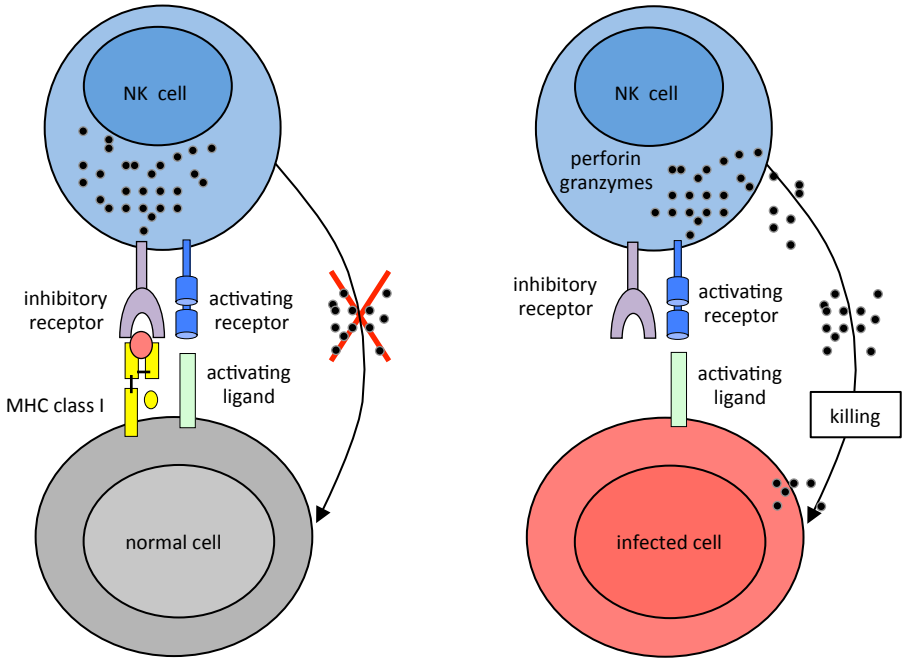

Figure 3. NK cell activation is regulated by the balance between activating and inhibitory signals. Upon virus infection, MHC class I ligands for inhibitory receptors are often reduced or lost. The NK cell only receives activating signals and cytolytic effector molecules are released, which results in target-cell killing.

Natural cytotoxicity receptors

The majority of human NK cells express two activating receptors, NKp46 and NKp30 that belong to the family of natural cytotoxicity receptors (NCRs). There is a third member of the NCR family, NKp44, the expression of which can only be measured on NK cells that are activated by IL-2 [46]. The NCR family belong to the Ig-superfamily and have a transmembrane domain that associates with immunoreceptor tyrosine-based activation motifs (ITAM) [47]. The ligands for the NCRs are largely unknown, however, the interaction of several viral and tumor associated molecules have been observed [48]. NKp46 and NKp44 have been claimed to interact with hemagglutinin (HA) on influenza virus and mediate cytolysis of influenza-infected NK cells [49, 50].

NKG2 receptors

Human NK cells also express CD94-NKG2A/NKG2C/NKG2E heterodimers, all belonging to the highly conserved C-type lectin family of killer lectin like NKG2-receptors (KLRs). Similar to the KIRs, these receptors also deliver activating (CD94-NKG2C/NKG2E) and inhibitory (CD94-NKG2A) signals depending on their motif in the cystoplasmic domain. NKG2A that has immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmatic domain induces inhibitory signals, while NKG2C induces activating signals through ITAM [51]. Both NKG2A and NKG2C heterodimers bind to HLA- E that are loaded with peptides derived from other HLA class I molecules, thereby monitoring the overall expression-level of HLA class I [51, 52].

Table 1. Simplified overview of NK cell-receptors, functions and ligands.

Receptor	Function	Ligand
CD16	activating	IgG
NKp30	activating	Unknown
NKp44	activating	HA
NKp46	activating	HA
NKG2A	inhibitory	HLA-E
NKG2C	activating	HLA-E
NKG2D	activating	MICA/MICB, ULBP1-4
KIR2DL1	inhibitory	HLA-C2
KIR2DS1	activating	HLA-C2
KIR2DL2	inhibitory	HLA-C1
KIR2DS2	activating	HLA-C1
KIR2DS4	activating	HLA-A11, C1, C2
KIR2DL3	inhibitory	HLA-C1
KIR3DL1	inhibitory	HLA-B
KIR3DS1	activating	HLA-B

NKG2D is another activating receptor, expressed on most NK cells, and is distinct from the other family members because it forms a homodimer in its active form. NKG2D recognizes a glycoprotein on human cytomegalovirus (CMV), and MHC class I polypeptide-related sequence A and B (MICA and MICB) (Table 1). NKG2D ligands are typically expressed on malignant or stressed cells, and multiple viral escape mechanisms are directed towards NKG2D. While the activating receptors of NKG2 and KIRs bind ITAMs, the activating NKG2D binds a non-ITAM sequence activating other pathways in the NK cell.

1.2.2 NK cell differentiation and education

NK cells are mainly identified through the surface marker CD56. They can be further subdivided into $CD56^{bright}$ and $CD56^{dim}$ NK cells depending on the intensity of CD56 that is expressed on the cell surface. Most $CD56^{bright}$ NK cells lack CD16, express the inhibitory NKG2A receptor, and have low expression of KIRs and cytotoxic molecules such as perforin and granzymes, and are considered as a more immature set of NK cells. In contrast, most $CD56^{dim}$ NK cells express CD16, have a variegated expression of KIRs, lower expression of NKG2A and contain intracellular granules with perforin and granzymes [36] (Figure 4). The function of NK cells is dependent on the stage of differentiation. Human NK cells continuously differentiate and this process is associated with functional changes [53-55]. $CD56^{dim}$ NK differentiation can be determined with the expression of CD57 together with the expression of NKG2A, where $CD57^{-}NKG2A^{+}$ cells lacking KIRs represent the least differentiated subset, and the $CD57^{+}NKG2A^{-}$ subset expressing multiple KIRs represents the most differentiated subset [53] (Figure 4). The late differentiated $CD57^{+}$ NK subsets are associated with

phenotypic and functional changes, such as a decline in proliferative capacity and responsiveness to cytokines together with increased ability to perform cytotoxic responses [54, 55].

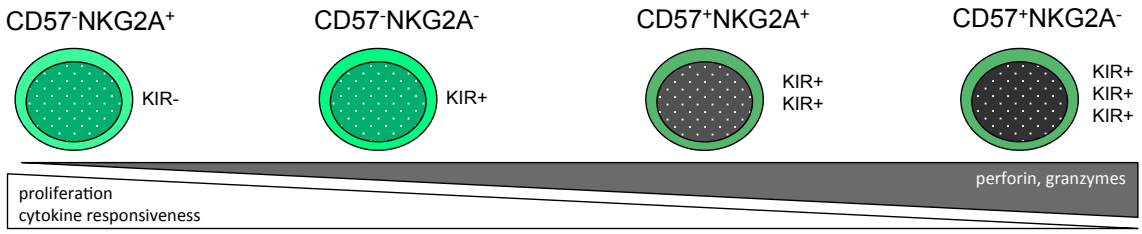

Figure 4. Schematic overview of CD56^{dim} NK cell differentiation. The least differentiated CD56^{dim} NK cells are CD57^{-}, have low numbers of KIRs, are more prone to cytokine stimulation and have a large proliferative capacity. The late differentiated CD57^{+} subsets are less responsive to cytokine stimulation, exhibit cytotoxic potential, and have a higher number of KIRs, thus they are more responsive to target cell killing.

