T cell differentiation by human parvovirus B19 non-structural protein 1 induced apoptotic bodies

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PREFACE

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Tiivistelmä

Uutta tietoa ihmisen immuunijärjestelmästä ja sen toiminnasta kerätään jatkuvasti. Synnynnäinen ja hankittu immunijärjestelmä toimivat yhteistyössä erilaisia patogeeneja vastaan. Dendriittisolut toimivat siltana synnynnäisen ja hankitun vastustuskyvyn välillä stimuloimalla T ja B lymfosyyttejä. Antigeenin esittely luokan II MHC molekyylin (*eng. major histocompatibility complex*) ja T solun reseptorin (TCR) välillä, sekä erillisen reseptorin ja ligandin tai sytokiini -signaalin välityksellä, CD4+ T solut stimuloituvat ja erilaistuvat. Erilaistuneita CD4+ T auttajasoluja (Th, *eng. T helper cell*) on eri alalajeja, jotka osallistuvat vaihtelevasti immuunipuolustukseen, esimerkiksi suojana erilaisia viruksia vastaan. Ihmisen parvovirus B19 (B19V) infektoi niin lapsia kuin aikuisiakin. Tartunta voi olla oireeton tai oireet voivat vaihdella flunssankaltaisesta vakavampiin tauteihin kuten parvorokkoon, nivelsairauksiin ja sikiön vesipöhöön. B19V on myös yhdistetty autoimmuunisairauksiin kuten nivelreumaan ja perhosreumaan (lupus). Patogeenisyyden mekanismeja B19V:een liittyvissä autoimmuunisairauksissa ei ole kuitenkaan vielä selvitetty. Kuitenkin tiedetään, että viruksen ei-rakenteellinen proteiini 1 (NS1) aiheuttaa isäntäsolussa tuhoa ja jopa apoptoosia. NS1 proteiinin kykyä laukaista immuunireaktio tarkkailtiin tässä työssä. Tutkimuksen tavoitteena oli kartoittaa T solujen ja dendriittisolujen kanssakäymistä B19V NS1 proteiinin aiheuttamien apoptoottisilla kappaleilla (ApoBodit) stimuloinnin jälkeen. Hypoteesina oletettiin, että NS1:n synnyttämät ApoBodit saavat aikaan immuunireaktion, jossa erityisesti autoimmuniteettiin viittaavat Th1 ja Th17 solut erilaistuvat. Seitsenpäiväinen kypsymisprotokolla perustettiin kaupalliselle dendriittisolulinjalle (MUTZ-3, sytokiini-riippuvainen ihmisen solulinja) ja kypsyminen viimeisteltiin 48 h kestävällä stimuloinnilla. Tämän jälkeen stimuloituja MUTZ-3 soluja kasvatettiin yhdessä T solujen kanssa 24 h, 72 h, ja 7 pv:n ajan. Virtaussytometriaa ja konfokaalimikroskopiaa käytettiin solunulkoisten merkkien tutkimiseen niin kypsytetyiltä MUTZ-3 soluilta kuin yhteiskasvatetuilta T soluilta kolmesta eri aikapisteestä. CD83 toimi kypsien dendriittisolujen solunulkoisena leimana, kun taas kuutta erilaista T solujen alalajien merkkiä käytettiin T solujen värjäyksessä: CD8a, CD4, CD183 Th1 soluille, CD194 Th2 soluille, CD196 Th17 soluille ja CD25 T säätelijäsoluille (Treg, *eng. T regulatory cell*). Myös sytokiinituotantoa tutkittiin 24 h ja 72 h aikapisteiltä. Dendriittisolujen kypsymistulokset osoittivat >10 % kypsymistä. Tämän lisäksi yhteiskasvatetut T solut osoittivat erilaistumista kaikista tutkituista T alalajityypeistä, vaikkakin Th2 soluja oli erilaistunut enemmän kuin muita alalajeja (Th1, Th17 ja Treg) 72 h aikapisteestä eteenpäin. 7 pv:ään mennessä Th1 ja Th17 solut olivat tasaisesti kasvaneet 2.5 % ja 1.5 % vastaavasti, kun taas Th2 ja Treg solut lisääntyivät 72 h aikapisteeseen asti mutta hupenivat 7 pv:n mennessä. Näiden lisäksi sytokiinianalyysi ilmensi tulehdusta edistävien ja hillitsevien sytokiinien tuotannon. Kuitenkaan kullekin T solujen alalajille tyypillisiä sytokiinejä ei tutkimuksessa havaittu. Tutkimuksen tuloksena voitiin kuitenkin todeta, että NS1 indusoimat ApoBodit stimuloivat tulehdusreaktion, jossa erilaistui variaatio T solujen alalajeja. Tämän lisäksi hypoteesi todettiin paikkansa pitäväksi, sillä Th1 ja Th17 soluja oli erilaistunut ja solujen määrä kasvanut kokeen eri aikapisteiden välillä. NS1 proteiinin aiheuttamia immuunireaktioita on kuitenkin tutkittava lisää, jotta B19V:een liittyvien autoimmuunisairauksien syntymisen mekanismit voidaan tunnistaa.

Avainsanat: B19V, NS1, ApoBodit, T solut, dendriittisolut, yhteiskasvatus, virtaussytometria

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Abstract

New information about the human immune system and its functions is constantly being gathered. The innate and adaptive immunities work in coherence against various pathogens. Dendritic cells (DCs) bridge the gap between the innate and adaptive immune systems by stimulating T and B lymphocytes. Antigen presentation between class II major histocompatibility complex (MHC II) and a T cell receptor, with an additional co-stimulation through receptor-ligand interaction or by a cytokine signal, stimulates CD4+ T cells to differentiate. A variety of differentiated CD4+ T helper (Th) cells are known to participate differently in diverse immune responses such as protection against viruses. For instance, human parvovirus B19 (B19V) infects children and adults alike. The symptoms vary from none to flu-like symptoms to more serious diseases such as erythema infectiosum (fifth disease), arthropathy and hydrops fetalis. Furthermore, B19V has been implicated in autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus. However, the exact mechanism of pathogenicity is yet unknown. Nonetheless, the non-structural protein 1 (NS1) is known to cause cell damage and even apoptosis in the host. Hence, examining the capability of NS1 to trigger an immune response was studied. The aim of this study was to investigate DC and T cell interaction after the stimulation with B19 NS1 induced Apoptotic Bodies (ApoBods). The hypothesis was that NS1 induced ApoBods stimulate an immune reaction *in vitro*, in which the autoimmune related Th1 and Th17 cells are differentiated. A seven-day maturation period for the commercial dendritic cells (MUTZ-3, the cytokine-dependent human cell line) was set up, and the maturation was finalized with a 48 h stimulation period with NS1 induced ApoBods. Afterwards, stimulated MUTZ-3 cells were co-cultured with T cells for 24 h, 72 h and 7 d. Flow cytometry and confocal microscopy were used to examine the extracellular markers on both the mature MUTZ-3 cells and the co-cultured T cells from each of the three time points. CD83 was the extracellular marker for mature DC detection, while six different T cell subtype markers were used for T cell labeling: CD8a, CD4, and CD183 for Th1, CD194 for Th2, CD196 for Th17, and CD25 for T regulatory (Treg) cells. Additionally, the supernatants from the 24 h and 72 h time points were studied for cytokine production. The results of the DC maturation experiment illustrated >10 % maturation of dendritic cells. Moreover, the co-cultured T cells demonstrated differentiation of each T cell subtype examined. The formation of differentiated Th2 cells after 72 h was at a higher quantity than the other CD4+ T cell subtypes (Th1, Th17, and Treg). At day 7, the Th1 and Th17 cells had steadily increased to 2.5 % and 1.5 %, respectively. Although Th2 and Treg cells had first increased at 72 h time point, both had decreased at 7 d. Furthermore, the cytokine analysis exemplified the production of pro- and anti-inflammatory cytokines. However, no signature cytokines for each T cell subtype were discovered. Still, it can be noted that NS1 induced ApoBods stimulated an inflammatory immune reaction, where a variety of T cell subtypes were formed. Moreover, the hypothesis was proven accurate, as Th1 and Th17 cells had differentiated, and the number of cells had increased throughout the experimented time points. Still, further studies on NS1 induced immune reactions are required to identify the exact mechanisms of initiation for B19V –related autoimmunity.

