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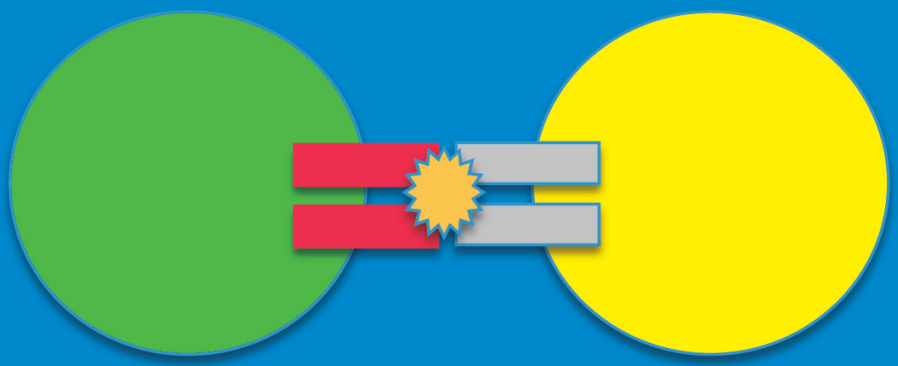
Immunobiology of Emerging Human DNA Viruses

Arun Kumar



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Immunobiology of Emerging Human DNA Viruses

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ACADEMIC DISSERTATION

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When you want something, all the universe conspires you to achieve it.
-*Paulo Coelho*

To my family

CONTENTS

ABSTRACT

Many unknown species of human DNA viruses have recently (2005-2013) been discovered by using modern molecular and bioinformatic tools. The clinical and pathogenic roles of these viruses are presently known only fragmentarily; however they were found in symptomatic patients, and some have been shown to cause severe infectious illness, or cancer. Some of these emerging DNA viruses are examined in this thesis: Human Bocavirus 1 (HBoV1), Merkel cell polyomavirus (MCV or MCPyV) and *Trichodysplasia spinulosa*-associated polyomavirus (TSV or TSPyV). Viruses like these are of fundamental importance in the genesis of not only of acute but also of chronic or late-onset illness. The immunobiology and pathogenesis of these new viruses along with the already known DNA virus (parvovirus B19 or B19) can be found by immunological and molecular methods.
For years it was thought that parvovirus B19, was the sole human-pathogen among its family members. In 2005 a new pathogenic species, HBoV1 (previously denoted HBoV), was discovered by random-PCR from a nasopharyngeal aspirate. The existing data strongly suggest that HBoV1 causes a respiratory illness in young children. The aim of our study was to increase our knowledge on HBoV1-specific Th-cell immunity by examining T-cell proliferation and cytokine responses in asymptomatic adults. HBoV1-specific response was compared to those elicited by B19. B19-specific Th-cell immunity appears to be more divergent (in terms of cytokine response patterns) than the HBoV1-specific one**.** The present study also suggests that interleukin-13 (IL-13) response induced by HBoV1 may contribute to the airway pathology like asthma or bronchiolitis.

A novel concept of $CD4^+$ T-cells with cytolytic potential ($CD4^+$ CTL) is emerging. Very recently, $CD4^+$ CTL have been implicated in the control of persistent viral infections, e.g., Epistein-Barr virus (EBV), hepatitis C virus (HCV) and HIV-1. While human parvovirus B19 can establish persistence, yet no data exist on the presence of B19-specific $CD4^+$ CTLs. Detection of vigorous B19-specific granzyme B (GrB) and perforin responses in seropositive individuals points to a role of $CD4^+$ CTL also in B19 immunity. Such cells could function within immune regulation and in the triggering of autoimmune phenomena such as Systemic Lupus Erythematosus (SLE) or rheumatoid arthritis (RA).

The newly discovered MCV resides in approximately 80% of Merkel cell carcinomas (MCC). The integration of MCV genome in-to the genome of host cell has been suggested to be the primary reason for this rare and aggressive skin cancer. Here we studied the T-cell immunity against this carcinogenic virus. We found that interferon-γ (IFN-γ) is the dominant cytokine among MCV-seropositive individuals and suggest that IFN-γ induced inflammatory response plays an important role in surveillance against MCV-induced disease. Our studies also suggested a role for IL-13 and IL-10 in anti-tumor immunity and immune regulation, respectively.

TSV, while exhibiting high seroprevalence in general population, has been detected in *trichodysplasia spinulosa* (TS) skin lesions, suggesting an etiological role in this disease. In order to characterize Th-cell immunity against TSV, and to permit its comparisons with

MCV-specific Th-cell immunity, we studied TSV and MCV-specific proliferation and cytokine responses in healthy volunteers and in one MCC patient. While an association between humoral and cellular responses was detectable with MCV, it was found to be weaker than the humoral and cellular responses detectable with TSV. Despite the significant homology in amino acid sequences of VP1, Th-cell crossreactivity was not evident between these viruses. As $CD8^+$ T-cells specific for MCV LT-Ag oncoprotein clearly provide an important defence mechanism against MCC, the MCV VP1-specific Th-cells may also be important in preventing the oncogenic process, by suppressing MCV replication with antiviral cytokines such as IFN-γ.

Parvoviruses (HBoV1 and B19) and polyomaviruses (MCV and TSV) induce effector $CD4^+$ T-cell responses that are best known for their ability to protect against viral infections. Besides helper functions, $CD4^+$ T-cell contribute to viral control and elimination by CD4-mediated cytotoxic effector functions. Thus, understanding of the $CD4^+$ T-cell immunity is of key importance in the development of vaccines and therapeutic agents against life threatening infectious pathogens.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred in to the text by their Roman numerals.

(I) **Kumar A**, Filippone C, Lahtinen A, Hedman L, Söderlund-Venermo M, Hedman K, Franssila R. Comparison of Th-cell immunity against human bocavirus and parvovirus B19; proliferation and cytokine responses are similar in magnitude but more closely interrelated with human bocavirus. Scand J Immunol 2011; 73(2): 135-40

(II) **Kumar A**, Kantele A, Hedman L, Hedman K, Franssila R. Granzyme B-mediated function of Parvovirus B19-specific $CD4^+$ T cells. (Submitted)

(III) **Kumar A**, Chen T, Pakkanen S, Kantele A, Söderlund-Venermo M, Hedman K, Franssila R. T-helper cell-mediated proliferation and cytokine responses against recombinant Merkel cell polyomavirus-like particles PLoS One 2011; 6(10): e25751.

(IV) **Kumar A**, Kantele A, Chen T, Kavola H, Sadeghi MR, Hedman K, Franssila R. *Trichodysplasia spinulosa*-associated polyomavirus (TSV) and Merkel cell polyomavirus: Correlation between humoral and cellular immunity stronger with TSV. PLoS One 2012; 7(9): e45773

ABBREVIATIONS

AMC	Acute myocarditis
APC	Antigen presenting cell
B19	Human parvovirus B19
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CMI	Cell mediated immunity
CTLs	Cytolytic (or cytotoxic) T lymphocytes
DCM	Dilated cardiomyopathy
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ds	Double stranded
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay (or EIA)
Elispot	Enzyme-linked immunosorbent spot
EMB	Endomyocardial biopsies
Fas L	Fas ligand
GrB	Granzyme B
HBoV	Human bocavirus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JCV	JC polyomavirus (or JCPyV)
KIV	KI polyomavirus (or KIPyV)
LT-Ag	Large T antigen
MCC	Merkel cell carcinoma
MCV	Merkel cell polyomavirus (or MCPyV)
MHC	Major histocompatibility complex
NK	Natural killer cells

nt	nucleotide
OBD	Replication origin binding
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
Rb	Retinoblastoma
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
ss	Single stranded
sT-Ag	Small T antigen
Tbet	T-box 21
TCR	T cell receptor
Th-cells	Helper T cells
TLR	Toll-like receptor
Tfh	T follicular helper cell
TNF	Tumor necrosis factor
Treg	T regulatory cell
TS	Thricodysplasia spinulosa
TSV	*Thricodysplasia spinulosa*-associated polyomavirus (TSV or TSPyV)
WUV	WU polyomavirus (or WUPyV)

INTRODUCTION

Virus infections lead to thousands of deaths annually worldwide and are responsible for billions of dollar economic burden globally. Viruses contain DNA or RNA as their genetic material, enclosed in a protein shell with or without outer lipid envelope. These small infectious molecules replicate only inside the living cell and exploit the DNA/RNA synthesis and protein production machinery of the cell (1). The initial detection of a pathogenic viral invasion triggers the host immune response to induce a complex defence mechanism aimed to limit the extent of infection and subsequently clear it. Immune system is comprised of a network of cells, tissues and specialized organs that work together to protect the host. The immune response to viral infections constitute of innate (non-specific) and adaptive (specific) defence mechanisms (2). Most viral infections are controlled by the innate immune system. However, if viral infection overtakes the innate immune system, the adaptive response must be functional. Humoral (antibody mediated) and cellular (lymphocyte mediated) immune responses are two arms of the adaptive immune response. B and T ($CD4^+$ and $CD8^+$) cells are the key players of adaptive immune system. $CD8^+$ T-cells control viral infection, by directly killing the infected cells while $CD4^+$ T-cells play a key role in command and control, closely interacting with cells of both innate and adaptive arms of immunity (3).

In order to develop efficient vaccines and therapeutic agents against viruses, the understanding of antiviral immune mechanisms is very necessary. Since human DNA viruses; HBoV1, MCV and TSV, are very recently identified, their clinical impacts beyond the first disease associations are just beginning to emerge: thus little is known about their infection kinetics and immunobiology. We established methods for the assessment of antiviral immunity for all these emerging DNA viruses and examined their pathobiology. This thesis is of enormous importance from both academic and clinical perspectives, as the characterization of protective immunity provides a basis for vaccine development.

REVIEW OF THE LITERATURE

Antiviral immunity

Viruses are obligate intracellular parasites that cause infection by invading cells of the body. The immune system has non-specific (innate immunity) and specific mechanisms that attack the virus during its life cycle. Antimicrobial peptides and innate immune cells, e.g., monocytes, macrophages and dendritic cells (DCs) mediate innate antiviral immune functions. Innate immune cells express receptors that recognize pathogen-associated molecular patterns (PAMPs) and trigger the activation of the innate immune response. Furthermore intracellular sensors of viral nucleic acids, e.g., Toll-like receptor 3 (TLR 3), TLR7, TLR8 and RIG-I-like receptor (RLR) family induce the production of various effector molecules, e.g., type I IFNs; cytokines such as IL-12 and IL-27 (4). These molecules act on natural killer (NK) cells to induce IFN-γ production. Chemokines, such as CXC-chemokine ligand 10 (CXCL10), also participate in inducing activation of $CD8^+$ T-cells and Th1 cells of adaptive immunity (4).

Specific antiviral immune mechanisms are both humoral and cellular. Specific antibodies protect against viral infections and play an important role in antiviral immunity, mainly during the early stages of infection. The most effective antiviral antibodies are neutralizing antibodies, which bind to viral envelope or capsid proteins and block the virus from entering into the host cell. T lymphocytes play a crucial role in the adaptive immune response. They include $CD4^+$ and $CD8^+$ T-cells, named after the glycoprotein co-receptor expressed on their cell surface. $CD4^+$ T lymphocytes are mainly considered regulators while $CD8^+$ T-cells are considered cytotoxic effectors of the immune response (5).

CD4 T cell immunity

$CD4^+$ helper T (Th) cells orchestrate immune responses against viral infections. $CD4^+$ T-cells help B-cells to promote antibody production and they are often required for the generation of memory and cytotoxic $CD8^+$ T-cells (3). Recent studies also suggest a role for $CD4^+$ T-cells in enhancement of innate immune responses and in mediating cytotoxic antiviral effector functions (6). $CD4^+$ T-cells also regulate/suppress immune responses both to control autoimmunity and to adjust the magnitude and persistence of responses (7). For mounting effector functions, $CD4^+$ T-cells recognize peptides derived from extracellular proteins presented by MHC classs II molecules on the surface of antigen presenting cells (APC). Pattern-recognition receptors (PRR) are host receptors that detect PAMPs and activate APCs to up-regulate the expression of MHC class II molecules, co-stimulatory molecules (e.g., CD80 and CD86) and pro-inflammatory molecules (e.g., tumor necrosis factors (TNFs), IL-6, IL-2, IL-1 and type I IFNs). In draining lymph nodes naïve virus-specific $CD4^+$ T-cells are primed by activated APCs migrated from the tissues and subsequently these T-cells differentiate to effector cells (6, 8). Different viral infections and many other factors, like dose and route of antigen infection and the targeted organ or cell type affect the priming (6). Differentiation of naïve $CD4^+$ T-cells in-to different subsets is

affected by specific subset of activated APCs, by antigen load, duration of antigen presentation and the pattern and amount of cytokines produced by different APCs (9). Differentiated $CD4^+$ T-cell subsets are classified on the basis of their ability to secrete different cytokines and the expression of specific transcription factors (10).