In order not to kill normal host-cells, most NK cells are equipped with at least one self-MHC-class I receptor to maintain self-tolerance. NK cells that express an inhibitory receptor (including inhibitory KIRs and NKG2A) for a self-HLA class I molecule are educated, and respond well to stimulation with HLA class I-negative target cells [56]. NK cells that lack expression of inhibitory receptors for self-HLA class I are considered "non-educated" and are hypo-responsive to stimulation with HLA class I-negative target cells [56, 57]. This suggests that non-educated NK cells may be particularly important in infections where HLA class I is not down-regulated by the virus, and where cytokines produced by other immune cells drive the NK cell response. Conversely, educated NK cells may be more important in the response against virus infected cells that have down regulated HLA class I from the cell surface.

1.2.3 NK cells in viral infections

Functional NK cells are crucial in the control of many viral infections. NK cell deficiencies in humans result in severe viral infections in childhood and adolescence [58]. In particular, it results in increased susceptibility to viral infections caused by herpes simplex virus (HSV), varicella zoster virus, cytomegalovirus and human papilloma virus [59-61]. NK cells are reported to control viral replication through killing of infected cells, and they also play an immune regulatory role in the development of adaptive immunity [62, 63]. Furthermore, NK cells can modulate the adaptive immunity directly through interacting with dendritic cells [62].

NK cell-responses have been studied both in the context of several chronic and some acute human viral infections, e.g. human immunodeficiency virus (HIV) [64], cytomegalovirus (CMV) [65, 66], hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) [67], hantavirus [68], as well as chikungunya virus [69]. In some

of these infections, it has been suggested that activated NK cells may be involved in the pathogenesis of the infection where the NK cells participate either by direct killing of infected cells, or by indirect killing through cyto- or chemokines, or by the recruitment of inflammatory cells into the tissues [70, 71]. Accumulation of NK cells expressing the activating NKG2C receptor are reported in some human infections i.e. with CMV, hantavirus, chikungunya virus [68, 69, 72] and the expansion of this subset is even greater in CMV positive individuals [66, 68, 73]. However, the expansion of NKG2C positive NK cells is not reported in all viral infections, as Epstein Barr virus (EBV) [74] and herpes simplex virus 2 (HSV-2) [75] where infection do not cause specific expansion of NKG2C NK subsets.

NK cells have also been studied to some extent in a number of human flavivirus infections including dengue fever [76, 77], West Nile fever [78, 79] as well as after live attenuated YFV vaccination with YFV 17D [80, 81]. In these infections, NK cells have been suggested to affect disease severity and outcome as well as to contribute to viral control, even though the underlying mechanisms remain unknown. For example, in the case of dengue virus (DENV) infection, a higher absolute number of NK cells count was reported in patients with a mild infection as compared to patients with more severe form of dengue hemorrhagic fever (DHF) [82]. The NK cell contribution in dengue fever is, however, not straightforward, since a higher percentage of activated NK cells early on during the infection in children developing severe DHF have been observed [83]. Cytotoxic activity in NK cells have also been shown to be increased in early disease stages of DENV infection, and greater cytotoxicity has been reported in the more severe forms of the disease [84]. In experimental systems, increased NK cell diversity was reported after exposure of human NK cells to West Nile virus (WNV) infected peripheral blood mononuclear cells (PBMC) [78] and primary activated human NK cells inhibited WNV infection of Vero cells *in vitro* [79]. NK cell responses are not well studied in the context of TBEV-infection. One study reports a decrease of the activation marker CD69 upon *in vitro* infection of PBMC with TBEV [85]. Patients with severe TBE (meningitis or encephalitis) have been reported to have higher percentages of NK cells in peripheral blood than in the cerebrospinal fluid (CSF) [86]. NK cells have also been reported to decrease one week post vaccination and then return to normal healthy control levels by the end of the acute period of neuroinfection (day 25-30) in patients febrile TBEV-infection [87].

1.3 T CELLS

T cells constitute one major part of the adaptive immune system. T cell responses establish an immunological memory to a pathogen after it has been cleared, and this leads to improved responses and often protection to subsequent encounters with that specific pathogen. T cells can be separated from other immune cells by the presence of the T cell receptor (TCR) expressed on the cell surface. The vast majority of T cells express alpha-beta TCRs (αβ T cells), but some T cells in epithelial tissues (like the gut) express gamma-delta TCRs (γδ T cells), which recognize non-protein antigens. The TCR displays exquisite antigen specificity because of V(D)J recombination that generates the highly diverse repertoire of TCRs. CD4 and CD8 are surface molecules that act as co-receptors to the TCR and they are expressed on different T cell subsets. CD4 has a domain that interacts with MHC class II molecules and therefore, T cells that have CD4 on their surface are MHC class II restricted. CD8 is expressed on cytotoxic T cells and interact with MHC class I molecules, thus CD8 T cells are MHC class I restricted.

MHC class II molecules are expressed predominatly by "professional" antigen presenting cells (APCs), for instance macrophages or DCs. These cells take up microbes through phagocytosis, degrade them, and load MHC class II molecules with peptides. MHC class II presented antigenic peptides thus mostly originates from extracellular material. The peptides (antigens) presented by MHC class II to the TCR are normally 15-20 amino acids long and the CD4 T cells responding to antigens have critical functions in activating and regulating immune responses via the production and release of various cytokines. Thus they bridge innate and adaptive immunity in infection and they are traditionally referred to as T helper cells. CD4 T cells can be divided into distinct subsets depending on their cytokine profile. In a simplified view, Th1 subsets activate cellular immune responses via the production of IFN-γ, TNF and IL-2, and Th2 subsets mainly produce cytokines supporting B cell activation [88]. Regulatory T cell subsets (T_{reg}) can be distinguished from other CD4 T cell subsets by expression of the transcription factor FoxP3. Their main function is to regulate immune responses and prevent autoimmunity. They suppress cytotoxic T cell responses after successful clearance of pathogen, thereby controlling the cytotoxic response to infection to avoid immunopathology [89, 90].

MHC class I molecules are expressed on nearly all nucleated cells in the human body and are normally loaded with endogenous peptides, i.e. peptides from within the cell. These peptides are usually 8-10 amino acids long. Similar to NK cells, the function of CD8 T cells is to kill infected cells through the release of perforin and granzymes, as well as releasing cytokines such as IFN-γ, TNF and IL-2 and recruit NK cells, macrophages and B cells to the cite of infection. T cells can also induce apoptosis though death receptors such as TRAIL and FASL [91]. Since viruses utilize the host cell

machinery, viral products are mainly presented by MHC class I molecules to the CD8 T cells.