Keywords: B19V, NS1, ApoBods, T cells, dendritic cells, co-culture, flow cytometry

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ABBREVIATIONS

5637 CM	Human bladder carcinoma cell line 5637 conditioned media
APC	Antigen presenting cell
ApoBods	Apoptotic Bodies
B19V	Human parvovirus B19
BSA	Bovine serum albumin
DC	Dendritic cell
FBS	Fetal bovine serum
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
GM-CSF	Granulocyte / macrophage colony stimulating factor
HepG2 cells	Human hepatocellular liver carcinoma cells
IFN-γ	Interferon-γ
Ig	Immunoglubulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LTα	Lymphotoxin-α
MEM	Minimun Essential Medium
MHC	Major histocompatibility complex
MUTZ-3 cells	Acute myelomonocytic leukemia cell line
NS1	Non-structural protein 1

P1 / 2 / 3	Passage number of virus cultures
PBS	Phosphate buffered saline
PE	Phycoerythrin
pen/strep	Penicillin-Streptomycin
RA	Rheumatoid arthritis
RORγt	Retinoic acid receptor –related orphan receptor γt
RT	Room temperature
*Sf*9 cells	*Spodoptera frugiperda*-derived cells
sICAM-1	Soluble intercellular adhesion molecule 1
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
TE	Transduction efficiency
TGF-β	Transforming growth factor-β
Th cell	T helper cell
TNF-α	Tumor necrosis factor-α
Treg cell	T regulatory cell

1 INTRODUCTION

The human body is constantly under attack from various pathogens. Most of the time, the immune system clears the enemy without the slightest hint of an intrusion. A crucial function of the immune system is the ability to identify between foreign and self. Therefore, to avoid an attack against self, tolerance is required. Occasionally the control of the immune system breaks down resulting in an assault upon self instead, hence, possibly leading to autoimmune disease (Sinha *et al.*, 1990).

Autoimmune diseases can arise from various reasons. Three nominators are commonly thought to have a causal effect on the development of autoimmune diseases: genes, immune reaction, and environment (for review see Ermann and Fathman, 2001). The original cause of a particular autoimmune disease can be difficult to pinpoint as the disease usually takes a longer period to develop. Additionally, different infectious agents can lead to varying symptoms of the same disease (Kivity *et al.*, 2009). However, autoimmune diseases are antigen specific, often tissue/organ specific as well, and the involvement of autoreactive lymphocytes and/or the encountering of autoantibodies are indicators of autoimmune diseases (for review see Marrack *et al.*, 2001).

Apoptosis or programmed cell death is a common event in the body. Apoptosis is vital in tissue homeostasis, given that it regulates the cell population without inflammatory signals (as in necrosis), thus, resulting in extensive cell removal without tissue damage (Kerr *et al.*, 1972). Moreover, the recycling of cell components makes it economical. Cells undergoing apoptosis form apoptotic bodies (ApoBods), which contain autoantigens such as histones, DNA, and RNA (Schiller *et al.*, 2008). In order to avoid reaction to autoantigens, the apoptotic cells and ApoBods are cleared quickly by phagocytizing neighboring cells (Kerr *et al.*, 1972). However, incomplete clearance of ApoBods can result in autoreactive immune cells and the break of tolerance (Lleo *et al.*, 2008). The players involved in maintaining tolerance are discussed in more detail below.

1.1 Overview of the human immune system

The immune system can be divided into two: the innate and adaptive immunities. The innate immunity is able to respond quickly to pathogens with a restricted set of receptors,

whereas, the adaptive one has a multiplicity of adaptable receptors with a several day delay in response time (for review see Turvey *et al.*, 2009). Moreover, both systems have cellular (hematopoietic and non-hematopoietic) and humoral defense mechanisms. Cells like neutrophils, macrophages and dendritic cells (DCs) are considered to be part of the innate immunity. The innate humoral defense consists of signaling molecules such as cytokines and chemokines, as well as, molecules that make up the complement (for review see Turvey *et al.*, 2009). The innate immune system both clears pathogens and activates the adaptive response. T and B cells, on the other hand, comprise the adaptive immunity. The antibodies produced by B cells provide the humoral response of the adaptive system. What the adaptive immune response loses in reaction time, it takes back in the specificity and memory of the defense (for review see Turvey *et al.*, 2009).

The exact mechanisms by which the immune system maintains tolerance or non-reactivity to self are not entirely certain. However, DCs are considered to be involved in various ways. Firstly, it is assumed that in lymph nodes resides a subset of DCs with high expression of major histocompatibility molecule (MHC) and low number of CD86 molecules that after receiving a signal from migratory DCs, which have ApoBod antigens, induce tolerance on lymphatic T cells (Steinman *et al.*, 2000). Moreover, the DC – T cell interaction leads to either T cell death or anergy, or T regulatory cell (Treg) induction that further regulates the T cells. Additionally, DCs are involved in the clonal deletion of autoreactive T cells (and B cells) (Steinman and Nussenzweig, 2002).

1.1.1 Dendritic cell and T cell interaction

Dendritic cells operate as a bridge between the two systems by stimulating T and B lymphocytes. Dendritic cells were discovered in 1973 from peripheral lymph organs in mice (Steinman and Cohn, 1973). DCs quickly became an interesting subject for study because of their ability to stimulate T cells. DC progenitors migrate from the circulation into non-lymphoid tissue where immature dendritic cells form (Cella *et al.*, 1997). Moreover, these immature DCs have a high capacity of antigen uptake by endocytosis, but poor T cell stimulation capability. Even though DCs’ endocytic activity is lower than macrophages’, the capability of mature DCs to stimulate T cells is much more proficient (Steinman and Swanson, 1995). Nonetheless, after antigen engulfment and processing, DCs mature into professional antigen presenting cells (APCs) and practically lose their

phagocytosis abilities (for review see Banchereau and Steinman, 1998). Mature DCs then migrate to T cell zones in the lymph organs and stimulate T cells (and B cells) (Gunn, 2003) by presenting the processed antigen on an MHC (for review see Steinman, 1991).

T cells require antigen stimulation and a secondary signal from a costimulatory molecule in order to mature and differentiate. A complex of T cell antigen receptors (TCRs) and MHC, as well as, costimulatory molecules such as CD40 ligand / CD40, CD28 / CD80 and CD86 located on T cells and DCs, respectively, is formed between T cell and DC that initiates T cell stimulation (for review see Banchereau and Steinman, 1998). In addition, adhesion molecules help sustaining the cell – cell contact as long as necessary (for review see Lanzavecchia and Sallusto, 2001). The cytokine signaling by the APC, naturally, has an effect on the T cell, as well, by helping the differentiation into a specific T cell subtype, as discussed more below.

T lymphocytes serve as the main pathogen eliminators of the adaptive immune system. T cells vary from cytotoxic killer cells to helper T cells (Th cells) and memory T cells and they can be roughly divided into cytotoxic CD8+ cells and helper CD4+ cells. However, there is a wide variety of subsets in each of the categories mentioned above, and it is highly probable that more subsets will emerge in the future as detection methods constantly improve. Currently, MHC class II (CD4) –related autoimmune diseases have been investigated more than MHC type I (CD8) –related (Sinha *et al.*, 1990), even though new insights into CD8 T cell involvement has been published in recent years (for review see Gravano and Hoyer, 2013). Therefore, CD4+ Th cells are of more interest here. Subtypes belonging to the CD4+ T helper cells are examined next.

1.1.2 CD4+ T helper cell subtypes

The discovery of different T helper subtypes has advanced the immunology field by providing more detailed insights into the immune system. Initially, only two subsets were discovered: Th1 and Th2 cells, because of the differences in the cytokine profiles and the varying immune reaction produced in different diseases (for review see Abbas *et al.*, 1996). Nowadays, there is a variety of CD4+ Th cell subsets, of which the focus here will be given to the four most studied ones: Th1, Th2, Th17 and Treg cells.