Th1 cells

Th1 cells are characterized by the production of IFN-γ and the expression of transcription factor T-bet (11). Th1 cells are mainly generated in response to viral infections and they secrete IFN-γ and induce other cells to secrete TNF-α and chemokines (12). IFN-γ is a major antiviral cytokine, produced not only by Th1 but also by cytotoxic $CD8^+$ T-cells and NK cells. It stimulates intracellular killing of microbes and presentation of antigens to $CD8^+$ and $CD4^+$ T-cells by up-regulating MHC class I and II molecules and it also has a direct antiviral effect (13). It is a critical extrinsic tumor-suppressor factor in immunocompetent hosts and it has several types of antitumor activities (14-16). B cell help of Th1 cells is limited by their tendency to kill B-cells (17).

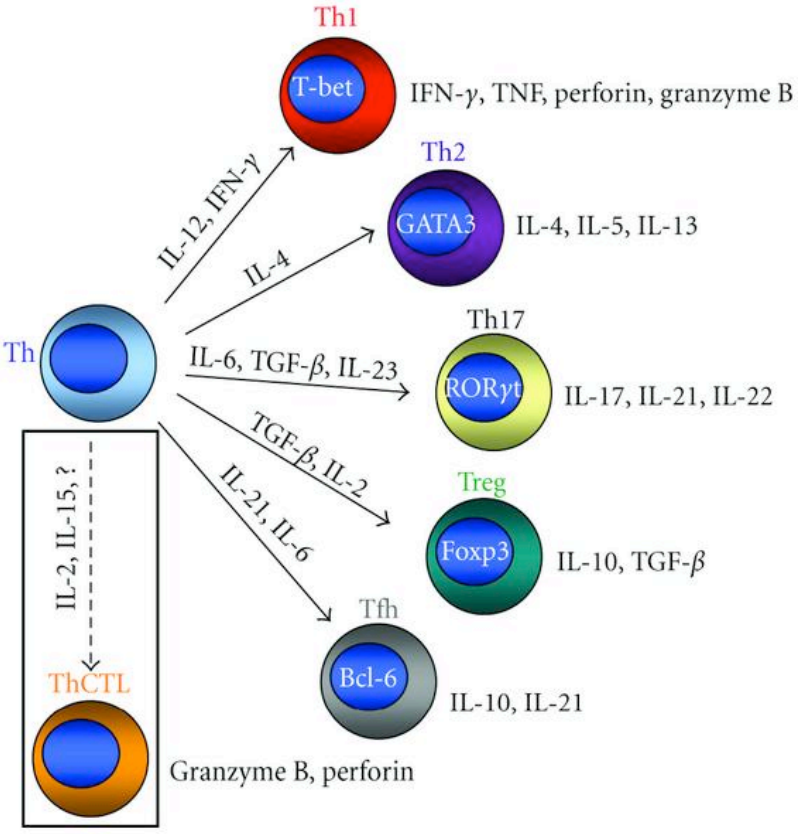


Figure 1: *CD4 T cell effector subsets. A CD4 T cell (Th) can differentiate into unique effector subsets determined in part by the cytokine milieu that is present when the cell encounters an antigen. Effector subsets are classified by the dominant transcription factor in concert with the cytokines that they express. From Marshall et a.l, 2011 (reproduced by the permission)* (11).

Th2 cells

Th2 subset is characterized by the expression of transcription factor GATA3 and secretion of IL-4, IL-5, IL-9, IL-10, IL-25 and IL-13 cytokines (18). IL-13 possesses several unique effector functions including regulation of gastrointestinal parasite expulsion, intra-cellular parasitism, airway hyper-responsiveness, allergic inflammation (19) and class switch to IgE and IgG4 (20). The role of IL-13 in regulating tumor growth depends on the tumor cell type. In some models inhibition of IL-13 or IL-13 receptors has promoted tumor growth (21, 22) whereas in others tumor growth has been inhibited (22, 23). In chronic B lymphocytic leukaemia (B-CLL) models IL-13 has been shown to block apoptosis of tumor cells (24, 25). Secretion of IL-13 has been found elevated in infections by some respiratory viruses and also participate in the pathogenesis of asthma (26). A recent report suggested that increased IL-13 secretion promotes Th2 cell differentiation, which leads to B cell activation (27). IL-10 is an important anti-inflammatory cytokine (28) and its major sources are Th2 cells and a subset of T regulatory cells (Treg) (29).

CD4 T follicular helper cells (Tfh)

These cells are characterized by the expression of transcription factor Bcl-6 and the secretion of IL-4 and IL-21 (6). Tfh cells are found in B-cell follicles of secondary lymphoid organs and important for the formation of germinal centers (30). These cells are best known for providing help to B cells by promoting generation of B cell memory and long-lived antibody-producing plasma cells (6, 30, 31). Hence, Tfh are likely important for generating long-lived antibody responses and protective immunity to most, if not all, viruses (6)

Regulatory CD4 T helper cells (Treg)

Treg cells are involved in the regulation of immune responses and their functions have been reported during viral infections in humans and animals (6). These cells also express FOXP3 transcription factor and secrete IL-10 (32). IL-10 increases B-cell growth, IgG secretion and essential for the maintenance of human germinal centre B-cells *in vitro* (33). Functional activities of Th1 cells, NK cells and macrophages are also inhibited by IL-10 during infection (these cell types are required for optimal pathogen clearance, and also contribute to tissue damage during infection)(28, 33, 34). The role of this cytokine on the immune response against cancer is controversial. As it can inhibit several key phenomena of adaptive immune responses, it has been considered to allow malignant cells to escape from immune surveillance (35, 36). By contrast, there is data to suggest that IL-10 might also favour immune-mediated cancer rejection (37-40).

Treg cells play an important role in controlling pathological conditions e.g., limiting collateral tissue damage, inhibiting autoimmune diseases and allergic diseases mediated by Th2 cells and suppressing anti-viral responses (32, 41). In a mouse model of herpes simplex virus (HSV) infections Suvas et. al. demonstrated that lesions were more severe and animals became more susceptible to infection upon depletion of Treg before infection (42).

However, in case of hepatitis C virus (HCV) and Human immunodeficiency virus (HIV) infections Treg cells appear to contribute to immune dysfunctions and are responsible for viral persistence and chronic tissue damage (32).

T helper 17 (Th17) cells

Th17 cells play an important role in host defence against various extracellular pathogens. Role of Th17 cells in viral infections is not well studied, however, in some mice models virus-specific Th17 responses have been investigated (43, 44). These cells are characterized by secretion of IL-17 and IL-22 cytokines and expression of ROR-γt transcription factor (6). IL-17 facilitates proinflammatory responses from various cell types by attracting and activating neutrophils (10). Studies have suggested that these cells induce inflammation during autoimmunity and IL-17 probably contributes to immunopathology during viral infections (45, 46). However, against some virus infections Th17 cells protect against virus infections (43). Secretion of IL-22 may contribute to tissue repair by regulating the expression of antimicrobial peptides, defensins (47).

$CD4^+$ Cytolytic T cells ($CD4^+$ CTL)

Besides shaping and coordinating different arms of adaptive immune system, $CD4^+$ T-cells can directly perform antiviral effector functions (10). Accumulating data suggest the significance of MHC class II-restricted CTLs in the pathogenesis of autoimmune diseases (48, 49) and in the control of chronic viral infections, such as Epstein-Barr virus (EBV) (50, 51), Cytomegalovirus (CMV) (52, 53), HIV (54, 55), as well as malignancies (56, 57). Cytolytic $CD4^+$ T cells share a common pathway with $CD8^+$ CTLs and NK cells and can kill target cells by two major mechanisms. One involves the interaction of Th-cell surface antigen Fas with the Fas ligand (FasL) on the target cell surface and activates caspase-mediated apoptosis programmes in the target cell (58). The other one is granule exocytosis pathway, which employs perforin and serine proteases called granzymes (59). These cytotoxic effector molecules trigger cell death by activating downstream apoptosis pathways (59). Granzymes, such as GrB, can also cleave other substrates besides caspases (59). This enzymatic activity may potentially contribute to autoimmunity by creating novel autoimmune epitopes from self-proteins (60). It can also mediate direct antiviral activity by cleaving essential viral proteins, as shown in adenovirus (61) and HSV models (62). Perforin is membrane disturbing and pore forming cytolytic protein synthesized in CTL and NK cells (54). Perforin-mediated cytolytic activity has been suggested for virus-specific $CD4^+$ CTL clones that recognize peptides derived from EBV latent membrane proteins, HIV-1 gag protein, poliovirus and dengue virus capsid protein (11, 63-65). Appay et al. showed that a low number of perforin producing $CD4^+$ T-cells are present in the blood circulation of healthy donors and that these cells expanded in donors with chronic viral infections (66).

CD8 T cell immunity

The main effector cells involved in specific antiviral immunity are $CD8^+$ CTLs. These cells recognize viral antigens presented on the cell surface associated with class I MHC molecules (67).

Two mechanisms are responsible for initiating all CTL-mediated apoptotic death of target cells: A) Directional delivery of cytotoxic proteins (perforin and granzymes) that are released from CTLs and enter target cells, B) Interaction of the membrane-bound Fas L on CTLs with the Fas receptor on the surface of target cells (2, 68). CTLs also release cytokines such as IFNs and TNF when they interact with the viral antigen (68). Either of these initiating events results in the activation of a signaling pathway that culminates in the death of the target cell by apoptosis. CTL response is not always beneficial, since the tissue destruction caused by CTL is sometimes greater than the damage done by the virus (68).

Parvoviruses

Parvoviruses are small (about 25 nm in diameter) and nonenveloped viruses. The genome of parvoviruses consists of linear and single stranded DNA (ssDNA) of size ≈5 kb. The family Parvoviridae is divided in-to two sub-families: Parvovirinae and Densovirinae. The family Parvovirinae is further divided in-to five genera: parvoviruses, erythroviruses, dependoviruses, amdoviruses and bocaviruses. The viral capsids are icosahedral and composed of 60 capsomeres. Virus particles of this family are resistant to inactivation by organic solvents because they lack lipids (1).

B19 Virus

Morphology

Cossart et al discovered B19 in 1974 and classified as a member of the *Erythrovirus* genus. It was found accidentaly, while evaluating tests for hepatitis B virus from an asymptomatic patient (69). The name of the virus originated from the identification of the tested sample: number 19 of panel B (69).

B19 is a small, nonenveloped virus with a ss DNA genome of 5.6 kb (5,596 nt long), and with a diameter of 20-25 nm. Internal coding sequence of 4,830 nt flanked by the identical terminal repeat sequences of 383 nt (70). The distal approximately 365 nt of the repeat sequences are imperfect palindromes and they form hairpin like structures and serve as primers in the replication of viral DNA (71-73). B19 mainly has two large open reading frames (ORFs), encoding for non-structural and structural proteins (74). In addition to these two ORFs, B19 genome also contains two additional small ORFs (74). Parvoviruses maximize their coding potential by using partially overlapping transcripts and all three reading frames. Virus particles of B19 consist of 60 units of capsomeres. The structure of parvovirus B19 has been explored by X-ray crystallography at 3.5 (75) and 8.0 A^{o} resolutions (76). Recombinant virus-like particles (B19 VP2 VLP) were used for the determination of structural properties because recombinant capsids are immunologically and structurally similar to the original viruses.

Structural proteins

The major capsid proteins (VP1 and VP2) encoded by genes on the right side of genome (70). Both proteins are encoded by overlapping reading frames; VP2 from nt 3125 to 4786, and VP1 from 2444 to 4786 (70). Viral capsids of B19 consists of major structural protein VP2 (58 kDa) and minor VP1 (83 kDa), which make 95% and 5% part of the capsids, respectively. B19 VP2 alone has the capacity to self assemble in insect and mammalian cell cultures and to make virus like particles that resemble native virions. ORFs of both proteins are similar except the presence of extra 227 amino acids at the N-terminus of VP1, called VP1u (VP1 unique region) (77). VP1u is essential for the infectivity and immunogenicity of B19 virus because of the presence of phospholipase A (PLA2) motif in this region and

the report that PLA2 activity is crucial for B19 infection (78, 79). VP2 mediate receptor binding and both VP1 and VP2 possess the capacity of inducing neutralizing antibodies (70, 80).