1.3.1 T cell development and differentiation

T cells mature in the thymus where they are exposed to endogeneous "self" peptides and undergo a process of positive and negative selection. Only T cells that bind MHC class I or MHC class II molecules with a suitable strength (not too strong or too weak) will survive the process. The earliest T cells are all negative for CD4 and CD8, but as they progress through development, they become double positive ($CD4^+CD8^+$). The $CD4^+CD8^+$ T cells that interact with MHC class II will down regulate CD8 from the surface and become single positive CD4 T cells, and CD8 will remain on the surface of the cells that interact with MHC class I. T cells leave the thymus as immature, naive T cells. The engagement and binding of the CD3/TCR complex to a peptide-loaded MHC molecule (pMHC) will activate a naive T cell, but efficient activation requires co-stimulation through dedicated co-stimulatory receptors. If the T cell do not receive the second co-stimulatory signal, it will undergo apoptosis [92]. This activation, or priming, by an APC, will allow the T cell to mature, divide and differentiate into effector and memory cells. A typical co-receptor, found on the surface of the T cell is CD28, which binds to CD80 or CD86 on the APC to initiate activation [93]. When a CD8 T cell becomes activated, it proliferates and this clonal expansion is supported by the T cell growth factor IL-2. The T cell differentiates into subsets of effector cells, many of which enter the blood and migrate to sites of infection. Following clearance of a pathogen, the effector T cell population contracts and the majority of the pathogen-specific T cells enter apoptosis. A small pool of pathogen specific T cells (5-10%) survives as memory cells [94]. The CD8 T cells destined to survive are sometimes referred to as long-lived memory precursor effector cells (MPECs) and tend to have a higher expression of the IL-7R (CD127) than the short-lived effector cells (SLECs) that do not survive [95]. Not all IL-$7R^{high}$ effector CD8 T cells become memory cells, so IL-7R alone does not indicate the fate of the CD8 T cell. The pool of memory cells is maintained in a cytokine-dependent manner mainly through the actions of IL-7 and IL-15 to promote memory T cell survival, independently of specific antigen [96]. Upon re-exposure to the pathogen, the memory cells quickly expand to large numbers of effector T cells (Figure 5).

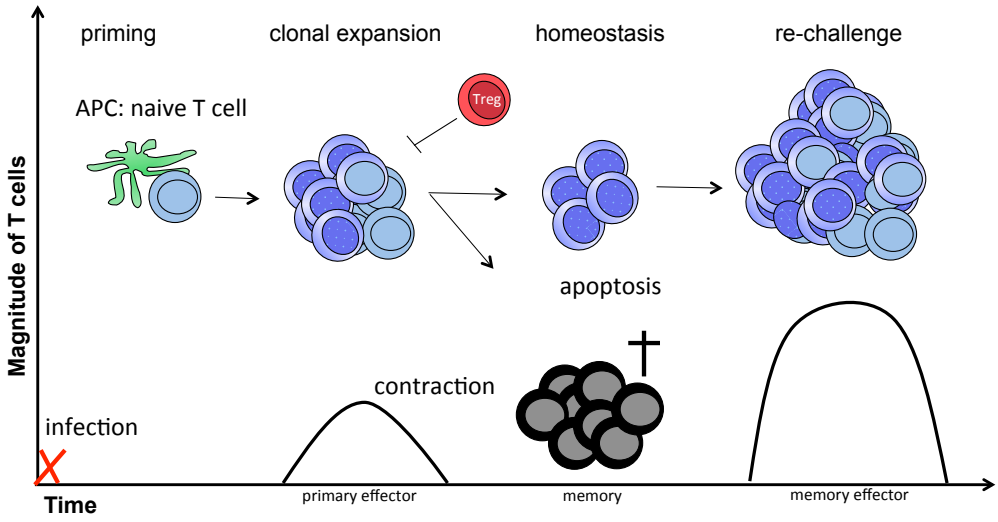

Figure 5. Primary anti-viral T cell responses go through a phase of rapid proliferation and clonal expansion. Following the clearance of the infecting pathogen, effector CD8 T cells undergo a contraction phase wherein the majority of pathogen-specific effector cells die by apoptosis, only 5–10% survive to further mature into memory CD8 T cells. A distinguishing feature of memory CD8 T cells is that they can rapidly contain a secondary infection through their ability to rapidly to produce a 'burst' of secondary T_{EM} cells that generate effector functions.

Memory T cell subsets were initially defined by the ability to mediate immediate effector functions, and the ability of the cell to migrate to secondary lymphoid organs [97]. Distinct stages of CD8 T cell differentiation have been defined by the expression of specific surface markers, such as the isoforms of CD45 and of the expression of the homing receptor CCR7, defining $CD45RA^{+}CCR7^{+}$ as naive (T_N), $CD45RA^{-}CCR7^{+}$ as central memory (T_{CM}), $CD45RA^{-}CCR7^{-}$ as effector memory (T_{EM}), and $CD45RA^{+}CCR7^{-}$ as effector memory RA (T_{EMRA}) CD8 T cells [97, 98]. The T_{CM} cells have higher sensitivity to antigenic stimulation, are less dependent on co stimulation and provide better feedback to DCs and B cells compared to T_N cells. T_{EM} cells require less co-stimulatory signals than the naive subsets and carry large amounts of perforin and granzymes, and both the CD4 and CD8 effector memory cells produce IFN-γ and other cytokines within hours upon antigen stimulation. The proportions of T_{CM} and T_{EM} in blood vary in the CD4 and CD8 T cells where TCM is predominant in CD4 and T_{EM} in CD8. A pool of CD8 T_{EM} cells expresses CD45RA, and T_{EMRA} cells carry the largest amount of perforin of the subsets. Over the past few years, another subset of memory T cells that resides in tissues has been identified; tissue-resident memory T (T_{RM}) cells. T_{RM} cells provide immediate protection against a secondary infection in extra-lymphoid tissues and are characterized by the inability to recirculate from tissue to lymph node to blood, and they have a high expression of CD69 together with high expression of CD103 (also known as αE integrin). T_{RM} are enriched for pathogens that they previously encountered in that specific tissue, and the TCR repertoire of skin T_{RM} cells differ from that of lung T_{RM} cells, and both are different from that of gut T_{RM} cells [99].

In the last decade, much attention has been directed towards the identification of transcription factors that regulate T cell differentiation of effector and memory T cells. Many transcription factors have been identified that have potentially important effects on gene expression in effector and memory T cells. They are believed to operate in pairs in opposing ways to facilitate effector versus memory T cell fates [100]. The T-box transcription factors T-bet and Eomesodermin (Eomes) have been shown to play important roles in determining the fate of CD8 T cells during infection [95, 101, 102]. High expression of T-bet promotes differentiation towards a more terminally differentiated profile [95, 103]. Eomes is homologous to T-bet in the DNA-binding domain, but it promotes the development of memory CD8 T cells rather than effector T cells [101, 104] (Figure 6). Genetic deletion of T-bet abrogates the formation of effector CD8 T cells, whereas the IL7R^{+} central memory subsets are sustained [95]. Additionally, IL-2 expression has been positively correlated with the expression of T-bet, which indicate a link between inflammation and accumulation of terminal effector cells [105]. Deletion of Eomes results in deficient memory T cells while the effector T cells are sustained [106]. Both T-bet and Eomes can be, and probably are, expressed at the same time in a cell but the gradient of these two will determine the fate of the T cell [95].