Since each differentiated T cell produces specific signals and extracellular markers, the categorization of Th cells into their respective subtypes involves several indicators such as, extracellular markers, cytokine profiles, and transcription factors (for review see Zhu *et al.*, 2010). The extracellular markers for each Th subtype are usually chemokine receptors that are expressed more based on each subtype as follows: CXCR3 (CD183) for Th1, CCR4 (CD194) for Th2, and CCR6 (CD196) for Th17 (for review see Hirahara *et al.*, 2013). The extracellular marker for Tregs is the IL-2α receptor (CD25) (Sakaguchi *et al.* 1995). The cytokines produced by a particular subset induce the polarization of the immune reaction by stimulating naïve T cells to differentiate into that precise subset, as well as, supporting the proliferation of already differentiated cells (Abbas *et al.*, 1996). Moreover, by producing certain cytokines and chemokines, the cells recruit specific immune cells to the required site. The mechanisms by which each subset induces its proliferation are constantly under investigation. The differentiation into a particular subtype is dependent upon the cytokine environment where the stimulation occurs, the antigen concentration, the APC participating, and the costimulation received (for review see Luckheeram *et al.*, 2011).

Th1 cells produce interleukin (IL) -2, interferon (IFN) –γ and Lymphotoxin (LT) –α as the main cytokines (for reviews see Abbas *et al.*, 1996 and Luckheeram *et al.*, 2011). Furthermore, IL-12 and IFN-γ, and to a lesser extent IL-18 and IL-27, are required for the differentiation into Th1 cell (for review Commins *et al.*, 2010). The transcription factor T-box expressed in T cells (T-bet) is considered to be the master regulator of Th1 cells, since it induces IFN-γ production, which inhibits Th2 and Th17 cell differentiation (for review see Zhu *et al.*, 2010). Moreover, signal transducer and activator of transcription (STAT) 1 and 4 are involved in Th1 cell differentiation. Diseases involving intracellular pathogens have Th1 cells as the main defenders, since the production of IL-2 and IFN-γ recruit and activate inflammatory leukocytes, as well as, promote cytotoxic CD8+ T cell differentiation (for review see Abbas *et al.*, 1996). Moreover, Th1 response has been implicated in organ-specific autoimmunity (for review see Luckheeram *et al.*, 2011).

Th2 cell differentiation requires IL-4, which is also one of the main cytokines the cells produce in addition to IL-5, IL-6, IL-10, and IL-13 (for reviews see Abbas *et al.*, 1996 and Commins *et al.*, 2010). GATA3 is the transcription factor master regulator of Th2 cells,

while STAT6 is, also, required (for review see Evans and Jenner, 2013). Th2 cells are involved in allergic inflammation and the clearance of parasitic infections by producing IL-4 and IL-5 that induce immunoglobulin E (IgE) class switching in B cells and the recruitment and activation of mast cells and eosinophils (for review see Abbas *et al.*, 1996).

Th17 cells were named because they are the only known cell line to produce IL-17 (for review see Romagnani, 2008). There is some controversy as to which cytokines are necessary for the development of Th17 cells because depending on the phenotype of the stimulated cell, different cytokines can produce Th17 cells (for review see Romagnani, 2008; Wilson *et al.*, 2007). However, at least transforming growth factor (TGF) –β, IL-6, IL-21, and IL-23 have been recognized to induce Th17 cells (for review see Zhu *et al.*, 2010). For Th17 cells, the retinoic acid receptor –related orphan receptor (ROR) –γt is considered to be the master regulator for lineage commitment (for review see Evans and Jenner, 2013). Furthermore, the close involvement of RORα and STAT3 are recognized in Th17 cells, as well. The cytokine profile of Th17 cells is proinflammatory as they produce IL-17A, IL-17F and IL-22 among others (for review see Littman and Rudensky, 2010). Therefore, Th17 cells can be found at inflamed sites. Also, Th17 cells are involved in the clearance of extracellular bacteria and fungi (for review see Luckheeram *et al.* 2011), as well as in autoimmune diseases (for review see Damsker *et al.*, 2010).

In addition to the T cell subtypes mentioned above, there are those that (try to) control the immune response: T regulatory cells. There are two types of Treg cells, the so-called natural Tregs that mature in the thymus and the induced or peripheral Tregs that can differentiate in the periphery with the proper stimulation (for review see Sakaguchi *et al.*, 2008). Natural Tregs require TGF-β and IL-2 in the absence of IL-6 for differentiation while the peripheral Tregs need IL-10 for differentiation (for review see Commins *et al.*, 2010). The forkhead box protein 3 (Foxp3) is the master regulator for Treg cells (for review see Evans and Jenner, 2013). CD4+ CD25+ Tregs suppress immune reactions to self, quasi-self and non-self; thus, deficiency in Tregs can lead to severe immunopathology, as well as, autoimmune disease (for review see Sakaguchi *et al.*, 2008).

Th cell differentiation involves several intricate pathways and mediators. As methods for investigation improve, more detailed knowledge is gathered in this matter. Table 1 below

summarizes the key factors involved in antigen –related Th cell differentiation described above.

Table 1. **Differentiation requirements for CD4+ T helper cells and the lineage specificity markers involved.** The main cytokines are in bold, and the master regulators of transcription are underlined.

Th subtype (extracellular marker)	Cytokines required for differentiation	Transcription factors involved	Cytokines produced
Th1 (CD183)	**IFN-γ**, **IL-12**, IL-18, IL-27	T-bet, STAT1, STAT4	**IFN-γ**, **IL-2**, **LTα**, TNF-α, GM-CSF
Th2 (CD194)	**IL-2, IL-4,** IL-19, IL-25, IL-33	GATA3, STAT6	**IL-4, IL-5, IL-10, IL-13, IL-25**, IL,-2, IL-9, TNF-α,
Th17 (CD196)	**TGF-β, IL-6**, IL-21, IL-23	RORγt, RORα, STAT3	**IL-17A, IL-17F, IL-21, IL-22**
Treg (CD25)	**TGF-β**, IL-2, IL-10	Foxp3, STAT5	**TGF-β**, **IL-10**, IL-35

1.2 Human parvovirus B19

Human parvovirus B19 (B19V) was discovered in 1975 (Cossart *et al.*, 1975). It belongs to the *Parvoviridae* family, genus *Erythrovirus* (King *et al.*, 2011). It is a small virus (~25 nm) with single stranded DNA (Shade *et al.*, 1986; Summers *et al.*, 1983) with an icosahedral capsid that consists of two main proteins VP1 and VP2 (Cotmore *et al.*, 1986). The virus has a number of non-structural proteins but the main one, non-structural protein 1 (NS1), is the largest (77kDa) and has numerous functions including site-specific DNA binding, ATPase and helicase activities, as well as, serving as a transcription promoter, to name but a few (Brown, 2000; for reviews see Heegaard and Brown, 2002 and Lehmann *et al.* 2003).

B19V causes a variety of illnesses. The viral infection in healthy adults can be non-symptomatic or manifest in flu-like symptoms (for review see Young and Brown, 2004). As the virus's target cells are erythroid progenitors, the infection can result in several hematological manifestations (Kerr, 2015). In children, B19V infection mainly causes erythema infectiosum (fifth disease), while adults usually suffer from arthropathy (Brown, 2000). Moreover, especially in pregnant women, B19V can trigger hydrops fetalis and can cause even fetal death through transplacental infection. Furthermore, B19V infection has been linked to various autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Takahashi *et al.*, 1998; Trapani *et al.*, 1999; for review see Lunardi *et al.*, 2008).

The NS1 protein has been linked to cytotoxicity and DNA damage in the host (Momoeda *et al.*, 1994; Kivovich *et al.*, 2011). NS1 protein can cause apoptosis in erythroid cells (Moffat *et al.*, 1998), as well as, induce the production of proinflammatory cytokines IL-6 and TNF-α (Moffat *et al.*, 1996; Kerr *et al.*, 2001). Through the intrinsic caspase 9 and 3 pathways, the NS1 protein has been demonstrated to induce apoptosis in hepatocytes (Poole *et al.*, 2004). Moreover, the resultant apoptotic bodies have been shown to include both NS1 DNA and self-antigens (Poole *et al.*, 2011; Thammasri *et al.*, 2013). As of yet, the exact causative agent for B19V –related autoimmune disorders is unclear. However, the multiple functions of the NS1 protein give reasons for suspicion towards the protein's involvement.