Non-structural proteins

The major non-structural protein NS1 (NS1), encoded by the genes located in the left side of the genome. This protein consists of 671 amino acids with a molecular weight of 77 kDa. NS1 is located in the nucleous of the infected cell (74) and has been shown to be cytotoxic and to block cellular proliferation (81, 82). NS1 also mediates several other important functions including, involvement in viral DNA replication (83), regulation of gene transcription (84), induction apoptosis in erythroid lineage cells by interaction with caspase 3 etc. (85).

Small proteins

Two additional small ORFs of B19 encode to small proteins of size 7.5 and 11 kDa (74), respectively. Precise role of these two proteins is still known, however, recent studies have reported that the 11 kDa protein participated in regulating the production rates of B19 capsid proteins (86). Also this protein induces apoptosis during B19 infection of primary erythroid progenitor cells. However, the role of the 7.5 kDa protein is not explored yet (87).

Pathogenesis and infection

Humans are the only known host for B19. The life cycle of B19 virus includes attachment of the virus to the host cell receptors, penetration (endocytosis), uncoating, DNA replication, ribonucleic acid (RNA) transcription, protein translation, assembly of virions, and finally cell lysis with release of the virions (70).

In 1983, Mortimer et al., has shown that B19 virus inhibits the process of erythropoiesis by infecting human erythroid progenitor cells in bone marrow and blood (88). Cellular receptor, P blood group antigen globoside (Gb4), has been found responsible for extreme tropism of B19 (89). Besides the cells of erythroid lineage globoside receptor is found also on platelets, heart tissues, lung, liver, endothelium, and kidney and on synovium (90, 91). Individuals lacking this receptor are resistant to B19 infection (92). Further on, it has been reported that only the presence of globoside receptor is not sufficient for the entry of B19 in-to cell, however, this receptor is necessary for cell infection. Therefore two additional co-receptors, α5β1 integrin (93) and Ku-80 (94) have been suggested which allow the entry of B19. Mature human red blood cells (RBCs) lacking the presence of α5β1 receptor but with a high expression of P antigen, only bound virus but do not allow viral entry. In contrast erythroid progenitor cells facilitate the entry of B19 because these cells express high level of both receptors (93). However, Ku80 was shown to enhance the entry of B19 suggesting that Ku80 mediate efficient B19 entry in cooperation with the other two receptors (94). B19 replicates in the nucleous of the host cells and completes the infection cycle, like other DNA viruses, in following steps: attachment to the host cell receptors,

internalization, transfer of genome to the nucleus, DNA replication, RNA transcription, assembly of the capsids, packaging of the genome, cell lysis and release of infectious virions (70).

Transmission and epidemiology

The transmission of B19 occurs generally by personal contact via aerosol or respiratory secretions. In one study, B19 was administered internasaly in-to voluntary healthy adults who subsequently became viremic and seroconverted (95). B19 can also be spread through blood and plasma products, organ transplantation and vertical transmission from mother to fetus during pregnancy (70, 96). The B19 receptor is abundant in the human placenta in early gestation, which might provide a pathway for the virus (97). B19 infection is a very common infectious agent in humans. B19 infections appear all through the year, but highest season is in late winter and spring, with major epidemics every few years (98). B19 circulates globally and serological studies have shown that B19 seroprevalence increases with age. Approximately, 15% preschool children, 50% adults and 85% elderly individuals show serologic evidence of past infection (99, 100). B19 infection is supposed to affect males and females in equal numbers, but in some studies females were more often B19-seropositive than males (101, 102). It is evident that persisting IgG antibodies provide a lifelong immunity and the presence of viremia and viral DNA in PBMC is very rare among healthy individuals. Although B19 has been found associated with a wide spectrum of diseases, large proportion of infections remains subclinical, both in children and adults. In some cases symptoms are nonspecific and cannot be differentiated from common cold and some of the clinical manifestations are mild and self-limited. B19 infection can be more severe in patients with shortened red cell survival, in pregnant women and in immunocompromised individuals (70).

Persistence in human tissues

B19 is capable of persisting in the circulation of immunocompetent and immunocompromised individuals (100, 103-105). The asymptomatic presence of B19 in healthy hosts is not generally recognized because such a phenomenon is mainly observed in cases associated with disturbed immune condition. B19 DNA has been shown to persist in bone marrow (70, 106), synovial tissues (107, 108), liver and kidney (109-111), brain (106), thyroid (112, 113), myocardium (114, 115) and skin (116, 117).

The exact mechanism of B19 persistence is not known. There are many thoughts about the viral persistence, e.g., Norja et al. (118) suggested that B19 genome can persist in different human organs for whole life and the genome can serve as the source of persistent replication. Another possibility proposed is that B19 could integrate into the human genome, as shown for other viruses (77, 119). Finally, it has been suggested that B19 DNA could retain in the synovium as a full length genome (107). If the persistent B19 DNA is not active, the most important issue will be whether the latent B19 infection can be reactivated under a certain condition, such as stress, immunosuppression or co-infections by other viruses.

Clinical manifestations

The clinical manifestations associated with B19 infections may range from benign to life threatening. Clinical symptoms are influenced by the infected individual's immunological condition (immunocompromised or immunocompetent), age and haematological status. There are many B19-associated syndromes reported, however, only few of them are well established.

Erythema infectiosum: The most common clinical presentation of B19 infection in immunocompetent individuals is erythema infectiosum or fifth disease or slapped cheek (120). Erythema infectiosum typically affects school-aged children. This disease is characterized by facial rash followed by appearance of rash on the trunk, rheumatic symptoms, mild fever and malaise (121). It has been suggested that the symptoms of B19 infection are due to the formation and deposition of immune complexes in the skin and other parts of the body. The rash associated with erythema infectiosum may be transient and recurrence may be provoked by exposure to sunlight, heat, emotion and exercise (121). Approximately 25-50% of these infections are asymptomatic (77, 122).

Arthralgia and Arthritis: Arthralgia and arthritis are common clinical complications found associated with B19 infections (123, 124). Approximately 50% (125) of adults and 10% (126) of children with erythema infectiosum suffer from joint manifestations. Arthropathy is more common in adult females (approx. 60%) compared to men (30%) (70) and characterized by polyarthritis typically involving metacarpophangeal joints, knees, wrists, or ankles (123, 125). B19-associated arthropathy usually resolves with in a few weeks, and even when symptoms persist for months or year, joint destruction does not occur (121).

It has been suggested that deposited antibodies against B19 in synovial fluid of joints, are responsible for joint pain (127). Sometimes arthropathy mimics classical rheumatoid arthritis (RA), however, B19-associated pathogenesis is unclear (128). To investigate the possible role of B19 in RA, primary human synovial fibroblasts were treated with B19 virus containing sera for 7 days (129). Incubation with B19 containing serum induced an invasive phenotype in fibroblasts and when the viremic serum was pre-incubated with neutralizing antibodies to B19 the effect was suspended (129). Presence of B19 DNA in synovial fluid (130), cells (131) and tissue (132) of affected joints also support the role of B19 in RA. Structural protein of parvovirus B19 was also seen in the synovial lymphocytes in patients with RA (133). Non-structural protein of B19 (NS1) induces the activation of IL-6 gene expression and it is known that over-production of IL-6 is responsible for the activation of autoreactive T cells and appearance of auto-reactive antibodies, including rheumatoid factors (84). IL-6 could also be responsible for bone destruction and osteoporosis (134). It has been suggested that B19-associated arthritis is genetically associated being more common in individuals with HLA DR4 or B27 (103, 135, 136). Secreted phospholipase A2 motif in the exposed B19 VP1 unique region may activate synoviocytes and accelerate the inflammatory responses in synovial tissues and thus contribute to the B19-associated atrhropathy (137).

Chronic parvovirus B19 infection has been shown to be associated with production of antibodies directed against auto-antigens, like human keratin and collagen type II and a direct correlation between arthritis and a clinical feature was found (138). There are many pieces of evidence suggesting that B19 infection is not associated with RA; there was no evidence of inflammatory arthritis in 54 patients with recent B19 infection after long term follow up (median 5 years) (139). It was also shown that the presence of B19 DNA in synovial membrane is not sufficient to confirm a link between virus and RA (140).

B19 and pregnancy: Pregnant women lacking B19 specific antibodies are at risk of acute B19 infection and subsequent transfer of the virus to the fetus (70). Fetal infection may be asymptomatic, but it has also been associated with fetal anaemia, spontaneous abortion and hydrops (141). Hydrops fetalis is a condition in the fetus defined by the presence of generalized fetal subcutaneous tissue accumulation of fluid (edema) in at least two fetal compartments (77). B19 has been found to be associated with fetal hydrops in about 8-17% cases and fetal death occur typically during the second trimester but sometimes also during the first trimester (142-144). Some reports also suggested that the asymptomatic infection in pregnancy carries a higher risk of transmission because it can be connected with weak immune response unable to prevent B19 replication (77). Many mechanisms for the development of B19-associated hydrops fetalis have been described, one report suggested that parvovirus B19 interrupts the process of erythrocyte production (erythropoiesis) and induces the development of aplastic crisis and heart failure (103).

Anaemia: In subjects with shortened red cell survival, the B19 infection may lead to aplastic crisis because B19 is erythrocytotropic and infects the red cell precursors in bone marrow (145). In healthy subjects, this condition is transient, but in immunocompromised individuals, e.g., subjects with HIV infection, congenital immunodeficience, or those receiving immunosuppressive therapy, anaemia may become chronic (146, 147).

Autoimmune diseases: Apart from RA, B19 has been found associated with many autoimmune diseases, e.g., systemic lupus erythematosus (SLE), juvenile idiopathic arthritis, Sjogren's syndrome, primary biliary cirrhosis, polymyositis, dermatomyositis, autoimmune cytopenia and vasculitis (148-150). Various studies suggested that mechanism of molecular mimicry between host proteins and viral protein is responsible for autoimmune disorders (138, 151, 152). Other possible mechanisms for the induction of autoimmunity included enhanced cytokine production by NS1 protein (84, 153, 154) and phospholipase A2-like activity of VP1u (78, 151).

Other diseases: Many studies proposed an association of B19-infection with heart related diseases (155-157). B19 might also be involved in pneumonia (158), nephritis (159), liver-associated diseases (160), and neurological disorders including meningoencephalitis, cerebellar ataxia, seizure and stroke (161, 162).

Diagnosis

Because of the difficulties in propagation of B19 virus in tissue culture laboratory the diagnosis of B19 is totally depend on the methods of antibody and nucleic acid detection (121). Detection of the B19 can also be done by electron microscopy in plasma and fetal tissues especially in case of high-titre viremia during acute infection. B19 proteins can also be detected in tissue by immunohistochemistry, however, this is a time consuming procedure (77, 163).

Most of the antibody based diagnostic assays rely on insect cell-expressed recombinant B19 virus-like particles. Acute B19 infection is usually diagnosed by the presence of B19 specific IgM usually after 10-12 days of infection and antibodies remain detectable up to 3-4 months or sometimes even longer (99). IgM antibodies against NS1 may take over six weeks to develop after the onset of illness, which explains the lower prevalence of anti-NS1 antibodies in persons with acute B19 infection (164). IgG antibodies may be detected about two to three weeks after infection and they can persist life-long and protect against reinfection. Seroprevalence of B19-specific IgG has been estimated to be as high as 30-60% among adult population (70). Children usually get infected after entering the school, yet 25% of the cases remain asymptomatic (70).

PCR (Polymerase chain reaction) is a very sensitive method for the detection of B19 DNA from serum and tissue samples. PCR based assays are important for the diagnosis of a persistent infection when antibody production is absent or low (165). Acute infection can be detected by both IgM ELISA and PCR from serum samples (165). Hybridization of PCR products with B19-specific probes improves sensitivity and specificity and confirms the results (166). However, PCR test alone, without serological tests, is not reliable, because it can be positive for several months after acute infection (141).

Treatment of B19 infections and vaccine development

There is no antiviral drug against B19 infection available. Among the immunocompetent individuals treatment is not required and infection can be self-controlled. Number of different options for the treatment of B19 infections can be used for different diseases and risk groups of patients. Patients with arthralgia may be treated with anti-inflammatory drugs. Among immunocompetent hosts sometimes non-sterdal anti-inflammatory drugs can be used for the treatment of arthralgia (103). Immunosuppressed patients or subjects with increased turnover of red blood cells, chronic anaemia or transient aplastic crisis may be treated with erythrocyte transfusions and intravenous immunoglobulin (IVIG) containing neutralizing antibodies. However, sometimes immunoglobulin therapy is not able to complete viral clearance (103, 121).