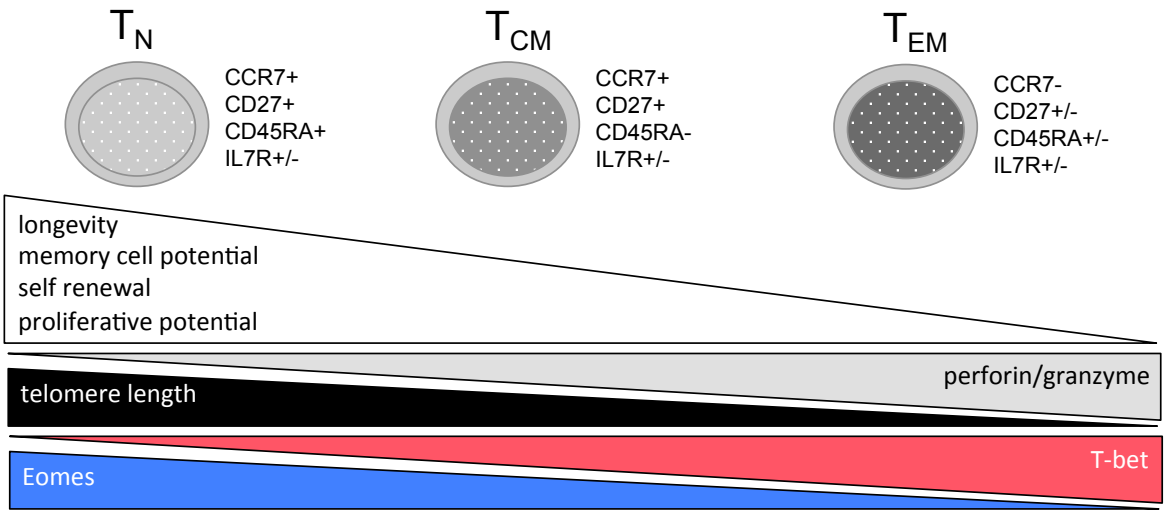

Figure 6. Schematic presentation of T cell subsets. The early CCR7^{+} T cells are positive for the co-receptor CD27, and in most cases positive for the IL-7Rα (CD127), have low content of perforin, granzymes, and T-bet. The mature CCR7^{-} subsets leave the lymph nodes and enter the periphery. They have a higher expression of perforin, granzyme and T-bet, shorter telomere lengths and are in most cases negative for the IL-7Rα.

1.3.2 T cell immunity against viruses

The ultimate immune response to a viral infection is initiated by a balanced innate response that limits viral replication, followed by a highly specific adaptive response that contains and eliminates the invading pathogen. In reality, however, many immune responses are less successful and persistent damage to the host as well as chronic infection may be the outcome.

CD4 T cells play an important role in the protective immunity against viruses, primed by infection or by vaccination. CD4 T cells can: (I) promote contact of CD8 T cells with DCs in secondary lymphoid tissue [107, 108]; (II) recruit lymphoid cells into draining lymph nodes [109]; (III) recruit innate or antigen-specific effectors to the site of viral replication [110, 111]. Furthermore, non-traditional cytotoxic MHC class II restricted CD4 T cells can occur and have been suggested to be important in viral control [112].

Due to difficulties in identifying the acute phase of viral infection in humans, most of current knowledge of antigen-specific T cell responses to acute primary viral infections comes from murine models, where responses to viruses such as lymphocytic choriomeningitis virus (LCMV) or vaccinia virus have been studied [113, 114]. Studies of human T cell responses to viral infections have to a large extent been focused on pathogens causing chronic infections with wide implications for human health such as HIV-1, EBV, HCV and CMV [115-119]. Such responses can be very robust, as exemplified by the massive clonal expansion of antigen-specific CD8 T cells [117, 119]. Based on these studies, it has also become clear that the resulting populations of human CD8 T cells display striking phenotypic differences, as determined by the expression profiles of surface markers [97, 98, 115]. The phenotype required to control the infection most likely differs depending on the antigen. Terminally differentiated CD8 T_{EMRA} cells dominate the response in persistent CMV infection [115, 119], whereas intermediate T_{EM} cells dominate the response in HIV-infected individuals [115]. It has further been reported that T_{EMRA} cells may be involved in protective immunity against HIV-1, since HIV-specific T cells with this phenotype were associated with viral control [120, 121].

Furthermore, during chronic infections with viruses such as HIV-1 and HCV in humans, the persistent exposure to viral antigens alters the function and gene expression of virus-specific CD8 T cells. Such changes are referred to as T cell 'exhaustion' because the antigen-specific T cells display a hierarchical loss of IL-2, TNF and IFN-γ, have impaired proliferation and, to some degree, decreased cytotoxic activity [122, 123]. The maintenance of antigen-specific T cells during chronic viral infection seems to be more dependent on the presence of the antigen and less dependent on IL-7 and IL-15, in contrast to the maintenance of the functional memory T cells that form after acute infection or in response to most vaccines [124]. Moreover, the cooperative expression of T-bet and Eomes in chronic infection is believed to be critical to sustain viral control since deletion of either one of them resulted in failure to control infection [125]. The expression patterns of T-bet and Eomes in CD8 T cells are not yet completely understood, and the analysis of these transcription factors during CD8 T cell differentiation may bring a novel molecular perspective to the phenotypic characterization of CD8 T cells and a deeper understanding of CD8 T cell differentiation during both acute and chronic viral infections.

In flavivirus infections, it has been discussed if the role of T cells is protective or pathogenic. For instance, CD8 T cells use a perforin-dependent mechanism to clear

WNV from infected neurons, despite the risk of immunopathogenesis [126]. T cells are believed to be protective in DENV infection, with higher frequencies of DENV-specific T cells observed in school children who subsequently developed subclinical infection, compared with those who developed symptomatic secondary infection [127]. Previous reports have shown that CD8 T cells play a key role in the pathogenesis of TBEV-infection. Cytotoxic T cell infiltrates have been linked to cell-death in infected human neuronal tissue [34] and mice that are deficient in CD8 T cells have a prolonged survival as compared to immuno-competent mice, or mice with adoptively transferred CD8 T cells [128]. Together, these data suggest that there may be a dual role of CD8 T cells, as they can be important in clearing viral infection but also mediate immunopathology within the CNS.

2 METHODS

The work in this thesis mainly relies on the analysis of advanced multicolor flow cytomery. The first flow cytometer was developed in the 1950s and have over the past decades become an extensively used technique to study immune cells [129]. An arsenal of receptors, chemokines, transcription factors and other molecules are used to determine the character of the cell. In this thesis, several multicolor immunofluorescence panels have been optimized to study the phenotype and character of NK cells and T cells in flavivirus infections.