2 AIMS OF THE STUDY

The immune system is a complex network of cellular and humoral signaling. Hence, knowing the exact mechanism of how a particular pathogen instigates disease can be challenging at times. Unraveling the cause for autoimmune diseases, where the body inflicts an assault upon itself, can be even more demanding. The pathogenicity of human parvovirus B19 is well documented. However, B19V as an instigator of autoimmunity is yet to be confirmed.

Setting up a novel protocol to examine the intricate interaction of NS1 ApoBods -stimulated mature dendritic cells and naïve T cells was an important goal for this study. Moreover, the specific aims of this study were:

1. To examine the formation of subsets of differentiated CD4 T cells after co-culturing with dendritic cells stimulated with B19V NS1 induced ApoBods.
2. To investigate the possibility of the initiation of an autoimmune –related immune reaction by B19V NS1 protein.

The hypothesis was that B19V NS1 induced ApoBods –matured DCs stimulate the differentiation of autoimmune related Th1 and Th17 cells.

3 MATERIALS AND METHODS

3.1 Producing apoptotic bodies

3.1.1 Cell cultures

Spodoptera frugiperda-derived cells (*Sf*9 cells) (ATCC-CRL-1711, Manassas, VA, USA) were used to produce recombinant baculoviruses expressing the parvoviral NS1 construct. The cells were grown in BioWhittaker® Insect-Xpress with L-glutamine (LONZA, Walkersville, MD, USA) medium in conical spinner flasks at 27 ºC, 120 rpm shaking. The density of the cells was kept at 0.5 x 10^6 cells/ml in total volume of 50 ml. The cells were subcultured every 2 – 3 days by counting the cells with a hemocytometer (Bürker, bright-line 0.100 mm, Marienfeld), spinning down (2600 rpm, 5 min) (Thermo Scientific SL 16R, Thermo Scientific, Germany) and resuspending to the proper amount into a new flask and continuing incubation in the above-mentioned manner.

Human hepatocellular liver carcinoma cell line (ATCC-HB-8065) (HepG2 cells) is a B19V non-permissive cell line (Poole *et al.*, 2004). Therefore, it was used both to produce apoptotic bodies (ApoBods) and to check the transduction efficiency (TE) of the viruses. HepG2 cells were grown in Minimum Essential Medium (MEM) (+Earle's, + L-glutamine, -$NaHCO_3$) (Gibco®, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco®, USA), 1% Penicillin-Streptomycin (pen/strep) (Gibco®, USA), and 1% L-glutamine (Gibco®, USA) at 37 ºC, 5% CO_2. HepG2 cells were seeded by trypsinization (0.5% trypsin / EDTA (Gibco®, Invitrogen, Carlsbad, CA, USA) in phosphate buffered saline (PBS), for 5 – 10 min at 37 ºC, at a cell density of 3 x 10^6 cells in 15 ml of media (75 cm^2 flask) or 10 x 10^6 cells in 25 ml of media (175 cm^2 flask).

3.1.2 Virus production and transduction efficiency measurements

Second passage (P2) recombinant baculoviruses that express the enhanced green fluorescent protein (EGFP) and the B19V NS1 protein (*Ac*EGFP-NS1) were produced from already prepared P1 viruses (by MSc Kanoktip Puttaraksa, JYU) as described earlier (Thammasri *et al.*, 2013; Kivovich *et al.*, 2010). Briefly, Bac-to-Bac® Baculovirus Expression system (Invitrogen) was used to produce the recombinant P1 baculoviruses. P2 viruses were prepared by infecting 2 x 10^6 *Sf*9 cells in 5 ml of media with 200 µl of

respective P1 viruses and incubated as *Sf*9 cell cultures as described above. At 48 – 72 h post-infection the samples showing the most prominent signs of infection (enlarged cells, uneven distribution of cells, loss of adherence and fragmented nucleus) were collected, centrifuged (5 min, 3000 rpm) (Thermo Scientific, Germany) and the supernatant transferred to a new tube with the addition of FBS until 2.5 % concentration. The P2 viruses were stored in dark at +4 ºC until further usage. P3 viruses were produced from the P2 viruses by adding 1.5 ml of viruses into 100 x 10^6 of *Sf*9 cells (total volume 50 ml) in spinner flasks. After 48 – 72 h the cells were counted and the samples containing 1.8 – 2.2 x 10^6 cells/ml were collected by centrifugation at 3000 rpm for 10 min. The supernatant was filtered through a 0.2 µm filter (Millipore, Billerica, MA, USA) followed by collection in the same manner as P2 viruses previously.

The viral TE was measured with BD FACSCALIBUR flow cytometer (Becton-Dickinson, NJ, USA) in accordance with a three-day procedure. First, 0.5 x 10^6 of HepG2 cells were seeded in 2 ml media in 6-well-plates and incubated overnight. Second, the cells were infected with 200 µl and 400 µl, respectively, of P3 viruses and incubated in dark, room temperature (RT) for 4 h. After the incubation, the cells were washed with warm PBS and continued incubation overnight in fresh media. Third, the cells were trypsinized and collected for flow cytometry (FC) analysis. Cell-Quest Pro software version 5.2.1 (Becton-Dickinson) was used in the FC data collection and analysis, whereas, FlowJo Flow Cytometry Analysis Software version 8.4.5 (Tree Star Inc., Ashland, OR, USA) was used for the statistical analysis. The optimal required TE of >70% was expected to be used in the ApoBods production (Section 3.1.3). However, a TE of 35 – 50 % was acceptable for use after increasing the virus volume to ensure efficient transductivity.

3.1.3 ApoBods production and purification

For the production of ApoBods, non-permissive HepG2 cells were split into 175 cm^2 flasks with 10 x 10^6 cells the previous day. On the ApoBods production day the cells were first washed with warm PBS before transducing with viruses (6 ml of 50 % TE) or staurosporine (from *Streptomyces sp*., Sigma) (20 µl in 20 ml of media), which was used as the positive control for ApoBods production. The cells infected with the virus were incubated at RT, in dark, on rocking for 4 h before washing again with PBS and continuing incubation with fresh media for 72 h, in dark, +37 ºC, 5% CO_2. The positive controls were

plainly incubated for 72 h after the addition of staurosporine. After 72 h incubation, the supernatant from the cells was collected and centrifuged (3 min, 1500 rpm) before filtering with gravity through 5.0 µm filter (Millipore, Billerica, MA, USA). The filtered supernatant was ultracentrifuged at 37 000 rpm for 1 h at +4 ºC (Beckman Coulter Optima L-90K Ultracentrifuge; rotor 70 Ti, Beckman, Ireland). In order to sustain the form of the ApoBods, the pellet was resuspended in 500 µl of Hank's Balanced Salt Solutions buffer (Gibco®, USA) supplemented with 3 mM $CaCl_2$ and stored at +4 ºC for a maximum of 7 d.

3.2 Dendritic cells and T cells

3.2.1 Cell cultures

Human bladder carcinoma cell line 5637 produces a variety of cytokines that act as growth factors for a variety of leukemia cell lines (Quentmeier *et al.*, 1997). Hence, these cells were grown for the purpose of producing 5637 conditioned media (5637 CM) for the MUTZ-3 dendritic cells. The cells were grown in Roswell Park Memoria Institute -1640 medium (Gibco®, USA) supplemented with 10% FBS and 1% pen/strep at 37 ºC, 5% CO_2, in tissue culture flasks. With a cell density of 2 x 10^6 cells in 75 cm^2 tissue culture flask, the cells were seeded by trypsinization (0.5% trypsin / EDTA (Gibco®, USA) in PBS), for 5 – 10 min at 37 ºC. The media from a confluent cell culture was collected after 48 – 72 h, any dead or floating cells and debris were removed by centrifugation (10 min, 1500 rpm) (Thermo Scientific, Germany) and filtering the supernatant through 0.2 µm filter (Millipore, Billerica, MA, USA). The resultant 5637 CM was stored at -20 ºC.