Empty virus-like particles are being produced and used as vaccine candidates for parvovirus B19. Previously two vaccine candidates based on B19 VLPs have been tested in phase I clinical trials (167, 168). The vaccines consisting of 25% VP1 and 75% VP2 have been tested evaluated for the induction of humoral and cell mediated immunity (167, 169,

170). These vaccine candidates were produced in insect cells by a baculovirus expression system. When these VLPs were administered with MF59 adjuvant, strong neutralizing antibody responses were occurred along with some side effects e.g. headache, fatigue etc (167, 169). Very recently, Novartis Vaccine developed a new B19 VLP vaccine candidate in *Saccharomyces cerevisiae* (171). This vaccine has been produced by co-expression of VP2 and either wild type VP1 or phospholipase-negative VP1 in a regulated ration from a single plasmid. Strong neutralizing response was found after mice immunization with vaccine candidate. The purity, homogeneity, yeast origin, and lack of phospholipase activity of these VLPs address potential causes of previously observed reactogenicity (171).

Humoral Immunity

Approximately 50% of adults and children are seropositive for B19, indicates that B19 is very common infection (99, 100, 103). B19-specific IgG and IgM antibodies directed against capsid proteins are produced after infection and provide a long-term immunity. B19-specific IgM antibodies are detected during the second week after infection (late in the viremic stage) and start declining at the second month after onset of the illness, however, in some cases IgM antibodies may be found for several months (70). B19-specific IgG antibodies start appearing after about 15 days of infection and persist for life long and protect against secondary infections (70). B19-specific IgA antibodies are also detected in IgG seropositive subjects (172). The basis of persistent infection is a defect in immunoglobulin production. Serum from patients with persistent infection lacks antibodies to B19 or contains a low level of non-neutralizing IgM or IgG antibodies (147). It has been shown that presence of antibodies to VP1u is important for the clearance of the virus (103). It has been shown that denatured capsid protein antigens, membrane protein spotting, and conformational viral like particles that IgGs recognizing the conformational epitopes of VP1 and VP2 remains for the life, but IgGs against linear epitopes of VP2 are signs of acute infection (173-175). Measurement of IgG avidity and epitope-type specificity (ETS) can be used to identify primary infection (141, 173-176). However, antibodies against NS1 have been reported only in acute and persistent infections (177-180). Interestingly, IgE antibodies also have been detected in an acute and recent B19 infection (181).

Cellular Immunity

Cellular immunity is the second arm of the specific immunity developed during B19 infection and plays an important role against the infection. Humoral immune responses are well characterized, however, cellular immunity induced against B19 have not been investigated widely.

***CD4 T cell Immunity*:** Only few reports described $CD4^+$ mediated responses against VP1 and VP2 (103, 170, 177, 182, 183). Till date most of the studies are based on *in-vitro* experiments for the characterization of antiviral CD4 T cell immunity.

In 1996, von Poblotzki et al., studied lypmphoproliferative responses by using prokaryotically expressed recombinant VP1, VP2 and NS1 proteins among healthy

individuals infected with B19. The results showed that B19-specific $CD4^+$ responses were HLA II-restricted and directed against the capsid proteins VP1 and VP2, however, responses against NS1 appeared only in two out of 10 subjects (184). Further on, immune response to baculovirus expressed virus capsid proteins, VP1 and VP2 among recently infected children with erythema infectiosum disease and healthy adults with no record of recent B19 virus infection were characterized. Compared to recently infected children IFN-γ response against VP1 and VP2, a very strong response among healthy individuals, suggesting that children with recent infection have defective IFN-γ responses and Th1-like immunity directed against B19 among healthy adults (177). It is known that VP1u is the major target for Th cells among recently infected children because stronger proliferation responses were detected with VP1 than VP2 (177). In this study authors did not explain the nature of the antigens (capsids or linear proteins) and absence of VP1u (separately) in T cell experiments, failed to explain the exact nature of antiviral T cell responses. In 2001 Franssila et al studied Th cell immunity by using B19 VP1/2 containing approximately 33% VP1 and 66% VP2, the ratios recommended for vaccine use. This study for the first time reported long term memory responses to B19 and showed Th-proliferation responses against B19 structural proteins in recently infected adults (182). The B19-specific T cell responses, in general, were most vigorous among the recently infected patients. However, such strong B19-specific proliferation was not confined within the acute phase, as 28% of the previously infected healthy individuals had B19-specific reactivity persisting at acute-phase levels, apparently for years or decades. These data indicate that B cells recognizing the VP1/2 capsids receive class II– restricted help from $CD4^+$ T lymphocytes (182). Reduction in IFN-γ responses during pregnancy among pregnant women with no clinical evidence of recent B19 infection, explain that weaker B19-virus immune response may increase suspectibility to fetal B19 infection (185).

Further on it was also shown that VP1/2 or VP2 alone are capable of inducing similar IFN-γ, IL-10 and proliferation responses in humans long after infection (186). Capsid protein VP2 contains epitopes capable of inducing Th-cell responses and B19-specific B-cells of previously infected subjects receive T-cell help via an epitope with in VP2 (170). Th cell immunity was also determined against prokaryotically expressed VP1u among recently and previously infected subjects. Compared to VP2 capsids, strong VP1u-specific IFN-γ and IL-10 responses detected in recently infected subjects suggest poor recognition of VP1u by PBMC (186). Subsequently, transiently transfected B-cells expressing VP1-unique region were used in an ELISpot to access T-cell immune responses against VP1u in PBMC. Significant numbers of IFN-γ secreting $CD4^+$ cells were detectable in PBMC of all individuals with recent, acute or persistent B19 infection, but not in PBMC of donors with past B19 infection and seronegative individuals (183). This data indicate that the presence of VP1u rgion is required for the maintenance of VP1-specific $CD4^+$ T-cells that are primed during acute infections. Along with previous findings this study also suggests the presence of several Th epitopes in VP1u. Overlapping peptides were used for the assessment of NS1- and VP1-specicifc immune responses among individuals with B19 persistence. Strong responses were found with structural proteins, however, responses with non-structural proteins were of lower magnitude (187). Possibility of liver involvement in persistent infection was reported in an immunocompetent individual suffering from acute hepatitis

and polyarthritis (188). Though, symptoms of arthritis and hepatitis resolved with-in a few weeks, B19 viral DNA in serum and $CD4^+$ T cell responses for VP1u were detectable more than 6 months after the onset of symptoms (188). B19 DNA is frequently detected in endomyocardial biopsies (EMBs) from patients with acute myocarditis (AMC) and dilated cardiomyopathy (DCM), but also in various healthy tissues (189-191). The biological and clinical relevance of B19-DNA persistence in myocardial tissue remains incompletely understood. In order to investigate the role of B19 in AMC/DCM in more detail, Lindner et al analysed B19-specific $CD4^+$ T-cell mediated immune responses in healthy individuals and patients with detectable B19-DNA in EMBs (192). Slightly lower B19-specific IFN-γ responses against VP2 and VP1/2 capsids were detected in AMC/DCM patients as compared to the healthy controls and no differences in virus-specific serology. Also correlation between the B19 DNA load and number of B19-specific T-cells in EMBs was not found significant. Data suggested that only detection of B19-DNA in EMBs is not sufficient to associate B19 with AMC/DCM (192).

T-cell immune responses against recombinantly expressed NS1 were reported among recently infected and B19-exposed individuals. Proliferation responses at higher frequency were eveident in recently infected patients with or without arthropathy. Interestingly, NS1 reactive lymphocytes were also found in three B19 seronegative patients, two of them were recently exposed to B19 but without clinical symptoms. Therefore, T-cell immune response to NS1 may indicate a recent infection rather than development of arthropathy (193).

CD8 T cell Immunity: Viral infections cause an immunological disbalance that triggers CD8 T-cell immune responses. These virus-specific T-cells play major role in clearing acute infections, limiting persistent infections and providing lifelong protective immunity. Emergence of innovative techniques for *in vitro* analysis of CD8 T-cell mediated immune responses has revealed an important role of these cells against viral infections (5, 194-196). Despite the importance of $CD4^+$ T-cells in B19 infection, little is known about the B19-specific $CD8^+$ T-cells. In B19 infection, NS1-specific $CD8^+$ T-cells may have important role. Tolfvenstam et al. in 2001 first initiated studies on CD8 T-cell immune responses against NS1 protein of B19 in seropositive individuals (197). In an HLA-B35 positive individual, vigorous cytotoxic T-cell response has been shown against a 9-mer-epitope derived from NS1 protein. This epitope was able to stimulate $CD8^+$ T-cells for IFN-γ secretion *ex vivo*, and these cells were very frequently detectable from B19-seropositive individuals. In order to determine the number of these NS1-specific $CD8^+$ T-cells, Elispot and HLA-B35 tetramer staining experiments were carried out for IFN-γ. These experiments showed a frequency of NS1-specific $CD8^+$ T-cells as high as approximately 300 spot forming cells (SFC)/10^6 PBMC, a frequency higher than HLA-A2-restricted influenza- and EBV-specific CTLs (197). These virus-specific $CD8^+$ T-cells also showed cytolysis, *in vitro* expansion and effector functions (197). The spotting of epitopes for $CD8^+$ T-cells also provide possibilities to analyse the potential role of such effector cells in B19 associated diseases. Because of the small size of B19 genome, it is possible to map completely epitopes inducing cellular immune responses.

By Elispot technique Klenermann et al. explored CD8 T-cell responses among asymptomatic B19-seropositive individuals by using peptide pools spanning the whole NS1 (198). Cellular responses were found against several peptide pools and suggested that NS1 may contain many $CD8^+$ T-cell epitopes and significant population of memory $CD8^+$ T-cells persist in normal B19-seropositive individuals (198). CTL responses against B19 were further studied by using peptides pools spanning whole VPu, VP2 and NS1 with PBMC obtained from 5 acutely infected subjects (199). Vigorous NS1-specific CD8 T-cells responses were observed in all 5 subjects and these responses were well maintained during follow up. This further explains that NS1 contained several epitopes for B19-specific $CD8^+$ T-cells. Interestingly, only 2 subjects showed CTL responses against VP2 and none against VP1u (199). Thus, $CD8^+$ T-cells are crucial for controlling parvovirus B19 infections. By multimeric HLA-peptide complex analysis of B19-specific $CD8^+$ T-cells were studied during and after acute infections among adult individuals (195). Frequency of B19-specific $CD8^+$ T-cells in peripheral blood of 11 adults with acute B19 infection continued to increase for many months following the resolution of symptoms. All acutely infected individuals showed B19-specific $CD8^+$ T-cell percentages ranging from 0.09% to 4.5% of total PBMC $CD8^+$ T-cells. Next, phenotypic makers and effector functions of these virus-specific $CD8^+$ T-cells were studied *ex vivo* (195). All acutely infected subjects maintained high level CD38 expression with strong expression of perforin and CD57 and down regulation of CD28 and CD27. Expression of CD57 increased over time in all most all acutely infected subjects. Most of these features were shown previously in antiviral T-cell responses to CMV, where expression of intracellular perforin, loss of co-stimulatory molecules CD27 and CD28 and loss of lymph node homing marker CCR7 reported (200-202). Importantly, these kind of cells characterized as; 'terminally differentiated' with sustained expression of CD57 (203). These results suggest that these cells are phenotypically mature and strongly activated *in vivo* ($CD38^+$) (195). Lower frequencies (around 0.05-0.5% of total $CD8^+$ T cells) of B19-specific $CD8^+$ T-cells with lower expression of perforin, CD38 and CCR7 were found when subjects were tested many years after infection. In these experiments NS1 gene appeared a major target of CD8 T-cell responses during acute B19 infections. This extensive study suggest that B19 persists in some form after acute infection and induce sustained activated CD8 T-cell responses and may contribute to the long term control of B19 virus (195). Polymorphic HLA class I present antigenic peptides to cytotoxic T-cells and mediate protective immunity (194). Particular HLA-I allotypes are associated with protective immunity phenotypes in infectious diseases and inflammatory diseases (194). Kasprowicz et al. (204) investigated CD8 T-cell mediated immune responses, in individuals positive for HLA-A$*$2402 (a common HLA1 allele in East Asia) with acute B19 infection. Out of 7 of these individuals, 6 exhibited vigorous $CD8^+$ T-cell mediated cytotoxic responses to NS1 (FYTPLADQF) epitope. PBMC of these individuals were also co-stained with an anti-CD8 antibody, B19/HLA tetramers and monoclonal antibodies against 11 different TCR Vβ (T cell receptor Vβ) chains. All responders showed highly focused TCR usage, both acute and previously infected patients used almost exclusively TCR Vβ5.1. These results can be exploited for vaccine design because HLA-A$*$2402 is a very common epitope with-in East Asian HLA types (204). Bluth et al. (205) reported that increased $CD8^+CD60^+$ T-cells in IgA-deficient individuals might regulate IgE memory responses and isotype switching. It

has been suggested that CD60 on activated T or B-cells may facilitate contact with other helper T-cells, influencing either T-T or T-B-cell interactions. Little is known about the role CD8 T-cell immunity in B19 associated heart diseases (190, 206). In 2008 Streitz et al (207) confirm expression of highly restricted TCR Vβ11 expression in a HLA A*02, A*11, B*07 patient with EMBs proven DCMi by using B19-NS1 peptide. These results suggested that B19-specific $CD8^+$ T-cells with effector functions are involved in B19 associated DCMi and dominant role of $CD8^+$ T-cells effector cells are crucial for anti-viral immunity (207). Thus, NS1 appears to be a major target of B19-specific $CD8^+$ T-cells and plays an important role against infection.