In order to identify virus-specific T cells with flow cytometry (as in **Paper III** and **IV**), T cell epitopes in the virus have to be identified if they are not already known. Epitope identification requires a systematic screening of the antigen, which can be difficult when the antigen has multiple conformations or binding domains. Peptide libraries are used to display multiple, linear peptide fragments in parallel to deduce specific epitopes. The peptide library is designed with overlapping peptide segments of the entire antigen, and T cell cultures are then added to the library. Binding can be assessed by flow cytometry and other cellular assays. Epitope discovery by this method has been especially useful for developing vaccines. Another method to identify T cell epitopes to a specific pathogen is to utilize online search engines, which consider HLA-type and possible peptide presentation by the MHC molecule on the cell surface. The traditional whole genome library have the advantage of being HLA-unbiased while predicative algorithms represent a more high throughput technology. In some cases, search engines may not be accurate in terms of identifying immunodominant epitopes, especially not in the unusual HLA-types. Nevertheless, predicative algorithms do not require the same amount of material, which is most often a restriction in human patient samples. Hence in this thesis, we have only predicted epitopes through search engines and not used the traditional peptide libraries (Figure 7).

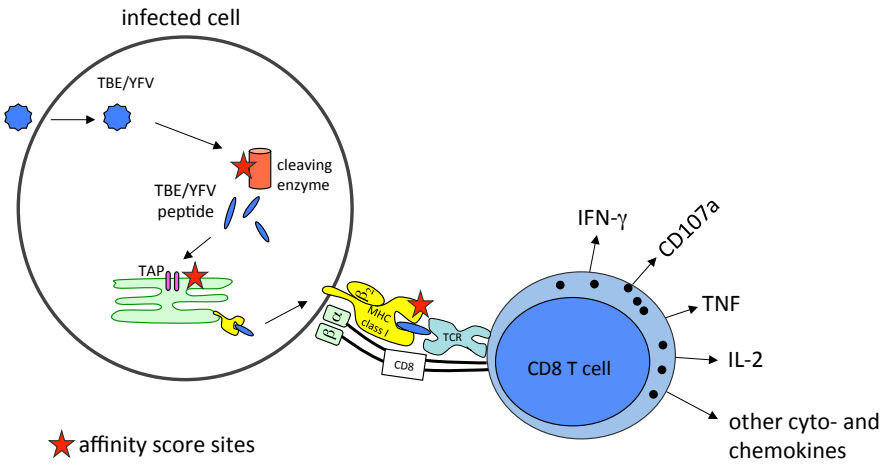

Figure 7. The NetCTL server integrates prediction of peptide-MHC class I binding, proteasomal C terminal cleavage and TAP transport efficiency. MHC class I binding and proteasomal cleavage prediction is performed using artificial neural networks while TAP transport efficiency is predicted using weight matrix.

3 RESULTS AND DISCUSSION

The studies in this thesis were performed on two cohorts. In one cohort, we utilized live attenuated YFV as a model for acute viral infection in humans by vaccinating 21 healthy individuals with YFV 17D (Stamaril), and collecting peripheral blood mononuclear cells (PBMCs), sera and whole blood before (day 0) and at days 1, 3, 6, 10, 15, and 90 after vaccination (described in **papers I** and **III**). The other cohort was collected in collaboration with Kaunas hospital in Lithuania, where PBMCs, cerebrospinal fluid (CSF), sera, and whole blood were collected from 20 patients with confirmed TBE (IgM positive for TBEV in serum). Samples were obtained within 3 days after hospitalization (day 0) and at days 7, 21, and 90 (described in **papers II** and **IV**).

3.1 HUMAN NK CELL RESPONSES TO FLAVIVIRUS INFECTIONS

3.1.1 Infection with TBEV induces high levels of pro-inflammatory cytokines

Upon infection with a virus or another pathogen, immune cells release cytokines and the assessment of these cytokines in sera may provide for a good immunological overview of an ongoing infection. Thus, our first approach was to assess cytokine responses caused by YFV 17D and TBEV infections. We focused on cytokines known to influence human NK cells (IL-2, IL-12, IL-15, IL-18, and IFN-α), as well as on pro-inflammatory cytokines and chemokines produced by NK cells (IFN-γ, TNF and Mip-1β).

No significant increases of cytokines in sera were detected in YFV 17D infection at any time point. However, we observed that NK cell-activating cytokines significantly increased together with elevated levels of the NK cell effector cytokines IFN-γ and TNF as well as the pro-inflammatory cytokines, IL-6 and IL-8, in TBEV-infected patients at the time of hospitalization (**paper II)**. These findings indicate that NK cells could be activated and involved in the immune responses, at least in patients with severe TBEV-infection. Hence, we set out to study the NK cells and their activation in detail in human infections with TBEV and YFV.

3.1.2 Flaviviruses activates NK cells

One of many factors that may influence the development of symptomatic flavivirus infection is NK cell numbers and function. For example, it is observed that early activation and expansion of NK cells after infections with WNV and DENV, has been associated with viral control and prevention of disease in mice [130, 131] and in humans [76, 82, 84].

We were interested in studying the characteristics and kinetics of the NK cell activation caused by YFV 17D and TBEV infections. To investigate NK cell activation induced by YFV 17D and TBEV, we analyzed the expression of Ki67 which is expressed in currently or recently dividing cells [132]. Infection with YFV 17D induced increased Ki67

frequencies at day 6 and 10 within both $CD56^{bright}$ and $CD56^{dim}$ NK cell populations (**paper I**). TBEV infection induced significantly increased Ki67 frequencies in $CD56^{dim}$ NK cells in the acute stage (day 0) as compared to the convalescent phase (day 90). In addition, activation of $CD56^{dim}$ cells preceded the activation of $CD56^{bright}$ cells that peaked at day 7 and 21 (**paper II**), which is distinct from that of infection with YFV 17D, and may indicate abnormalities in the NK cell response pattern in patients infected with TBEV.

To analyze how NK cell activation induced by YFV 17D related to viral replication and IFN response, plasma viral load and levels of type I/III IFN were measured. Viral RNA was detected at day 6 in a majority of the donors, concurrent with an increase in the levels of type I/III IFN in plasma (**paper I**). Levels of type I/III IFN in plasma at day 6 correlated positively with NK cell activation, measured as CD69 expression at day 6 and Ki67 expression at day 10 (**paper I**). YFV 17D co-incubated with PBMC induced up-regulation of CD69 on NK cells, whereas purified NK cells did not. This up-regulation of CD69 on NK cells by YFV 17D could be abrogated by the inhibition of the JAK signaling pathway with Pyridone 6 (**paper I**). These results indicated that the presence of other cell types is required for activation of NK cells during infection with YFV 17D, and that activation was dependent on type I/III IFN. Despite the evidence of NK cell activation caused by TBEV infection, we did not detect significant alterations in the frequency of total NK cells among lymphocytes (**paper II**). One possible explanation may be that the activated NK cells are recruited to the CNS, or other tissues where the virus may be active. CD4 T cell activation (**paper IV**) correlated with total NK cells at day 0 (**paper II**) in TBEV infection. No such correlation could be observed with CD8 T cells and NK cells. This observation suggests that innate responses may rely upon early interactions between NK cells and CD4 T cells in infection.

To conclude, symptomatic TBEV-infection cause a distinct activation pattern of NK cells, in which activation of $CD56^{bright}$ NK cells occurs after that of $CD56^{dim}$ NK cells, about one week following hospitalization (**papers I** and **II**). This further indicates that the kinetics of the NK cells also may be an important factor in the prevention of severe disease.