Two different immune cells were used in this study. Firstly, acute myelomonocytic leukemia cell line (MUTZ-3 cells) (ACC 295, DSMZ, Braunschweig, Germany), a cytokine-dependent dendritic cell (CD34+ CD14+) equivalent for *in vitro* studies, were grown in MEM-α medium (Gibco®, USA) supplemented with 20% FBS, 20% 5637 CM and 1% pen/strep, at 37 ºC, 5% CO_2. The cells were maintained at 0.4 – 1 x 10^6 cells/ml and subcultured every 4 – 6 d. Secondly, T lymphocyte cells (ATCC CRL-8066) were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco®, USA), which was supplemented with 20% FBS, 1% pen/strep and 1% L-glutamine at 37 ºC, 5% CO_2.

The cells were maintained at 0.2 – 2 x 10^6 cells/ml and subcultured every 7 d. Additional media was added to both cell types every 3 – 4 d depending on the cell density.

3.2.2 *DC maturation experiment*

A seven-day maturation procedure for DCs was obtained from Masterson *et al.* (2002). The initial experiments yielded poor results, which resulted in the execution of two duplicate assays in the efforts to optimize the DC maturation protocol. In order to mature MUTZ-3 DCs the cells need to be supplemented with a cytokine mixture: granulocyte-macrophage stimulating factor (GM-CSF), IL-4 and TNF-α (Masterson *et al.*, 2002; Santegoets *et al.*, 2006). Hence, MUTZ-3 DCs were grown (without 5637 CM) for 7 d in the above-mentioned cytokine mixtures with varying concentrations. Group A received the original concentrations obtained from Masterson *et al.* (2002): 100 ng/ml of GM-CSF (Sigma-Aldrich, USA), 10 ng/ml of IL-4 (Sigma-Aldrich) and 2.5 ng/ml of TNF-α (Gibco®, USA). The concentrations of GM-CSF and IL-4 were increased for group B as follows: 150 ng/ml of GM-CSF and 20 ng/ml of IL-4. The appropriate concentrations for each cytokine were determined in collaboration with Heini Brander.

The cytokines were added on days 3 and 6. Simultaneously, a 50% addition of fresh media was placed in the cells. Maturation was finished with a 48 h stimulation period. Both lipopolysaccharide (LPS) and a high dose of TNF-α have been used to stimulate full maturation of MUTZ-3 DCs (Masterson *et al.*, 2002; Santegoets *et al.*, 2006). On day 7 the media was changed, and a high dose of TNF-α (75 ng/ml) was added. Moreover, in the second assay 200 ng/ml of LPS was also used to test the maturation of the DCs in this manner. CD83 is an extracellular marker for mature DCs (Zhou and Tedder, 1996). Therefore, phycoerythrin (PE) anti-human CD83 antibody (BioLegend, USA) was used to measure the maturation of the DCs with a flow cytometer (Beckton-Dickinson) using the staining procedure provided by the manufacturer. Briefly, a total of 0.1 x 10^6 cells were used in the immunolabeling. The cells were washed twice by centrifugation at 2000 rpm for 5 min (Eppendorf centrifuge 5415D, Eppendorf AG, Hamburg, Germany) with 1.5 ml of 1% BSA/PBS before staining. A total volume of 200 µl of washing buffer with 5 µl of antibody was placed on the cells and incubated for 30 min on ice in dark. After incubation the cells were again washed once and re-suspended in 500 µl of washing buffer. 10 000 events were analyzed with the flow cytometer analogously to the manner explained in

Section 3.1.2. After which the cells were fixed in 4% paraformaldehyde in RT for 15 min and mounted on glass slides with 8 µl of DAPI (Prolong Diamond Antifade Mountant with DAPI, Molecular Probes, USA). The non-stimulated cells were considered as background while analyzing the values in Excel (Microsoft Office) and were, therefore, removed from the final values in Figure 1.

3.2.3 First co-culture assay

Before beginning the co-cultures, DCs were matured for 7 d in the above-mentioned manner with group B concentrations of cytokines. Additionally, IL-7 is a crucial cytokine for T cell survival and homeostasis, as well as, keeping naïve T cells alive in a resting state (Tan *et al.*, 2001). Therefore, T cells were prepared for co-culture by culturing the cells in 10 ng/ml of IL-7 for 7 d. On days 2 and 6 a 50% addition of fresh media and a new set of IL-7 were placed on the cells.

The first assay included duplicate experiments and there were a total of four samples in each: as positive control MUTZ-3 dendritic cells stimulated with high dose TNF-α; normal MUTZ-3 DCs grown in cell culturing DC media were used as negative control for the assay, while MUTZ-3 DCs without stimulation functioned as negative control for the cells; MUTZ-3 DCs stimulated with NS1 induced ApoBods were the tested sample. The matured cells were placed into fresh media by removing the supernatant (1500 rpm, 3 min) (Thermo Scientific, Germany) and the stimulation was added to those samples that required it: 75 ng/ml of TNF-α and the NS1 ApoBods. Thus, on day 7 the ApoBods were added by first removing the buffer by centrifugation (Eppendorf centrifuge 5415D, Germany) with max speed (16 100 rpm) for 10 min. Next the ApoBods were resuspended in MUTZ-3 media (without 5637 CM) and added to the cells. All of the samples were incubated for 48 h at 37 ºC, 5% CO_2.

Stimulated DCs and T cells were first co-cultured for two time points (24 h and 72 h) in duplicate experiments. First, both cells were counted with trypan blue (Sigma-Aldrich, USA) and divided into 6-well-plates in 1:4 (DC to T cell) ratios in T cell IMDM media (supplemented with 20% FBS, 1% pen/strep and 1% L-glutamine). Next, the cells were incubated at 37 ºC, 5% CO_2 for the required time points before FC analysis.

3.2.4 *Second co-culture assay*

There were a total of six samples used in the second assay. It was noticed that using TNF-α –matured DCs as a positive control in the co-cultures did not result in differentiated T cells (Figure 2). Therefore, two different positive controls were tested in the second assay: for CD8+ reaction the wild type Baculovirus (1:10) was used to stimulate the DCs and for a CD4+ reaction a strain of *Borrelia burgdorferi* (1:40) (a gift from MSc Bettina Hutz, JYU) was used. Additionally, MUTZ-3 DCs stimulated with staurosporine induced ApoBods were used as a control for ApoBod production and null immune reaction control. The rest of the samples remained the same. The stimulation of DCs was done as previously in the first assay, and the T cells were treated with IL-7 prior to co-culture, as well. The DCs and T cells were co-cultured similarly as described above with an additional 7 d time point.

3.3 Immunolabeling and flow cytometry analysis of differentiated T cells

The co-cultured T cells were immunolabeled in each assay with BioLegend (USA) PE anti-human antibodies for five different CD extracellular markers: CD8a, CD183, CD194, CD196 and CD25. For the CD4 marker, a fluorescein isothiocyanate (FITC) anti-human CD4 antibody (BioLegend, USA) was used. At the appropriate time points the cells were collected and spun down (3 min, 1500 rpm) (Thermo Scientific, Germany). The supernatants were collected and stored at -20 ºC. The cells were re-suspended in the staining buffer (described below), and the samples were divided into separate 2 ml Eppendorf tubes for each stain and one for unlabeled cells.

The first co-culture assay had a staining procedure in which the cells were handled on ice, in dark and 0.2×10^6 cells were used for each stain. The staining buffer was 1% bovine serum albumin (BSA) (Sigma) in PBS. The cells were washed once with 2 ml of staining buffer by centrifugation (Eppendorf Centrifuge 5415D, Germany) for 5 min at 2100 rpm, before and after the addition of an antibody. A 1:50 ratio was used to prepare the antibody/staining mixture with total volume 100 µl per each sample. The stained cells were incubated on ice, in dark for 30 min. After washing only once, the cells were re-suspended into 500 µl of staining buffer and analyzed with a flow cytometer in a similar manner as mentioned in Section 3.1.2. Excel (Microsoft Office) was used to create tables and graphs

of the results. The values from the non-stimulated cells were treated as background and were removed from the final figures.

The staining method for the second co-culture assay was altered in several ways in order to gain a more optimal procedure. Firstly, the number of cells was increased to 0.3 x 10^6 cells. Secondly, a detergent was added into the staining buffer to remove unspecific binding (0.2% of Tween® 20, Sigma-Aldrich) and the cells were washed twice between each step. Thirdly, the amount of antibody was decreased to 1:100 and the incubation time was increased to 1 h. Table 2 below summarizes the adjustments made to the staining procedures between the assays before the FC analysis.