Human Bocavirus 1

Morphology

In 2005, a new member of the family Parvoviridae was discovered by Allander et al. in clinical specimens from children with respiratory tract infections by random-PCR and large-scale sequencing techniques (208). The new virus was named human bocavirus (HBoV) because the deduced amino acid sequence showed that the closest relatives of this virus were bovine parvoviruses and canine minute virus of the genus *Bocavirus* (208). Later in 2009 three new species of human bocavirus were discovered named HBoV2, 3 and 4 (209-211). After the discovery of these three additional species of human bocavirus the HBoV was denoted as HBoV1. HBoV1 is a nonenveloped virus of icosahedral symmetry and diameter of about 25 nm. HBoV1 contains genetic material as a single stranded DNA molecule of size of about 5 Kb. The viral genome encodes two non-structural proteins (NS1 and NP1) and two major structural proteins (VP1 and VP2) (208). Capsid proteins VP1 and VP2 are identical except the presence of an additional 129 amino acid peptide, VP1 unique (VP1u), in VP1. Like parvovirus B19 (78), VP1u of HBoV possesses a phospholipase A2-like activity (PLA2) (212). Multifunctional NS1 protein possesses DNA binding and transcription activation domains and plays essential roles in virus replication. Possible role of NP1 has been shown in a very recent study, which suggests that NP1 may involve in cell cycle arrest and apoptosis (213). Phospholipase activity is thought to help in parvoviral infectivity by mediating the transfer of the viral genome from endocytic compartments to the nucleous of the host cells to initiate replication (79).

Clinical Features

Human parvovirus B19 has been for years the only parvovirus known to be pathogenic in humans. HBoV is the second known parvovirus species pathogenic to humans. A number of cases have been reported, to establish a causal link between HBoV and respiratory disease (214-216). Clinical symptoms of HBoV1-related acute respiratory illnesses including common cold, asthma, acute wheezing, bronchiolitis, pneumonia and acute otitis media, are reported in various studies (217). HBoV1 has been detected in co-infections with other respiratory viruses such as, rhinovirus, RSV, human adenovirus, human metapneumovirus and influenza virus. It is very hard to clinically differentiate between respiratory tract infections caused by these viruses and HBoV1 (218). Hypoxia and neutophilia are some of the symptoms found more severe with HBoV1 compared to RSV among children with lower respiratory tract illness (219). Some of the findings also reported the presence of HBoV DNA in 1-10% fecal samples of children with gastroenteritis (220-223) and in 1.5% of adults (224). HBoV1 has been detected in 45% of fecal samples with HBoV1- positive respiratory samples (225). However, so far no clear clinical association between HBoV1 and gastroenteritis has been detected.

Epidemiology

Globally HBoV1 has been detected in nasopharyngeal, serum, fecal and urine samples (209, 214, 226-230). Presence of HBoV1 DNA has been detected in 2-19% of patients with upper or lower respiratory tract disease (208, 214, 217, 231, 232). HBoV1 infections are mainly detected in early childhood year around but most commonly during winter (216, 217, 231, 233). Presence of HBoV1 DNA among adults is less frequent compared to children (224, 226, 228, 234, 235). Seroepidemiological studies have shown that >90% of children at the age of over three years are seropositive for HBoV1 (217, 236). Persistence of low-level antibodies can be found until the age of 6-12 months after that all most all children become seropositive until the age of 6 years (216, 231, 236, 237). Most of the adults are exposed to this virus and 100% seroprevalence clearly indicates that HBoV1 infections are very common (216, 238, 239).

Cellular immunity

In addition to humoral immunity, cellular immunity also plays an important role against viral infections. Because of difficulties in the cultivation of the virus, recombinant VLPs were used for the study of cellular immune response against HBoV1. There is not much data available on T-cell immune responses against HBoV1. Lindner at al. first investigated cellular immune responses in healthy individuals by stimulating PBMC with HBoV1-VP2 VLPs and reported production of IFN-γ secreted by $CD4^+$ T-cells (240). Compared to RSV-related bronchiolitis, children with HBoV1-related bronchiolitis have shown increased levels of the Th1 and Th2 cell cytokines, e.g., IFN-γ, IL-2 and IL-4 (241). However, the concentrations of IL-10 and TNF-α were lower in children with HBoV1-induced than RSV-induced bronchiolitis. These findings suggest that HBoV1 can elicit typical virus-induced immune responses involving both Th1 and Th2 cells; however Th-2 polarization was not evident (241). Previous studies strongly suggest that HBoV1 induce respiratory symptoms and therefore the role of cytokines involved in respiratory pathology should also be investigated.

Diagnosis and treatment

The most reliable diagnostic methods for HBoV1 infection are quantitative PCR and EIA-based techniques. Clinical significance of HBoV1 can be determined by quantitative PCR, as higher viral loads correlate with acute infections, fewer co-infections and increased disease severity (216, 229, 237, 242). Recombinant virus-like particles of HBoV1 have been utilized for the serodiagnostic assays for HBoV1 specific IgG and IgM antibody detection (216, 238). Although HBoV1-4 cross-react, reliable seroprevalence of HBoV1 can be determined by depletion of HBoV2–4 reactive antibodies (243). For more accurate diagnosis of HBoV1, a method to measures IgG avidity by EIA has been developed by Hedman et al. (244). IgG EIA can be used for the determination of primary and secondary infection, acute and past infections and for immune activations as well (244).

No specific antiviral agent or vaccine is available for the treatment of HBoV1 infections. The course of HBoV1 disease is often self-limiting and uncomplicated; however, standard precautions and preventive measures should be applied to limit the transmission of the virus (217).

Polyomaviruses

Polyomavirus is the only genus belonging to family Polyomaviridae. Members of this family are small (40-45 nm in diameter), nonenveloped and containing a single molecule of circular dsDNA genome of size of about 5 kb (245). Polyomaviruses are widespread in the human population. Polyomaviruses cause persistent latent infections that are usually asymptomatic and polyomavirus-associated diseases occur mainly after reactivation of the virus in immunocompromised individuals (246). Till date 12 polyomaviruses have been identified (Table 1).

Table 1. *Human polyomaviruses and associated clinical manifestations (modified by permission from DeCaprio et al. 2013* ((247).

Polyomaviruses and Year of discovery	Short Name	Source of Isolation	Disease Association
BK polyomavirus, 1971 (248)	BKPyV or BK	Urine	Polyomavirus associated nephropathy, haemorrhagic cystitis
JC polyomavirus, 1971 (249)	JCPyV or JC	Brain, urine	Progressive multifocal leukoencephalopathy
Karolinska Institute polyomavirus, 2007 (250)	KIPyV or KI	Nasopharyngeal tissue	None
Washington University polyomavirus, 2007 (251)	WUPyV or WU	Nasopharyngeal tissue	None
Merkel cell polyomavirus, 2008 (252)	MCPyV or MCV	Skin	Merkel cell carcinoma (MCC)
Human polyomavirus 6, 2010 (253)	HPyV6	Skin	None
Human polyomavirus 7, 2010 (253)	HPyV7	Skin	None
Trichodysplasia spinulosa-associated polyomavirus, 2010 (254)	TSPyV or TSV	Skin	Trichodysplasia spinulosa
Human polyomavirus 9, 2011 (255)	HPyV9	Skin, urine, blood	None
Malawi polyomavirus, 2012 (256)	MWPyV	Stool, wart	None
St Louis polyomavirus, 2012 (257)	STPyV	Stool	None
Human polyomavirus 12, 2013 (258)	HPyV12	Liver, cecum, rectum	None

Merkel Cell Polyomavirus

Merkel cell carcinoma (MCC) is an aggressive tumor of neuroendocrine origin (259). MCC is very rare and a lethal skin cancer with a mortality rate greater than 30%. MCC occurs mainly in elderly individuals and UV exposure may be a risk factor (259). In 2008 Feng et al. discovered Merkel cell polyomavirus (MCV) by digital transcriptome subtraction (DTS), a technique that can identify foreign transcripts by using human high-throughput cDNA sequencing data (252). MCV was found monoclonally integrated in-to the genome of approximately 80% of human MCCs (252). Circular genome of MCV is about 5.4 kb long and contains early (small T antigen (sT-Ag), the large T antigen (LT-Ag), the 57kT antigen) and late genes encoding (capsid proteins VP1, VP2 and VP3) (252, 260-262). Major structural protein VP1 of MCV interact with host cell surface and after endocytosis the viral DNA is transported to the endoplasmic reticulum and then finally to nucleus for replication (263, 264). A recent study suggested that minor capsid protein VP2 facilitates post attachment stage of MCV entry into some cell types (265). Furthermore this study indicated that VP3 is absent in MCV-infected cells and is not found in native MCV virions and mutation in VP3 gene did not significantly affect MCV infectivity (265).

Early genes are critical for the initiation of viral DNA replication and they are produced by alternative splicing (262). MCV LT-Ag contains many conserved domains, required for polyomavirus genome replication. These domains are present across different polyomaviruses such as DnaJ and LXCXE retinoblastoma (Rb) protein binding motifs, a OBD domain, an ATPase/helicase domain and a helicase domain (262). It has been shown that LT-Ag of SV40 is an oncoprotein, inactivates retinoblastoma (pRB) and p53 protein (266). Studies also reported that LT-Ag also arrests the cell cycle by inhibiting G1 checkpoint (267). Mutated MCV LT-Ag has been found from MCC tumors and mutations were present in pRb binding domain (this domain is critical for tumorigenesis and consequently affect replication). Thus non-mutated MCV LT-Ag undergo cell lysis or death and cells with mutation are allowed to survive and responsible for uncontrolled growth of infected cells (260). Mutations in LT-Ag can also affect cytotoxic T-cell immunity, as epitopes for cytotoxic T-lymphocytes are present on LT-Ag (268).

The sT-Ag encodes a domain mediating protein phosphatase 2A (PP2A) binding (262) and enhances the functions of LT-Ag (269, 270). Additionally, the sT-Ag promotes mitogenesis, cell proliferation and cellular transformation by reducing the hyperphosphorylated eukaryotic transcription initiation factor 4E-binding protein 1 (4E-BP1) turnover in a PP2A or DnaJ domain independent manner (271). Its inhibition halts the cell cycle progression in MCC cell lines but does not cause cell death (271). Mutation in VP1 also disrupts viral capsid formation (270, 272).

Recently, an association of MCV infection with chronic lymphocytic leukaemia (CLL) was reported, yet the causal association remains to be proven (273-275).

Epidemiology

MCV is considered to be a commonly occurring human virus and infection is generally asymptomatic (253). Serological studies have shown that 50-80% of adults display MCV-specific antibodies by using recombinant viral capsid proteins as antigens (276-279). MCV primary infection is typically acquired during early childhood and the prevalence of IgG antibodies was found to be 9% at 1-4 years and increased up to 35% at 13 years of age among children (280).

Presence of MCV DNA was reported in buffy coats of healthy blood donors pointing to latency/persistence in peripheral blood leukocytes (281). Detection of viral DNA in cutaneous swabs from healthy individuals suggests that virus is shed chronically from normal skin and MCV is likely a ubiquitous virus (253, 282). MCV DNA has also been detected in relatively low amount compared to skin in respiratory tract, saliva, urine, lymphoid tissues and gastrointestinal tract (283-289). Possible route of MCV transmission may be fecal-oral, cutaneous, mucosal and respiratory as suggested by existing data; however, the exact mechanism is unknown.

Humoral immunity

Detection of antibodies against MCV suggests that host immune system respond against virus in infected individuals (277, 278, 280). Most of the reports have shown that IgG antibody titers to capsid proteins of MCV are high in MCC patients, compared to general population (277, 278, 290). As reported, MCC tumors do not express capsid proteins, suggesting that the occurrence of high antibodies titers in MCC infected patients is not because of increased capsid antigen production (277, 279). A possible reason behind this could be an increased replication of MCV in patients with MCC (291, 292). In support of this hypothesis it is reported that MCV DNA levels in skin swabs from patients were found significantly higher than in controls (282) and in second report positive correlation between MCV antibody titre and DNA levels in skin biopsies was found (292). Therefore, poorly controlled MCV infection and high viral load promoted by defective immune response could possibly be the factor behind high antibody titer.