3.1.3 NK cell differentiation is affected by TBEV and YFV 17D infections

$CD56^{dim}$ NK cell differentiation can be determined by the parallel analyses of NKG2A and CD57 (Figure 4). To determine whether NK cell differentiation was affected by infections with YFV 17D and TBEV, we analyzed the expression of Ki67 in combination with CD57 and NKG2A in $CD56^{dim}$ NK cells as well as the expression of NKG2C. YFV 17D infection induced increased expression of Ki67 in $CD57^{-}$ $CD56^{dim}$ NK cells at day 10. Both $CD57^{+}$ and $CD57^{-}$ subsets responded with similar kinetics (**paper I**). TBEV infection induced significantly higher frequencies of Ki67 expression in the late differentiated $CD57^{+}$ subsets at day 0 compared to day 90 (**paper II**). Thus, infection with YFV 17D caused activation of subsets that are more responsive to cytokines ($CD57^{-}$

subsets) while infection with TBEV induced activation of subsets that respond better to target cells ($CD57^+$ subsets). This may indicate that activation of the less differentiated $CD56^{dim}$ NK cell subsets provides protective immunity during flavivirus infection.

Expansion of $CD56^{dim}$ NK cells expressing the activating receptor NKG2C has been reported in CMV infection [72, 133, 134] as well as in other viral infections [68, 69]. As occurs in EBV and HSV-2 virus infections [74, 75], we did not observe any expansion of NKG2C-positive NK cells induced by YFV 17D or TBEV infections (**paper I** and **II**), despite most of the donors being CMV seropositive. Nevertheless, it cannot be excluded that $NKG2C^+$ NK cells expand numerically at the site of local infection. For instance, in patients with celiac disease, intraepithelial T cells in the gut lumen, i.e., the site of inflammation, become activated and express high levels of NKG2C, whereas the corresponding T cells in peripheral blood remained unaffected [134].

3.1.4 $CD56^{dim}$ NK cell responses to target cells are impaired in TBEV infection

Many viruses down-regulate MHC class I on the surface of the infected cell to evade cytotoxic T cell responses, which may result in NK cell killing [135]. Flavivirus infected target cells are reported to be protected from lysis by NK cells, and it has been suggested that this is a consequence of virus-mediated MHC class I up-regulation [136, 137]. At least two distinct mechanisms of immune evasion have been reported in flavivirus-infections. First, flaviviruses increase MHC class I expression on infected cells [138], and even though increased MHC class I expression may result in enhanced T cell responses, the evolution of flaviviruses has probably been driven more by selective escape to innate immunity rather than adaptive T cell immunity [139]. Second, the viral protein NS5 can inhibit early phosphorylation events in the IFN signal-pathway by acting as an IFN agonist [140], which has been observed in studies with TBEV and Langat virus. Similar strategies may be utilized by other flaviviruses such as WNV and JEV [141, 142]. Furthermore, it has been shown that JEV is able to inhibit CD8 T-cell responses by interfering with MHC class I antigen presentation [143].

Antiviral responses by NK cells can, as mentioned above, be activated through cytokines like IL-12 and IL-18 or by the recognition of viral-induced changes on infected target cells. We observed that infection with YFV 17D induced increased expression of IFN-γ and CD107a in $CD56^{dim}$ NK cells after stimulation with IL-12 alone, IL-12 and IL-18 in combination or in response to K562 target cells (**paper I**). The functional responsiveness peaked at day 6 and returned to baseline levels at day 15 after vaccination. TBEV infection induced reduced degranulation and cytokine production of $CD56^{dim}$ NK cells in response to K562 target cells at day 0 as compared to day 90 (**paper II**). Surprisingly, the capacity to produce IFN-γ in response to IL-12/IL-18 stimulation was intact throughout infection with TBEV (**paper II**).

We found that, in contrast to infection with YFV 17D, TBEV infection induced a dampened NK cell response against target cells, but the responsiveness to cytokine activation remained. This further supports the hypothesis that viral escape is directed towards NK cells and moreover, that TBEV escape mechanisms in humans may be directed towards MHC class I target cell recognition since no dysfunctional changes could be observed in NK cells activated with cytokines.

3.1.5 Effects of NK cell differentiation on the response to flavivirus infections

Increased NK cell differentiation is associated with expression of multiple KIRs on single NK cells; therefore we asked whether the number of inhibitory KIRs affected the responsiveness of NK cells in these infections. We divided the NK cells into subgroups of cells expressing zero, one, two or three KIRs. NK cells that express zero or one KIR are considered as more responsive to cytokines and less responsive to target cells, whereas NK cells expressing two or three KIRs are considered as late-differentiated and more responsive to target cells. We could not detect any differences in activation in NK cells that express zero, one, two or three KIRs over time after YFV 17D infection (**paper I**). Conversely, TBEV infection induced significant activation of the NK cells expressing 0 KIR in the late differentiated $CD57^{+}NKG2A^{-}$ and $CD57^{+}NKG2A^{+}$ subsets at day 0 as compared to day 90 (**paper II**).

Explanations for the difference in the activation patterns in these subsets caused by YFV 17D and TBEV infections may be either to due to the mild infection YFV 17D causes, or that TBEV alters the immune response so that the NK cells become more responsive to cytokines instead of infected target cells.

3.2 HUMAN T CELL RESPONSES TO FLAVIVIRUS INFECTIONS

3.2.1 T cells are activated in flavivirus infections

There are many challenges in studying virus-specific T cell activation in human viral infections. Epitopes are required to identify the nature of virus specific cells either through MHC class I tetramers, or through *in vitro* activation. However, T cell epitopes have not yet been identified for all viruses and further, one virus-specific epitope does not represent the magnitude of all virus specific cells. To circumvent this problem, Miller *et al.* identified a set of general activation markers (CD38, HLA-DR and Ki67), the measurement of which indicate the magnitude of the virus-specific T cell response, independent of epitope- or HLA-specificity [27].

Activation of immune cells is required to create protective immunity, and inhibition or alterations in the activation can affect acquired immunity against the pathogen. Thus, as a first approach, we wanted to study T cell kinetics, magnitude and activation of the virus-specific T cells in YFV 17D and TBEV infections. Based on the previous study of Miller *et al.* [27], we identified the virus-specific cells using the activation marker CD38,

in combination with Ki67. Activation was measured at day 0, 7, 21 and 90 in TBEV-infected individuals, and before (BL) along with days 10, 15, and 90 post YFV 17D administration to previously unvaccinated healthy individuals.

Indeed, we detected a clear CD8 T cell activation in both infections (**papers III** and **IV**). Infection with YFV 17D induced CD8 T cell activation that peaked around 15 days post vaccination and TBEV infection induced a later activation that peaked about one week after hospitalization, i.e., the beginning of the second phase of disease. The activated ($CD38^+KI67^+$) cells expressed high levels of perforin, granzyme B, HLA-DR, PD-1, T-bet and Eomes together with low levels of Bcl-2, thereby defining these as cytotoxic effector cells (**paper IV**). We did not observe a difference comparing activated CD8 T cells in the two infections (Figure 8). This indicates that severity of disease may not always affect the magnitude of the antiviral CD8 T cell response. Previous studies have suggested that nonspecific CD8 T cells can be activated and perform cytotoxicity in the absence of TCR engagement [144, 145], referred to as bystander activation. No CMV of EBV bystander activation is reported in YFV 17D infection [27], and to address the possibility of recurring CMV in TBEV infection we studied Ki67 expression in CMV-specific CD8 T cells. The Ki67 expression was low in the CMV-specific population and remained so throughout the course of infection in all tested donors, showing that no CMV-bystander-activation occurred (**paper IV**). This finding suggests that TCR engagement probably is the primary form of activation of the antiviral CD8 T cells in response to TBEV and that bystander activation of CMV-specific T cells is not a major feature in TBEV infection.