Table 2. **Differences of immunolabeling procedures between co-cultures.**

Assay	Cells	Washing buffer	Wash times between steps	Antibody ratio	Antibody incubation time
1st co-culture	0.2 x10^6	1 % BSA/PBS	once	1:50	30 min
2nd co-culture	0.3 x10^6	1 % BSA/PBS + 0.2 & Tween	twice	1:100	1 h

3.4 Confocal imaging

After the DC maturation and co-culture experiments, the cells were fixed and stained with DAPI as mentioned previously in Section 3.2.2. DCs and T cells were imaged with Nikon A1R laser scanning confocal microscope with NIS-elements (AR software, Plan APO VC 60x oil objective, numerical aperture 1.4) in order to verify both the maturation of DCs and the differentiation of T cells, separately. The excitation wavelengths used were 405 nm, 488 nm and 561 nm for DAPI (blue), FITC (green) and PE (red) stains, respectively. Additionally, differential interference contrast (DIC) images were taken to illustrate the morphology of the cells.

An open access image processing software Fiji (Schindelin *et al.*, 2012) was used in the processing of the images. Brightness and contrast were adjusted for the images, as well as, the average intensities of stacks were combined to form one representative image of a stack for DAPI and PE/FITC channels. Additionally, uneven illumination was corrected for DIC images.

3.5 Cytokine production analysis

The cytokine production from the second assay of co-cultured cells was tested for NS1 ApoBods samples from 24 h and 72 h time points. The non-stimulated samples from the same time points were used as negative control. A commercial kit (Proteome Profiler Array Human Cytokine Array Panel A, R&D Systems, USA) was used in the experiment. The kit could detect 36 cytokines and chemokines in total: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, complement component 5/5a (C5/C5a), CD40 ligand, granulocyte colony-stimulating factor, GM-CSF, macrophage migration inhibition factor (MIF), soluble intracellular adhesion molecule 1 (sICAM-1), interferon-γ (IFN-γ), CCL1, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL8, CXCL10, CXCL11, CXCL12, plasminogen activator inhibitor 1 (PAI-1), TNF-α and soluble triggering receptor expressed on myeloid cells 1 (sTREAM-1). Manufacturer's instructions were followed while handling the kit and ChemiDoc XRS System (Bio-Rad, USA) was used to image the membranes for 15 min. ImageJ macro Dot Blot Analyzer plugin (Gilles Garpentier, University of Paris) was used in the analysis of the membranes. There were two separate signals for each cytokine in each membrane. The Dot Blot Analyzer detected the signal intensity of each blot and the mean value for each cytokine was calculated. The results were normalized according to the six positive controls in each membrane after the background subtractions. Additionally, a paired student's t-test was done to analyze the possible significances between both the non-stimulated cells versus NS1 ApoBod –stimulated cells and the 24 h versus 72 h time points of the same samples in Excel (Microsoft Office).

4 RESULTS

4.1 DC maturation

For a DC to effectively present antigen to a T cell, it must mature to become a professional APC. Here a maturation protocol was set up based on Masterson *et al.* (2002). Two different concentrations of cytokines were used in maturing DCs for 7 d. Group A received the original concentrations suggested by Masterson *et al.* (2002): 100 ng/ml of GM-CSF, 10 ng/ml of IL-4 and 2.5 ng/ml of TNF-α. For group B, the concentrations of GM-CSF and IL-4 were increased to 150 ng/ml and 20 ng/ml, respectively. The concentration for TNF-α

remained the same. The maturation was finished with a high dose (75 ng/ml) of TNF-α for 48 h. Additionally, in the second assay LPS (200 ng/ml) was tested as a stimulant for maturation. The cells were first counted with trypan blue to ensure the viability and proper amount of cells. Figure 1A demonstrates the percentages of DC maturation as analyzed by FC for 10 000 events.

A

7 days in cytokine mixture	Stimulation for 48 h		
	no stimulation	high dose TNF- α	LPS
1st assay			
Group A	5.03	6.78	
Group B	4.42	**10.88**	
2nd assay			
Group A	2.1*	8.5*	5.2
Group B	3.04	**12.26**	5.95

*Only 1005 and 3030 cells analysed in no stimulation and high dose TNF- α samples, respectively.

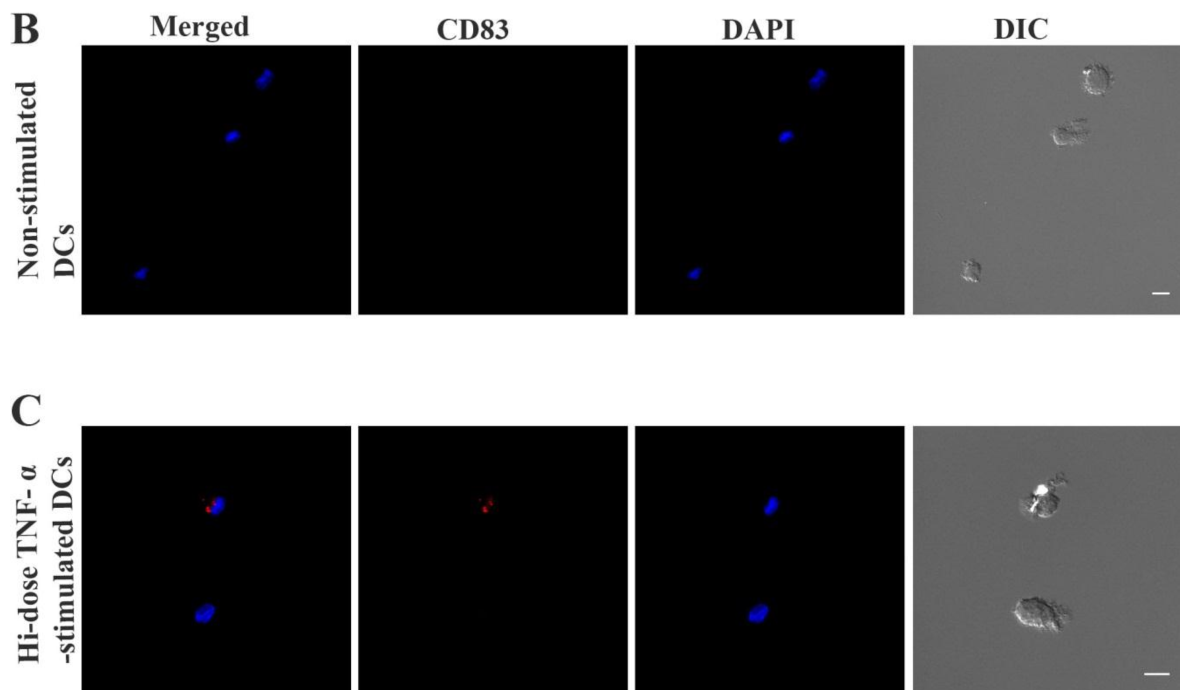


Figure 1. **Maturation of dendritic cells.** A illustrates the percentages of mature dendritic cells gathered from two assays. The cells were first grown in a cytokine mixture for 7 d in order to produce pre-effector cells and then stimulated to fully mature with the addition of either high dose TNF-α (75 ng/ml) or LPS (200 ng/ml) for 48 h. The cytokine concentrations for Group A were 100 ng/ml GM-CSF, 10 ng/ml IL-4, 2.5 ng/ml TNF- α, while Group B received 150 ng/ml GM-CSF, 20 ng/ml IL-4, 2.5 ng/ml TNF- α. In both assays, Group B with the high dose TNF-α stimulation produced more matured cells than Group A or if the stimulation was left out. Additionally, LPS did not work as a stimulant for the cells. The extracellular maturation marker CD83 (red) was measured by flow cytometry for 10 000 cells. The background (values from no stimulation) has been subtracted from the samples. Panels B and C exemplify the confocal images of

non-stimulated dendritic cells and cells stimulated with high dose TNF-α, respectively. DIC (grey) images illustrate the cell morphology while DAPI (blue) dyes the nucleus. The red color demonstrates the extracellular dendritic cell maturation marker CD83 seen in panel C. The scale bar is 10 µm.