Approximately 40% of MCC patients are seropositive for MCV T antigens; however, among general population these antibodies occur in only 0.9% and low titre (293). Therefore anti-MCV T antigen antibodies are more specifically associated with MCC than those against viral capsid proteins, but they are incapable of protecting against the disease progression and may serve as a biomarker of the disease (293). As neutralizing MCV antibodies occur in high titers among patients, they apparently fail to prevent MCC tumorigenesis (277). It is therefore possible that cell mediated immunity (CMI) may be involved in protection against MCV- induced malignancy.

Cellular immunity

Cellular immunity is believed to play a central role against viral infections and in tumor elimination (50, 294-296). It has been shown that LT proteins are expressed in active MCV infection in MCC patients and that these proteins elicit humoral immune response (293).

Antiviral T- cell immune response against cancer-associated viruses (50, 294) and other polyomaviruses (297-299) have been shown in many studies. Intratumoral infiltration of $CD8^+$ T lymphocytes is an independent predictor of better survival prognosis among MCC patients (268). Gene expression profiling notified over-expression of genes encoding for granzymes, chemokines, CD8 receptor molecules and lymphocyte-activation molecules in MCC tumors with favorable prognosis (268, 291). Almost 100% survival was also seen in MCC patients with vigorous intratumoral T-cell infiltration as compared to 60% survival among patients with no intratumoral infiltration. These studies suggested the important role of cellular immunity in MCC the patients and increased rate of MCC in immuno-compromised patients. Iyer et al. investigated T-cell immunity against MCV among MCC patients and control subjects (300). They reported the presence of MCV-specific $CD4^+$ or $CD8^+$ T-cells from MCV-positive (2 of 6) but not from virus-negative MCC tumors (0 of 4). Interestingly, T-cell responses among PBMC were also detected by using broad range of peptides derived from capsid proteins (2 epitopes) and oncoproteins (24 epitopes), in 52% of MCC patients and 38% of control subjects, respectively. Whether cellular responses modulate the clinical course of MCC is not known. By HLA-LT-Ag tetramer the authors showed that virus-specific $CD8^+$ T-cells were markedly enriched among TILs as compared to blood in one patient, suggesting intact T-cell trafficking into the tumor (300). Next they detected tetramer-positive $CD8^+$ T-cells in the blood and found that these cells failed to produce IFN-γ when stimulated *ex vivo* with LT-Ag peptide, suggesting non-responsiveness. The findings to date, however, do suggest that MCC tumors often develop despite the presence of T-cells specific for MCV. A very recent investigation showed that MCV-specific $CD8^+$ T-cells dynamically fluctuate with tumor burden and with viral oncoprotein-specific antibody titre (301). These T-cells express a high level of exhaustion molecules PD1 (programmed death 1) and Tim-3 (T-cell immunoglobulin and mucin-domain 3). This may explain why MCV-specific $CD8^+$ T-cells fail to control MCC and suggest that the inhibition of these pathways may be used for therapeutic approach. NK cells also have anti-cancer effector functions and might play role against MCV infections. Mishra et al. investigated the protective role of the NK and γδ T-cells against polyomavirus-induced tumors in a mouse model (302).

Presently, there is no vaccine available against MCV infection. Very recently DNA-based vaccines have been developed by utilizing immuno-dminant epitopes of MCV LT and sT-Ag (303, 304). Vaccination caused prolonged survival, decreased tumor size and increased LT-specific $CD8^+$ T-cells in tumor-bearing mice.

Trichodysplasia spinulosa-associated polyomavirus

Haycox et al. in 1999 reported a case of folliculocentric viral infection in an immunocompromised patient and designated the name 'trichodysplasia spinulosa (TS)' for the condition (305). TS is a very rare disease of skin and characterized by follicular papules and keratin spines known as spicules widespread on the face (particularly on nose and chin) and ears and some other parts of the body may be affected as well. Sometimes alopecia of the eyebrows and lashes can be detected and in some cases facial distortion may also occur. Histopathology of the affected skin shows distended and abnormally matured hair follicles with high numbers of inner root sheath cells containing excessive amounts of trichohyalin features (305-308). Keratin spines of size 1-3 mm originate from these abnormal follicles. Organ transplant recipient patients with immunosuppressive conditions and patients suffering from haematological malignancies are particularly affected by TS (305-307, 309, 310).

Electron microscopy studies showed the presence of virus-like particles in skin biopsies of TS patients, suggesting an etiological role of virus for this disease (305-308). Recently, the research group of Meriet Feltkamp identified in the TS lesions of a heart transplant recipient a new human polyomavirus, designated as TS-associated polyomavirus (TSV) (254). Electron microscopy revealed that TSV particles are non-enveloped, small (28-46 nm in diameter) and icosahedral shaped (309). There are five ORFs present on 5232 bp long circular genome of TSV. The genome has putative 'early' genes encoding sT and LT-Ag and the putative 'late' genes encoding structural genes VP1, VP2 and VP3 (254).

Diagnosis and immunity

Several research groups developed diagnostic methods based on the detection of antibodies, nucleic acid or polyomavirus like particles (309, 311-314). The existing data suggest that TSV infections are frequent among the general population (~ 70% seroprevalance) and that the primary infections often occur in childhood and seroprevalence increases with age (311, 313-315). Further investigation of TSV infections was shown by analysis of TSV particles by EM in paraffin embedded tissues (309, 316). Kazem at al. reported an active polyomavirus infection from TS lesional and non-lesional skin samples from TS patients by quantitative PCR and by immunofluorescence for expression and localization of viral proteins. All TS lesion skin samples were positive for TSV DNA; as compared to 2% control samples and abundant expression of TSV VP1 protein in the hair follicle cells of the lesional skin sample demonstrates a direct link between TSV infection and the disease (317). This and several other studies revealed a statistically significant association between the presence of TSV DNA and symptomatic TS (309, 312, 316, 317).

AIMS OF THE STUDY

1. Comparison of Th-cell immunity against two members of the family Parvoviriade (HBoV1 and B19)
2. Investigation of the cytokine pattern after the parvoviruses (HBoV1 and B19) and polyomaviruses (TSV and MCV) infections.
3. Investigation of the cytolytic potential of $CD4^+$ T-cells against B19 infections
4. Characterization of lymphocyte proliferation and cytokine responses against MCV and TSV
5. Comparison of Th-cell immunity and investigation of cross-reactivity between two newly discovered polyomaviruses (MCV and TSV)

MATERIALS AND METHODS

Study Subjects (I-IV)

Altogether randomly selected 174 asymptomatic subjects (age range 19-59 years) were studied: 50 B19-seropositive (20 and 30 for study I and II, respectively) and 38 seronegative (16 and 22 for study I and II, respectively), 45 MCV-seropositive (15 and 30 for study III and IV, respectively) and 36 seronegative (15 and 21 for study III and IV, respectively), 30 TSV-seropositive and 21 seronegative (study IV). All subjects were seropositive for HBoV1 (I-IV).
A 48 years old MCC cancer patient was also studied. The patient was HIV negative, and had no immunosuppressive medication or other known immunodeficencies. Ethical approval was obtained from University of Helsinki ethics committee and informed consent also obtained from every subject

Antibody assays (I-IV)

IgG for HBoV1, parvovirus B19, MCV and TSV in plasma were measured by in-house EIAs employing VLPs as antigens (182, 216, 280, 311).

Antigens for T-lymphocyte culture (I-IV)

The B19, HBoV1 VP2 and MCV, TSV VP1 VLPs were expressed with recombinant baculoviruses in High 5 and Sf9 cells and purified by CsCl gradient ultracentrifugation (182, 216, 280, 311). After extensive dialysis the protein was concentrated and purified further by using 50 and 100 KDa MWCO centrifugal filters (Amicon Ultra, Millipore, Billerica, MA). Electron microscopy with negative staining showed VLPs. The antigens were further characterized by silver staining (SilverXpress, Invitrogen) immunoblotting (western and dot) with seropositive human sera. A B19-VP2 specific murine monoclonal antibody R92F6 was also used for B19 VLP blotting.
As control antigens, we used Tetanus toxoid (TT; National Public Health Institute Helsinki, Finland), in-house prepared and heat inactivated *Candida albicans* and Phytohemagglutinin-P (PHA) (Sigma-Aldrich).

Endotoxin assays (I-IV)

Endotoxin in the antigen preparations was measured by the Limulus amebocyte lysate assay (QCL-1000; Cambrex Biosciences, Walkersville, MD, USA), and it was less than 2 EU/mg with MCV, TSV, HBoV1 and B19 VLPs antigens.

Isolation of PBMC (I-IV)

Blood was drawn to mononuclear cell separation tubes (Vacutainer CPT, Becton Dickinson, Franklin Lakes, NJ) containing 0.45 ml sodium citrate. The tubes were

centrifuged at 1500 x g for 30 minutes and washed two times with 1X PBS. PBMC were separated within 2 hrs of blood sampling followed by counting.

Lymphocyte culture (I-IV)

Lymphocyte culture was conducted as described previously (170, 177, 182, 186). Briefly, isolated PBMC were resuspended in the RPMI-1640 medium (Sigma) containing 20mM HEPES, 2mM L-glutamine, streptomycin (100 μg/ml), penicillin (100 U/ml), 50 μM 2-mercaptoethanol and 10% human AB serum (Cambrex Biosciences, USA) and were cultured with the antigens.

Proliferation assays (I-IV)

Counted PBMC and antigens in triplicate were placed in 96 well U-bottom plates (Coster, Corning Inc., Corning, NY). Cells (200,000/well) were cultured for 6 days (37ºC and 5% CO_2) and pulsed for the last 16 hours with 1μCi of tritiated thymidine (specific activity 50 C/mmol, Nycomed Amersham, Buckinghamshire, UK). Thymidine incorporation was measured in a liquid scintillation counter (Microbeta, Wallac, Turku, Finland). The data were expressed as counts per minute (Δ cpm): Δ cpm = mean cpm (test antigen) - mean cpm

Cytokine assays (I-IV)

PBMC culture supernatants were harvested after 3 days for IFN-γ, GrB and perforin and after 5 days for IL-10 and IL-13, and were stored at -20 ºC. Cytokine production in the supernatants was analyzed by IFN-γ, GrB (e-Biosciences and MABTECH AB), perforin (MABTECH AB, Sweden), IL-10 (Pharmingen, San Diego, CA, USA) and IL-13 (Invitrogen corporation CA, USA) kits, according to the manufacturers' instructions. Background (media) cytokine production was subtracted from total to yield antigen specific cytokine production.

Depletion of CD4- and CD8-positive cells (II, III and IV)

PBMC were depleted of $CD4^+$ or $CD8^+$ T-cells by using magnetic beads coated with CD4- or CD8-specific monoclonal antibodies (Invitrogen Dynal AS, Oslo, Norway), according to the manufacturer's instructions. Then, 200,000 pure $CD4^+$ or $CD8^+$ cells were cultured with the antigens as described (170).

Positive selection of CD4- and CD8-T cells (I)

PBMC were first depleted of $CD4^+$ T-cells and then of $CD8^+$ T-cells by using magnetic beads coated with CD4- and CD8-specific monoclonal antibodies (Invitrogen Dynal AS, Oslo, Norway), according to the manufacturer's instructions. Positively isolated $CD4^+$ and $CD8^+$ T-cells were detached from beads by using Detach-A-Beads (Invitrogen Dynal AS, Oslo, Norway). 100,000 pure $CD4^+$ or $CD8^+$ cells were then cultured with the antigen presenting cells (CD4 and CD8-depleted PBMC) exactly as described for PBMC.

Flow Cytometry analysis (I-IV)

Purity of the depleted or positively selected cell populations was analyzed by BD Accuri C6 and FACScan flow cytometer (Becton Dickson, USA). The cells were washed twice with PBS and incubated for 30 min at +4 °C with MultiMix triple-colour cocktail of FITC, RPE and APC labelled monoclonal antibodies for CD8, CD4 and CD3 respectively (DakoCytomation, Denmark). Anti-isotype antibodies (DakoCytomation, Denmark) were used in parallel, for specificity control. Flow cyometry results showed > 95% depletion efficiency after CD4 and CD8 depletion or positive selection, respectively.

Antibody blocking assays (I-IV)

MHC class II restriction of the T-cell responses was further studied by HLA class II-specific MAbs (HLA-DR, DP, DQ) (IgG2a, clone Tu39; BD PharMingen), or isotype control MAb (IgG2a, clone G155- 178; BD PharMingen). These antibodies were used at 5 μg/ml, according to the manufacturer's instructions.