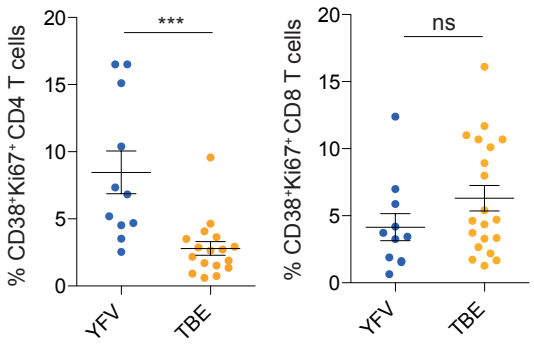

Figure 8. T cell activation induced by YFV 17D and TBEV infections. A) $CD38^+KI67^+$ CD8 T cells in YFV 17D infection (day 15) and TBEV infection (day 7) were compared at the time of the maximum peak (left panel). $CD38^+KI67^+$ CD4 T cells induced by YFV 17D infection (day 10) or TBEV infection (day 7) were compared at the time of the maximum peak (right panel). Statistical analysis was performed using Mann-Whitney T test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

We were further interested in studying the CD4 T cells and the activation induced by infections with YFV 17D and TBEV. We used the same parameters as in CD8 T cells and observed a peak of activation in CD4 T cells at day 10, preceding that of CD8 T cells, in YFV 17D infection (**paper III** and Figure 9). CD4 T cell activation occurred at day 7 in TBEV infection, along with activation of CD8 T cells (**paper IV**). Phenotypically, the activated ($CD38^+KI67^+$) CD4 T cells, as with the CD8 T cells, expressed high levels of HLA-DR and PD-1, together with low levels of Bcl-2. Not unexpectedly, the expression of perforin and granzyme B in the activated CD4 T cells were lower compared to that of CD8 T cells (7% vs 55% in perforin expression and 17% vs 82% in granzyme B expression). Furthermore, infection with YFV 17D showed

a higher percentage of CD38⁺Ki67⁺ CD4 T cells as compared to TBEV infection (Figure 8). The role of CD4 T cells in TBEV infection is difficult to interpret, as the presence of IgM and IgG antibodies at the time of hospitalization suggests that the CD4 T cell response is either biphasic or started before the onset of symptoms. Nevertheless, the activation of CD4 T cells with the right magnitude and kinetics may be a requirement for viral clearance and protective immunity.

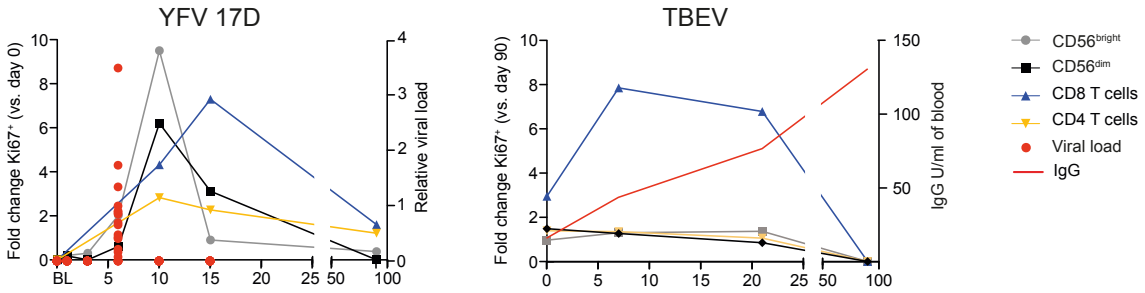

Figure 9. Collected data of the cell-mediated immune responses against YFV 17D and TBEV infections.

3.2.2 T cell-specific responses to flavivirus infections

CD8 T cells have a range of functions to control viral infections, and the functional properties of the specific T cells may play a major role in viral clearance. For instance, it has been observed that high levels of polyfunctionality in the CD8 T cells response to HIV-1 infection is associated with slow disease progression [146]. Adding a further layer of complexity, the magnitude of the CD8 T cell response has been shown to correlate with selection of viral-escape mutants during acute infection, rather than the level of polyfunctionality [147]. DENV-specific memory T cells are reported to influence the outcome of disease severity. T cells from patients with mild/sub clinical DENV-infection preferably produced granzyme B upon antigen stimulation, while those who were hospitalized with the more severe form produced both TNF and IFN-γ, or TNF alone [148].

To assess the functional profile of YFV 17D- and TBEV- specific CD8 T cells, we constituted a pool of potential peptide epitopes predicted by the NetCTL algorithm [149]. The predicted peptide-pools were then used to activate virus-specific CD8 T cells *in vitro*. YFV 17D specific cells show a diverse response pattern that was dominated by mono-, bi-, and triple-functional cells with respect to intracellular expression of IFN-γ, IL-2, MIP-1β, TNF and surface expression of CD107a. The responses tended to become less polyfunctional over time, since monofunctional cells dominated the specific cells at day 90 (**paper III** and Figure 10). The CD8 T cell response to TBEV infection was almost undetectable in patients at the time of hospitalization. Similar to NK cells (**paper II**), significantly lower expression of CD107a, Mip-1β and TNF at days 0 and 7 was observed, as compared to days 21 and 90. Furthermore, TBEV specific CD8 T cells

primarily showed responses of a monofunctional nature that lasted over time (**paper IV** and Figure 10).

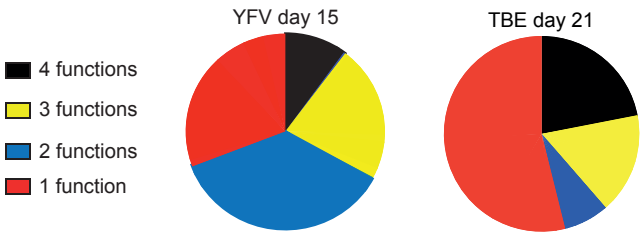

Figure 10. Functional properties of YFV 17D-specific and TBEV-specific CD8 T cells. Pie charts indicate the mean composition of the total response in CD8 T cells with regard to their capacity to express one, two, three or four functions at day 15 after vaccination with YFV 17D (left panel) or at day 21 after hospitalization in patients infected with TBEV (right panel).

Because the YFV 17D provides a high level of protection, our observation that the response is dominated by mono-, bi-, and triple-functional cells would be consistent with a model in which polyfunctionality with four of five functions is not a strict requirement for a protective CD8 T cell response. TBEV-specific CD8 T cells showed low production of cytokines and was primarily mono-functional and could possibly reflect functional inhibition and delay of the T cell caused by TBEV.