Greater values of DC maturation were obtained from group B after high dose TNF-α –stimulation than group A or if the final stimulation was done with LPS (Figure 1A). Ultimately a yield of 10-12 % (highlighted yellow in Figure 1A) of maturated DCs was gained in this procedure. Moreover, LPS should work as a similar stimulant as TNF-α, but here a great deal of the cells had died and only a little over 5 % had matured. Yet, the difference in maturation between group A and group B stimulated with LPS was lower than when stimulated with TNF-α. Additionally, in the second assay, the number of cells analyzed in group A for non-stimulated and TNF-α –stimulated samples was well below the required 10 000 cells. The cells were lost during the staining steps. However, as the values were percentages of the total events counted and based on the first assay, the ratio was expected to stay in a similar range as it was now.

Confocal images were taken after the FC analysis to ensure the maturation of the DCs (Figure 1B and 1C). However, as there were only 0.1 x 10^6 cells used in the FC analysis a great deal was lost during the staining procedure. Hence, in Figure 1B and 1C only a couple of cells were seen under the microscope. Still, a CD83 signal (red) was spotted on one of the cells (Figure 1C). The DIC (grey) images demonstrate the morphology of the cells. The mature cells were larger than the naïve cells.

Overall, DCs matured more with higher concentrations of GM-CSF and IL-4, and a high dose TNF-α –stimulation than with any other combination that was tested. Therefore, the group B cytokine concentrations were chosen to be used in the maturation of DCs in the co-culture assays.

4.2 First co-culture assay and the need for optimization

To test the immunological reaction caused by DCs stimulated with NS1 induced ApoBods, the stimulated mature DCs were co-cultured with naïve T cells for 24 h and 72 h. Extracellular T cell markers CD4, CD8, CD183, CD194, CD196, and CD25 were analyzed with a flow cytometer to examine which T cell subtypes had differentiated. A protocol for co-culturing was developed and tested out in duplicate experiments. Before staining the cells, the co-cultures were checked for viability with trypan blue and the 72 h

cells were counted to ensure the proper amount of cells (0.2 x10^6) for the staining. Figure 2A below exemplifies the percentages of differentiated T cell subsets from the two experiments. The values from the non-stimulated cells were considered as background and were subtracted from the sample values.

A

Cell type	Extracellular marker	1st experiment (%)		2nd experiment (%)	
		24 h	**72 h**	**24 h**	**72 h**
Cytotoxic T cells	CD8	0	2.69	1.99	0.81
Helper T cells	CD4	0	2.55	2.07	0.11
Th1 cells	CD183	2.73	22.49	3.2	1.61
Th2 cells	CD194	3.37	29.91	5.8	5.6
Th17 cells	CD196	0.29	1.49	3.76	0.5
Treg cells	CD25	4.3	21.4	8.6	4.0

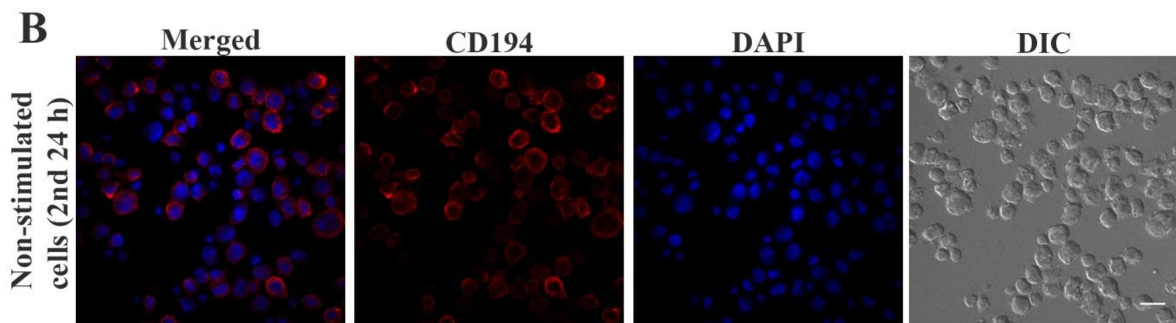


Figure 2. **Immunolabeling of differentiated T cells from the first co-culture assay**. The co-culture protocol was tested out in duplicate assays. A illustrates the percentages of differentiated T cell subsets from the NS1 ApoBod –stimulated sample gathered from flow cytometry analysis of 10 000 cells. The background has been removed. The values for CD183, CD194 and CD196 at 72 h appear questionably high. In the efforts to assure the functionality of the protocol, the samples were stained with DAPI (blue) and mounted on glass slides for microscopy observations. In B, the confocal images from non-stimulated cells from the second assay (24 h time point, CD194 stain) exemplify the need for adjusting the protocol. Scale bar 20 µm.

The 24 h time points presented similar differentiation percentages, although the second trial had somewhat higher values, which indicated that the co-culture was working since T cells were being differentiated. The percentages of CD4 T cells in both experiments and time points, however, appeared questionably low, when considering that the rest of the CD –stains (CD183, CD194, CD196 and CD25) are subsets of CD4+ cells. In addition, the CD183, CD194 and CD25 stains in the first 72 h time point showed suspiciously high values when compared to the values from the second experiment and even the 24 h time points.

Confocal images were taken to verify the FC results. The images in 2B exemplify the unspecificity of the CD194 stain binding, as well as, the need for adjusting the procedure as the cells are from the negative control (non-stimulated sample) from the second 24 h analysis. Hence, the conclusion was drawn that the co-culture protocol was working, but there were too much unspecific binding left from the staining procedure, which resulted in too much of false signal seen in both the FC analysis and microscopy images. Therefore, adjustments in the immunolabeling and FC analysis procedures were needed.

4.3 Second co-culture assay

The DC and T cell FC protocol was adjusted in several ways after the initial assay as was explained in Sections 3.2.3, 3.2.4 and 3.4. Both FC analysis and microscopy imaging were done to all of the samples. The percentages of differentiated T cell subtypes from NS1 and staurosporine ApoBods –stimulated samples, with the background removed, from three different time points, 24 h, 72 h and 7 d are presented in Figures 3, 4, and 5, respectively.

At 24 h all but Th1 cells were differentiated between 0.5 – 2 % for NS1 ApoBods –stimulated samples (Figure 3). When comparing NS1 and staurosporine ApoBods –stimulated samples, the NS1 ApoBods had higher percentages of differentiated T cells in all subtypes except Th17 cells (neither had Th1 cells). The staurosporine ApoBods had stimulated the autoimmune related Th17 cells over 3 % while the NS1 ApoBods ones had 0.51 %. Moreover, staurosporine induced ApoBods had close to none Th2 and Treg cells (0.07 % and 0.04 %) when NS1 ApoBods had 1.04 % Th2 cells and 1.98 % Treg cells. Hence, both stimulations were differentiating a variety of T cell subtypes, though the staurosporine ApoBods had an overall weaker stimulatory capability.

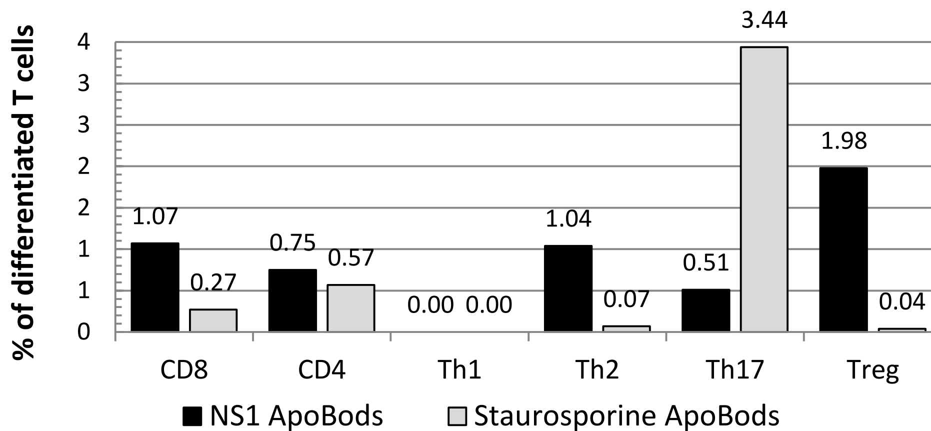


Figure 3. **The percentages of differentiated T cells from 24 h time point of 2nd co-culture assay.** NS1 and staurosporine-induced ApoBods –stimulation differentiated CD8, CD4, Th2, Th17 and Treg T cell subtypes at 24 h.