Statistical methods (I-IV)

Responses among seropositive and seronegative subjects were compared by the Mann-Whitney U test. Paired responses were evaluated by using the Wilcoxon Signed Rank test. The distribution of responders having Δ cpm > 5000 against each antigen was studied using Fisher's Exact test. P values < 0.05 were considered significant. All analyses were done with an SPSS statistical program version 15.0 (IBM corporation, New York, USA).

RESULTS AND DISCUSSION

Comparison of Th-cell immunity against HBoV1 and parvovirus B19 (I)

Comparison of cellular immune responses against HBoV1 and B19

Recombinant VLPs mimic original viral particles and are widely used for the diagnostics and development of vaccines against many viruses (216, 280, 311, 318, 319). In this part of the study we compared Th-cell immunity among 36 asymptomatic individuals against HBoV1 and B19 VP2 VLPs. Among them 20 were seropositive and 16 seronegative for B19 and all were seropositive for HBoV1. Previous studies showed that B19 and HBoV1 VLPs elicit virus-specific Th immune responses (186, 240). HBoV1-specific proliferation and cytokine responses were frequently detected among 36 HBoV1-seropositive subjects. In this study we for the first time studied IL-13 and IL-10 responses along with previously studied IFN-γ against HBoV1. To characterize the cell populations secreting cytokines and mediating proliferation responses, we incubated positively selected $CD4^+$ and $CD8^+$ T-cells with the antigens. Proliferation and cytokine responses were found only with $CD4^+$ T-cells, not with $CD8^+$ cells (Fig 3 of Study I).

When we studied HBoV1-specific proliferation and cytokine responses among 20 B19 seropositive individuals with HBoV1 VLPs, proliferation and IFN-γ responses were readily detectable, but the average B19 VLPs-specific proliferation and IFN-γ responses were stronger than those detected with HBoV1 VLPs. IL-10 reactivity was very similar with both antigens, and average IL-13 responses were stronger with HBoV1 VP2 than with B19 VP2. However, individual variability in responses was extensive causing large SDs (Table 2).

Table 2. *Th-cell proliferation and cytokine production in 20 B19-seropositive individuals in response to HBoV1 and B19 VP2 VLPs.*

Antigens	Proliferation Δ cpm[a]	IFN-γ pg/ml	IL-10 pg/ml	IL-13 pg/ml
HBoV1 VP2	8989 ±9705	30 ±42.6	10.9 ±20.6	73.3 ±128.4
B19 VP2	13860 ±11655	70.3 ±114.3	11.2 ±18.5	51.2 ±60.8

Results are shown as mean ± SD.

[a] *Δ cpm: antigen-specific cpm minus background cpm*

Next we investigated how HBoV1 and B19-specific cytokine and proliferation responses correlate among the 20 B19 seropositive subjects (Table 3). As shown, all HBoV1-specific proliferation and cytokine response pair showed significant positive correlations ($p< 0{\cdot}033$). HBoV1-specific IL-13 responses showed particularly strong correlation with the

other HBoV1-specific responses: p=0·001 with IL-10 and <0·0001 with IFN-γ and with proliferation. Interestingly, although the response patterns appeared to be very similar with B19 and HBoV1 antigens, no significant correlations could be found between any B19-specific proliferation and/or cytokine response pairs (p≥ 0·059) (Table 3).

Table 3. *P-values of correlation of HBoV1-specific and B19-specific (in parentheses) proliferation and cytokine responses among 20 B19-seropositive subjects.*

Variable	IFN-γ	IL-10	IL-13
Δ cpm[b]	0.003 (0.059)[a]	0.033 (0.890)[a]	≤0.0001(0.602)[a]
IFN-γ pg/ml		0.002 (0.154)[a]	≤0.0001(0.150)[a]
IL-10 pg/ml			≤0.001(0.611)[a]

[a]*P-values of correlation of proliferation-cytokine and cytokine-cytokine response pairs after Wilcoxon Signed Rank Test*

[b]*Δ cpm: antigen-specific cpm minus background cpm*

Despite different epidemiology pattern of both viruses (102, 216, 238, 320) we found cytokine responses of similar magnitude against HBoV1 and B19 VLPs. Many subjects in this study did not induce B19-specific responses for all cytokines and proliferation, which explains that the response pattern for B19 was stastitistically not significant. Where as strong inter-dependent T-cell responses were found with HBoV1. Therefore, at the collective level, B19-specific Th-cell immunity appears to be more divergent than the HBoV1-specific one. This possibility needs to be studied further with B19 and HBoV1-specific Th cell lines and intracellular cytokine staining.

IL-13 and respiratory symptoms

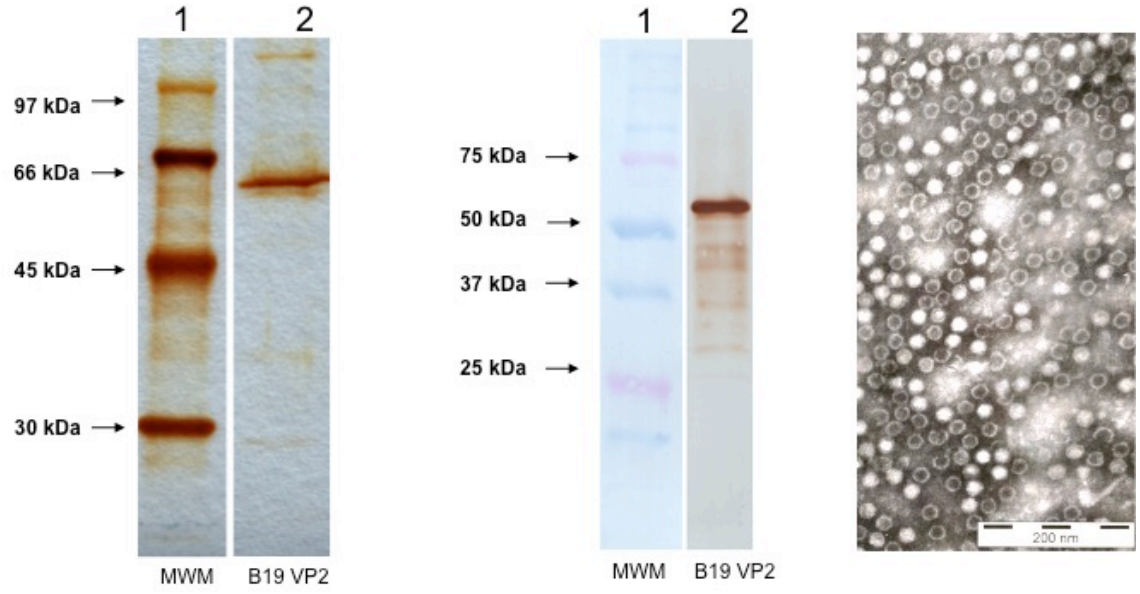
Th2 cells secrete IL-13 and like IL-4, it is a switch factor for IgE and IgG4 synthesis and it also mediates many other important effector functions (18-20). Secretion of IL-13 is elevated in infections by some respiratory viruses, e.g., Rhinovirus, and also participates in the pathogenesis of asthma (19, 26). Dominance of IL-13 responses against HBoV1 and B19 is one of the major finding of this study. There is enough literature available which suggest that IL-13 is an important contributor to respiratory symptoms and pathology including asthma (19, 321). A recent study proposed a link between HBoV1 and asthma exacerbations in young children (322). This hypothesis is supported by our present study because when we compared Th1 (IFN-γ) and Th2 (IL-13) cytokine responses against HBoV1 and B19, it appeared that HBoV1 predominantly induce Th2 cytokines (P= 0.014).

Cytolytic CD4+ T-cell mediated immune responses against parvovirus B19 (II)

Further, we explored $CD4^+$-mediated T-cell responses by studying cytolytic activities of these cells. Since B19 has been observed to establish a chronic infection and to be linked with autoimmunity and even malignancies, it was of interest to explore whether such $CD4^+$ CTLs could also emerge after B19 infection. The present study focused primarily on GrB, not only because of its critical role in cytolysis, but also because of emerging data on its function as an inducer of autoimmunity (60).

Granzyme B response against B19

B19 (Fig 2) and control antigens (HBoV1 and *Candida albicans)* were found to induce PBMC to secrete GrB and IFN-γ in 30 B19 seropositive and 22 seronegative subjects. B19-specific GrB ($P \leq 0.0001$) and IFN-γ ($P \leq 0.0001$) responses were much stronger among the B19 seropositive than among the seronegative subjects at both antigen concentrations (1.5 and 0.5 μg/ml) (Table 1 of Study II). Next we studied correlation between the B19-specific IFN-γ and GrB responses. A strong correlation ($P < 0.0001$) was found in the B19 seropositive group at both antigen concentrations, whereas the respective correlations were less significant among the seronegative subjects ($P \leq 0.024$). No correlation was found between the HBoV1- and the B19-specific GrB responses. Thus, also in the B19 model, the strength of the GrB response is associated with the strength of antigen-induced Th- cell activation, which has been observed previously with other viruses (10, 54, 323). The observed B19-specific GrB responses suggest that $CD4^+$ T-cells secreting GrB may contribute to the control of B19 by guarding against reactivation in cases where helper function is provided by other viruses



(A) (B) (C)

Figure 2: Characterization of B19 VP2 antigen (A) Silver staining of B19 VP2 capsid protein in 10% PAGE (B) Western Blotting with B19-seropositive human serum (C) Electron microscopy of B19 VP2 particles purified by density gradient ultracentrifugation

Perforin response against B19

In order to mediate cytolysis, GrB needs delivery into target cells by perforin (59). Therefore it was also important to study B19 –specific perforin responses. Perforin responses were studied in 7 B19 seropositive and 3 seronegative subjects. B19-specific perforin responses were detectable only in B19 seropositive subjects, whereas PHA (control antigen) elicited strong responses in all and *Candida albicans* antigen in all but one subject (Fig 2 of Study II). In these 7 subjects we found a strong correlation between B19 induced perforin and GrB responses. This finding is a further evidence for the cytolytic nature of B19–specific GrB response and explains by the common exocytosis of perforin and GrB in cytotoxic granules.

With B19 VLPs, both GrB and perforin responses were confined within the $CD4^+$ T-cells, as shown by T-cell subset depletion and by blocking of HLA -class II presentation (Fig. 4 and 5 of Study II). Blocking experiments confirm that GrB and perforin secreting cells are HLA-class II-restricted.

Various autoimmune phenomena, including the induction of autoantibodies and autoimmune diseases such as RA and SLE have been linked to B19 infections (128, 149). However, the pathogenetic mechanisms of B19-induced autoimmune diseases are not fully understood. Several mechanisms have been proposed: activation of the IL-6 and TNF-α promoters by B19 NS1 protein during persistent infection, molecular mimicry between a B19 VP2 epitope and autoantigens, such as collagen II (84, 138, 153) and the

phospholipase activity of B19 VP1 unique domain with a subsequent activation of synoviocytes and induction of anti-phospholipid antibodies (137, 324). Recently, B19 NS1 has been shown to induce apoptotic bodies containing self-antigens potentially associated with autoimmunity (325).

We propose an alternative (or additional) mechanism for B19-triggered autoimmunity: an excessive activation of B19-specific $CD4^+$ cells secreting GrB in genetically predisposed individuals. GrB has been shown to cleave autoantigens and create unique fragments recognized by autoantibodies (326-328). In line with this, $CD4^+$ T-cells with cytolytic potential have been described in patients with RA and SLE (48, 49).

T-cell mediated immunity against MCV (III)

This study is one of the pioneer reports in the field of anti-viral T-cell immunity against MCV. We were the first to show Th-cell proliferation and cytokine responses against structural proteins of MCV by using VLPs among asymptomatic individuals.

Proliferation and cytokine responses against MCV and control antigens

First we compared proliferation and cytokine responses among 15 MCV-seropositive and seronegative individuals. MCV-specific proliferation and cytokine responses were much stronger than the responses of the seronegative subjects, at both 0.25 µg/ml and 2.5 µg/ml concentrations of antigen (Table 4). With the control antigens (*Candida albicans* and HBoV 1) the cytokine responses (≤ 0.106) were very similar among the MCV-seropositive and -seronegative subjects.