3.2.3 Memory T cell formation in flavivirus infections

The pattern of when, and for how long, an antigen is recognized by the T cells in primary CD8 T cell responses to acute virus infections in humans is incompletely understood. To study this pattern in YFV 17D infection, we pre-selected and generated seventy-eight peptides of YFV 17D epitope candidates predicted to be presented by the most common HLA-types (HLA-A1, -A2, -A3, -A24, -B7, or -B8) using the NetCTL search engine. The responding cells were identified as proliferating ($CFSE^{low}$), IFN-γ^{hi} after a seven-day expansion assay. A CD8 T cell response was detected against as much as 73% of the predicted candidate epitopes. Furthermore, we discovered that the magnitude of responses against targeted epitopes differed markedly. Some HLA-A2, and HLA-B7 responses to their respective peptide were as strong and dominate in effector (day 15) and memory stages (day 90). Several other specific responses were less strong and some responses were transient, being detectable at day 15 but undetectable at day 90 (**paper III**). Some HLA-A2 positive donors showed expansion upon stimulation with the immunodominant YFV 17D HLA-A2-specific peptide before vaccination (**paper III**), which indicates the existence of a preexisting pool of YFV 17D "specific" cells, possibly induced by another pathogen.

Since there is a lack of knowledge about primary human immune responses, we were interested in studying the phenotype of the virus-specific cells in detail. We identified single peptides from the previous peptide pools that induced cytokine responses in response to infections with YFV 17D or TBEV. The selected peptides were then used to generate HLA-A2 (both YFV 17D and TBEV) and HLA-B7 (YFV 17D) tetramers specific for YFV 17D and TBEV.

After YFV 17D administration, the HLA-A2 and HLA-B7 specific cells had detectable frequencies throughout the course of the response. During the effector response against YFV 17D infection, the most prevalent phenotype of tetramer-defined CD8 T cells of both A2- and B7 specificities was T_{EM} PD-1$^+$ at day 15, but at the memory stage at day 90 the specific cells had shifted to a T_{EMRA} PD-1$^-$ phenotype (**paper III**). The HLA-A2 tetramers identified detectable frequencies of TBEV-specific specific cells at day 7 and 21 after hospitalization, whereas tetramer-positive cells were barely detectable at day 0 in TBEV infection (**paper IV**). The most dominant phenotype at day 7 of the TBEV-specific CD8 T cells was T_{EM} CD57$^-$PD-1$^+$. This phenotype contracted rapidly and was less prevalent at the convalescent phase (days 21 and 90) (**paper IV**).

In memory T cell subsets, the expression pattern of T-bet and Eomes may differ depending on the antigen specificity. For instance, polyomavirus BK-specific cells display a T-bet intermediate and Eomes low phenotype. In the same donors, CMV-specific cells are both T-bet and Eomes high, whereas influenza-specific cells are T-bet^{high} and Eomes^{low} [150]. Since the activated (Ki67$^+$CD38$^+$) CD8 T cell population in TBE patients showed a distinct pattern of increased expression of T-bet and Eomes (**paper IV**), we measured the expression of these transcription factors in TBEV-specific CD8 T cells in combination with Ki67. We observed a triple positive (Eomes$^+$Ki67$^+$T-bet$^+$) dominant phenotype among the TBEV-specific CD8 T cell population at day 7, which retracted to become almost undetectable in the convalescent phase (**paper IV**). In the convalescent phase of disease, Eomes$^-$Ki67$^-$T-bet$^+$ CD8 T cells dominated the TBEV-specific pool. This suggests that simultaneous up-regulation of Eomes, Ki67, and T-bet occurs in the effector phase of the T cell response against TBEV. In the memory stage, however, Ki67 and Eomes are down regulated and T-bet alone is dominant in the memory cells. This is consistent with the phenotype reported in influenza-specific cells [150]. Thus, long-term human memory T cells specific for cleared infections such as influenza or TBEV may have a shared Eomes^{low} profile, which is distinct from T cells specific for persisting antigens such as CMV.

Patients with severe TBEV infection might fail to clear the virus, and the responses we observed are most likely of dysfunctional nature. Despite the fact that no virus can be detected at any time in the TBE patients, specific CD8 T cells arise one week after symptom debut. This suggests that the virus (or parts of it) can prime and induce specific CD8 T cells. Activation of CD4 T cells as well as NK cell activation, together with the seroconversion of IgG suggests that some adaptive activation occurred earlier in the TBE patients. This is further confirmed by the correlation of activated CD4 T cells with NK cells (**paper II**).

In DENV infection, DENV-NS1 is secreted and detected in blood and the levels of NS1 in blood correlates with disease outcome. NS1 is further shown to induce inflammation and endothelial permeability in mice regardless of viral presence and interestingly, this could be prevented by NS1 vaccination [151]. Hypothetically, because NS1 is secreted

by DENV, TBEV may in a similar way secrete viral proteins that in this case can pass through the blood brain barrier. Further and importantly, a vaccine directed to TBEV-NS1 (or other viral proteins) could possibly prevent or dampen the symptoms caused by TBEV.

4 CONCLUDING REMARKS

YFV 17D is the ideal virus for the human immune system. It is optimized so that the immune system can clear the virus without symptoms (in most cases) and a life-long protective memory is created. Herein, it is used as a model system to capture the early primary immune responses in a controlled model of acute viral infection in humans. Knowledge of the immune responses gained from YFV 17D is then utilized to shed light on the immune responses in a natural infection with TBEV. Below are some major conclusions presented.

- Both YFV 17D and TBEV infections induce activation of NK, CD4 and CD8 T cell subsets, and the activation caused by TBEV exerts a distinct immune activation-pattern from that induced by YFV 17D (**papers I-IV**).
- The activation of NK cells induced by YFV 17D infection relies on NK cell differentiation, and is likely primed by type I/III IFNs (**paper I**).
- Infection with TBEV induces a significant increase of cytokines in sera in parallel with an increase of less educated-late differentiated $CD56^{dim}$ NK cells at the time of hospitalization (**paper II**).
- NK cell function *in vivo* is dampened by TBEV infection (**paper II**).
- Infection with YFV 17D induces CD4 T cell activation that precedes CD8 T cell activation (**paper III**), but TBEV infection induce simultaneous activation of CD4 and CD8 T cell subsets (**paper IV**)
- YFV 17D-specific CD8 T cells are mainly mono, bi and triple functional (**paper III**), while TBEV-specific CD8 T cells primarily excert mono-functional properties (**paper IV**).
- TBEV-specific CD8 T cells may also, as NK cells, be functionally dampened at the onset of symptoms, as the production of effector cytokines is significantly lower at the early timepoints after hospitalization, as compared to the convalescent phase of disease (**paper IV**).
- TBEV-specific CD8 T cells show a distinct transcriptional profile at the peak of activation that contracts and becomes almost undetectable in the convalescent phase (**paper IV**).

Data presented herein offers an evaluated framework that adds to the understanding of the cell-mediated immune regulation in flavivirus infections in humans. The application of this framework might yield a better understanding of the role that cell-mediated immunity plays in other viral infections. Furthermore, it may be helpful for the insight in pathogenesis caused in viral infections as well as the design of new treatment regimens and the development for better vaccines to TBEV.

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