Table 4. *Comparison of MCV-specific proliferation and cytokine responses among MCV seropositive and –seronegative subjects at 2.5 and 0.25 (in parenthes) µg/ml antigen concentration.*

MCV serostatus	Proliferation ΔCPM[a]	IFN-γ pg/mL	IL-10 pg/mL	IL-13 pg/mL
Positive	9562±7419 (*5878±5959*)	280.7±197.6 (*159.8±234.0*)	51.2±56.0 (*23.3±30.5*)	56.5±54.9 (*23.5±28.2*)
Negative	3086±3918 (*534±660*)	51.8±60.6 (*6.5±8.40*)	22.7±21.8 (*4.50±4.80*)	20.5±26.4 (*4.0±5.40*)
P	0.001 (*<0.0001*)	<0.0001 (*0.006*)	0.041 (*0.026*)	0.019 (*0.021*)

Results are shown as mean ± SD.

[a] *Δ cpm: antigen-specific cpm minus background cpm*

Despite the presence of humoral immunity, generation of cellular immunity is essential for tumor elimation. Central role of T-cell immunity has been reported against cancer-associated viruses and other polyomaviruses (294, 297, 329, 330). In this study $CD4^+$ T-cells appeared to be the main responding cells among PBMCs, as MCV-specific responses were restricted among seropositive subjects, indicating that the responding cells are able to establish memory. Furthermore, HLA class II-specific antibodies blocked MCV-specific proliferation, IFN-γ, IL-10 and IL-13 responses, whereas the depletion of $CD4^+$ cells abrogated them in this study. Previously, Iyer et al., characterized T-cell responses against peptides derived from structural and non-structural proteins of MCV and detected virus-reactive $CD4^+$ and $CD8^+$ cells in the blood of MCC patients and control subjects (300). Interestingly, despite the higher number of $CD3^+$ or $CD8^+$ T-cells in MCV-positive than in MCV-negative MCC patients, favourable survival was found in both groups indicating beneficial role of intratumoral T-cell infiltration (331). Another study has found a high

intratumoral CD8 count to be associated with a favourable outcome in MCC (268). Present study (III) and the previous one by Iyer et al., suggest that MCC tumors often develop despite the presence of virus-specific $CD4^+$ or $CD8^+$ T-cells. Elderly people and individuals with immune-compromised conditions, organ transplantation and CLL have been found associated with high a risk of developing MCC (332-335). Defective immunity is thought to play a role in the increased incidence of MCC in these populations, as previously reported that MCV contains potent epitopes responsible for inducing cytotoxic T-cell immunity (263, 268).

IFN-γ emerged as the dominant MCV-specific cytokine; yet MCV-specific IL-10, IL-13 and proliferation responses were readily detectable among sero-positive individuals. As IFN-γ possesses antiviral and tumor suppressing functions, $CD4^+$ cells are important mediators of MCV-specific T-cell immunity. Very little is known about the role of other sub-sets of immune cells against MCV, however, protective role of NK cells and γδ T-cells against polyomavirus-inuced tumors in mouse models have been reported recently (302).

Presence of seronegative responders

We also encountered some responder subjects in seronegative group. The presence of MCV- seronegative responders suggests that B-cell immunity against MCV is not always persistent, or that a degree of cross-reactivity in the VP1 Th-cell epitopes may exist between MCV and some hitherto unidentified virus. VP1 proteins of other polyomaviruses are possible candidates. For instance, the VP1 protein of a recently discovered TSV virus has as high as 57% amino-acid similarity with that of MCV (254). Alternatively, some MCV strains might be of aberrant B-cell antigenicity. One such MCV strain, termed “350”, having critical double mutations at VP1 positions 288 and 316, has been described to date (290). VP1 of strain “350” is not recognized by sera strongly reactive with VP1s of MCV strains lacking these mutations.

Th-cell mediated immunity against TSV and comparison of MCV-specific cellular immunity (IV)

TSV-specific proliferation and cytokine responses in the TSV-seropositive and -seronegative individuals

This is the first study to investigate TSV-specific Th-cell immunity. In this study we investigated how TSV-VP1 VLPs stimulate Th cells from asymptomatic individuals to proliferate or to secrete IFN-γ, IL-10 and IL-13. TSV-specific IL-10 responses were found similar in seropositive and seronegative groups, whereas proliferation, IFN-γ and IL-13 responses appeared to be stronger among the TSV-seropositive than -seronegative subjects (Table 1 of Study IV). However, these differences did not prove statistically significant. Antibody blocking and depletion experiments confirmed that the source of cytokines and proliferation were $CD4^+$ T-cells (Fig. 4 and 3 of Study IV)). In contrast to findings with TSV, the MCV-specific responses were consistent with our previous study (Study III).

The effect of humoral responses on Th-cell responses

We investigated whether there would be a correlation between the strength of TSV-specific humoral response, described as optical density x 1000 (ODx1000) and the Th-cell mediated responses. A significant positive correlation was found between humoral response and TSV-specific proliferation, IFN-γ and IL-10 in the 30 TSV seropositive subjects; with IL-13 the correlation did not reach significance (P= 0.098). Further we divided these 30 TSV seropositive subjects into two groups of equal size (15 subjects) according to their TSV-IgG ODs (Fig. 5B of Study IV): subjects in group A had ODs under and those in group B had ODs above the median OD (1720) of the 30 TSV seropositive subjects. Group A had TSV-specific responses similar to that found in the seronegative controls. In group B, by contrast, both the TSV-specific proliferation (P= 0.007) and IFN-γ (P= 0.013) responses were stronger than in the seronegative controls; no difference was found in the TSV-specific IL-10 and IL-13 responses, or responses with the *Candida albicans* control antigen. Significantly stronger TSV-specific proliferation, IFN-γ and IL-10 responses were found in group B than in group A. However, with MCV the correlations between humoral and Th-cell responses were less evident among the seropositive subjects.

Interestingly; however, the vigour of TSV-specific humoral responses had a significant impact on TSV-specific Th-cell responses, and subjects with the highest antibody responses not only had significantly stronger Th-cell responses than the seronegative controls, but also significantly stronger responses than the TSV-seropositive subjects with low TSV-specific IgG level. At least two different possible explanations could account for this. First, subjects with low TSV-IgG and Th-cell responses level may have contracted the TSV infection long time before the subjects with stronger responses became infected. Consistent with this, antibody and Th-cell responses are known to decline with time (336). Second and more interesting explanation is that subjects with high TSV-specific responses could have had more reactivations or even reinfections, each having a potential to boost both humoral and cellular responses (337). In conclusion, as association between the virus specific Th-

cell responses and antibody responses was found also with MCV, yet it was less evident than with TSV.

Study of cross-reactive T cell responses against MCV and TSV

TSV and MCV share approximately more than 57% of amino acids (Table S3 of Study IV) in their VP1 protein, and both viruses may possibly induce cross reactivity immunity. Van der Meiden et al. and Chen et al. confirmed that antibodies against TSV-VP1 do not cross-react with MCV-VP1 by serological assays (311, 314). In order to rule out the possibility of cross-reaction at T-cell level we divided the subjects in different groups (Table 3 of Study IV) on the basis of their serostats: $MCV^{+}TSV^{+}$, $MCV^{-}TSV^{+}$, $MCV^{-}TSV^{-}$ and $MCV^{+}TSV^{-}$. When we compared Th-cell responses in groups $MCV^{-}TSV^{+}$ and $MCV^{+}TSV^{-}$, significantly stronger responses with TSV (P= 0.007) and MCV (P= 0.003) occurred while comparison in other groups showed no significant difference. This suggests, that some unique epitopes are present in VP1 region of TSV and MCV, which induce these responses.

Next, we compared MCV and TSV-specific responses between these groups, significantly stronger TSV-specific proliferation, IFN-γ and IL-13 responses were detected in $MCV^{+}TSV^{+}$ than in $MCV^{-}TSV^{-}$ group. TSV-specific IL-10 responses and those to *Candica albicans* control antigen were similar in both groups, ($P \geq 0.18$), respectively. Interestingly, with the MCV antigen Th-cell responses were stronger even in the $MCV^{+}TSV^{-}$ than in the $MCV^{-}TSV^{+}$ control group.

Data from subgroup experiments showed that if there is any crossreactivity between TSV and MCV, it must be relatively low, since the MCV-serostatus had a significant impact on TSV-specific responses only when double (TSV^{+} MCV^{+}) seropositive subjects were compared with double ($TSV^{-}MCV^{-}$) seronegative subjects. Furthermore, some of these double seronegatives showed significant Th-cell responses to both viruses, despite being seronegative to both. This could imply that B-cell immunity against these antigens would persist a shorter time than T-cell immunity, or that VP1 proteins of MCV and TSV would contain Th-cell epitopes cross-reactive with some other agents, such as other human polyomaviruses (Table S3 of Study IV). Because a high level of cross-reactivity is an essential feature of the T-cell receptor (338-341), it is also possible that the Th-cells of our seronegative subjects had originally been primed by Th-cell epitopes differing largely in sequence from the MCV or TSV VP1 proteins. The most interesting possibility is that in some subjects MCV and/or TSV infection induces cytotoxic $CD4^{+}$ cells which would kill antigen-presenting B-cells and cause eradication of virus specific antibody response (342, 343).

MCV and TSV-specific IFN-γ and IL-10 responses in the MCC patient

Th-cell mediated immune responses were studied in a subject with Merkel cell carcinoma. Similarly to the healthy controls in group $MCV^{+}TSV^{-}$ described above, also this patient was seropositive for MCV and seronegative for TSV, and much stronger IFN-γ and IL-10 responses were found with MCV than with TSV (Note S1 of Study IV). The kinetics of his

MCV-specific IL-10 response was similar to that in healthy controls (5d response higher than 3d response); with TSV-antigen a reverse pattern was detected. An early and exceptionally strong IL-10 response was detected with TSV-antigen, suggesting that T-cell epitopes within TSV were recognized as altered peptide ligands (344) or alternatively, these common epitopes activated regulatory T-cells in this patient (345). In both cases an IL-10 oriented T-cell response is known to occur (344, 345).

Taken together, Th-cell immunity appears to be much better maintained against the major structural protein (VP1) of MCV than TSV. As TSV and MCV infections appear to occur around same age (280, 311, 314), the time span from the primary infection should not explain this. Possibly MCV becomes reactivated in the human body more readily than TSV, boosting Th-cell immunity more efficiently. Comparison of TSV and MCV responses within the $MCV^{+}TSV^{-}$ subjects (including the MCC patient) and within the $TSV^{+}MCV^{-}$ subjects clearly showed that VP1 proteins from both viruses contain unique Th-cell epitopes which are not shared. However, as the differences between MCV and TSV responses were more significant in the $MCV^{+}TSV^{-}$ than the $TSV^{+}MCV^{-}$ group, MCV VP1 appears to contain more unique virus-specific epitopes than TSV-VP1 does. Alternatively, Th-cell epitopes within MCV VP1 have higher affinity for MHC II than Th-cell epitopes of TSV VP1.

Finally, as $CD8^{+}$ cells specific for MCV T-Ag oncoprotein clearly provide an important defense against established MCC (300), the MCV VP1-specific Th-cells may be important in preventing the full process of oncogenesis, by suppressing MCV replication with antiviral cytokines such as IFN-γ. If this mechanism exists, also the MCV-crossreactive Th-cells should have a cross-protective role, and subjects with these cells might be less susceptible to develop MCC.

CONCLUSIONS AND FUTURE DIRECTIONS

These studies suggest that humoral immunity alone is not sufficient to prevent diseases caused by these emerging viruses, and it appeared that cellular immunity has a key importance in controlling the diseases.

The following conclusions can be drawn from the present study:

- B19-specific immunity appeared more divergent than HBoV1 and IL-13 may be responsible for respiratory symptoms in HBoV1 infections.

- B19-specific $CD4^{+}$CTLs may induce autoimmune diseases among B19-infected individuals.

- IFN-γ is the dominant cytokine among MCV seropositive adults and plays an apparently important role in surveillance against MCPV-induced disease. Our studies also suggested the role of IL-13 and IL-10 in anti-tumor immunity and immune regulation, respectively.

- Th-cell immunity appears to be better maintained against MCV than TSV after infection. The study also suggests that there is no cross-reactivity between these two viruses at the T-cell level.

The significance of our project is both academic and practical, as the characterization of protective T-cell immunity provides a basis for vaccine development and immune-based therapies.

It is believed that B19 has some role in triggering rheumatoid arthritis (RA). In the future this phenomenon should be investigated by analysing T-cell immunity in patients with RA. B19 has been found to be associated with many autoimmune diseases, therefore this direction must also be included in future studies. The *ex-vivo* generated B19-infected erythroid progenitor cells may be exploited to further investigating the role of NK and cytotoxic T-cells in controlling B19 infections. Interestingly, HBoV1 has been found to be associated with asthma exacerbations in young children (322). We suggest that a study of HBoV1-specific IL-13 responses in (young) asthmatics and in age-matched controls might further elucidate the possible role of HBoV1 in asthma.

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