All T cell subtypes, except CD8 cells, from the NS1 ApoBods sample, had increased by 72 h, whereas only Th1, Th2 and Tregs were increased in the staurosporine ApoBods sample (Figure 4). Moreover, Th2 cells had increased dramatically to 14.83 % and 20.33 % in NS1 and staurosporine ApoBods –stimulation, respectively, which could be an indication of still lingering, unspecific binding from the staining procedure. Therefore, confocal images were taken to check for the unspecificity (Figure 6B). However, the increase in Th1, Th2, Th17 and Treg cells provide further evidence of an inflammatory reaction stimulated by the NS1 ApoBods.

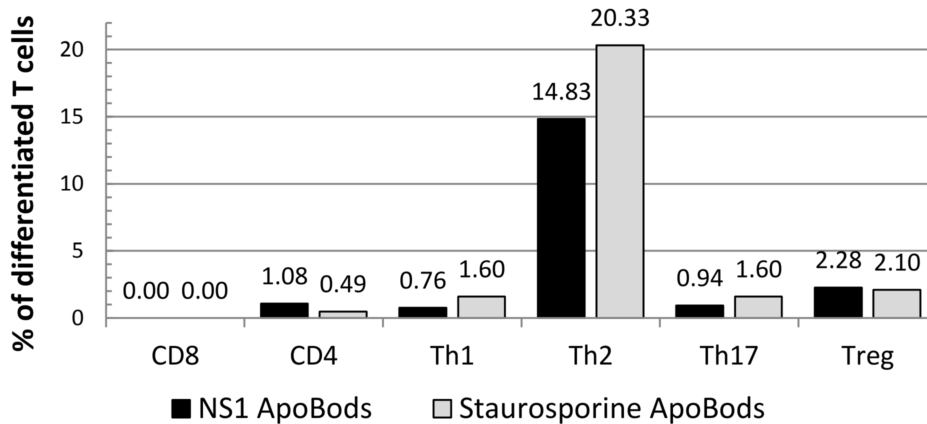


Figure 4. **The percentages of differentiated T cells from 72 h time point of 2nd co-culture assay.** At 72 h CD4, Th1, Th2, Th17 and Treg cells were differentiated in both NS1 ApoBods and staurosporine ApoBods samples, while no CD8 cells were produced.

At 7 d, the percentages for each T cell subtype had diminished in the staurosporine ApoBods sample, which indicated a reduced immune reaction as it should be (Figure 5). Similarly, CD4 stain, Th2 and Treg cells were diminished in the NS1 ApoBods sample, although the percentage of Th2 cells was still high at 6.97 %. However, the percentages for the autoimmune related Th1 and Th17 cells had increased to 2.48 % and 1.48 %, respectively, indicating a continuing inflammatory reaction.

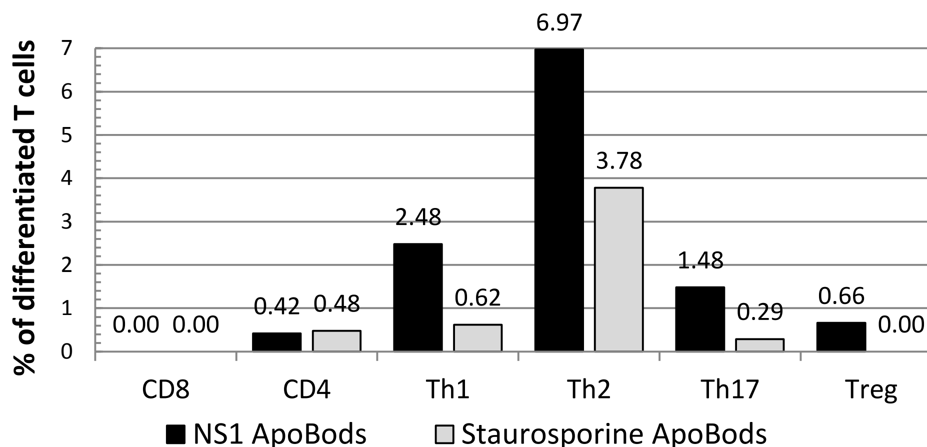
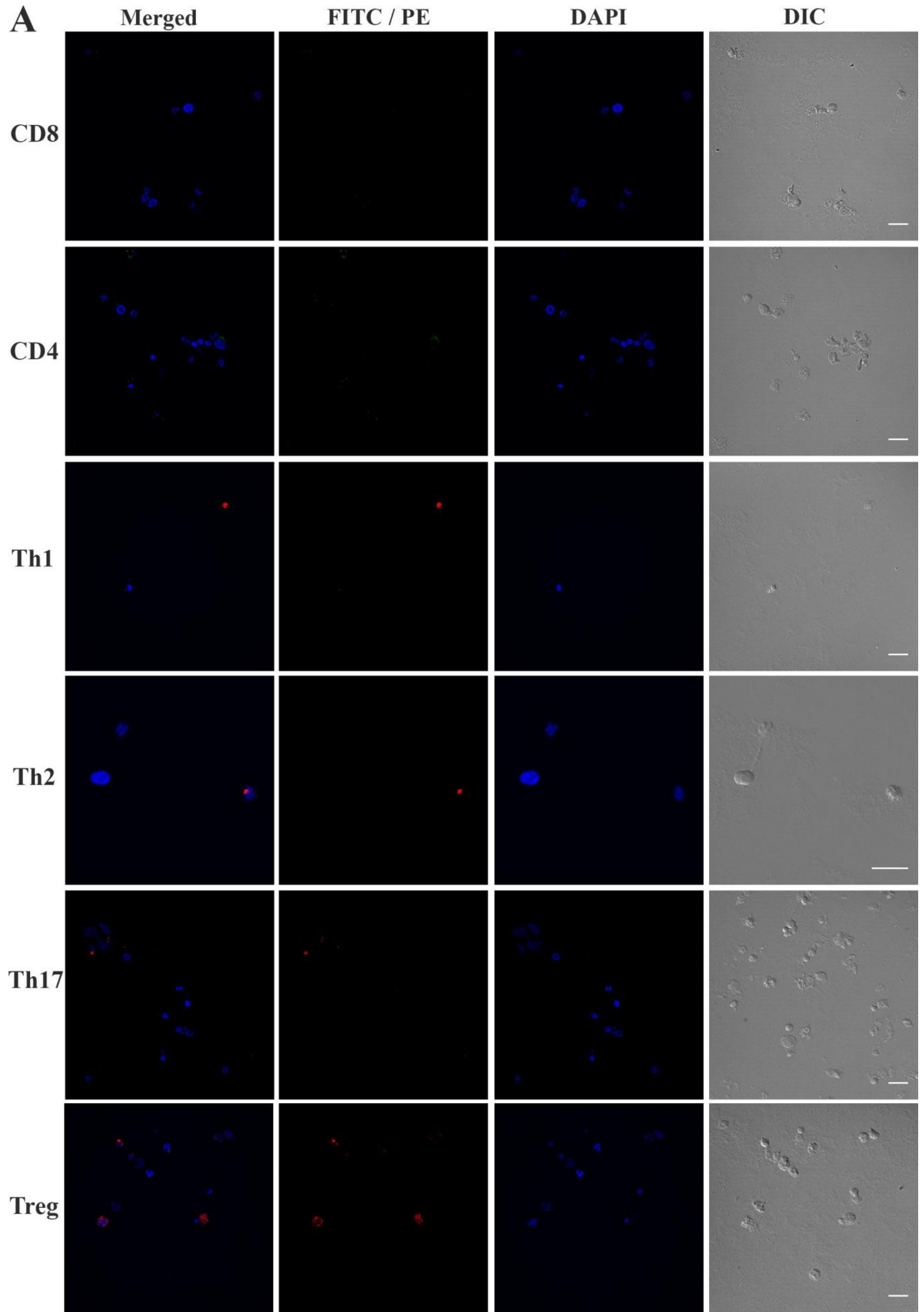
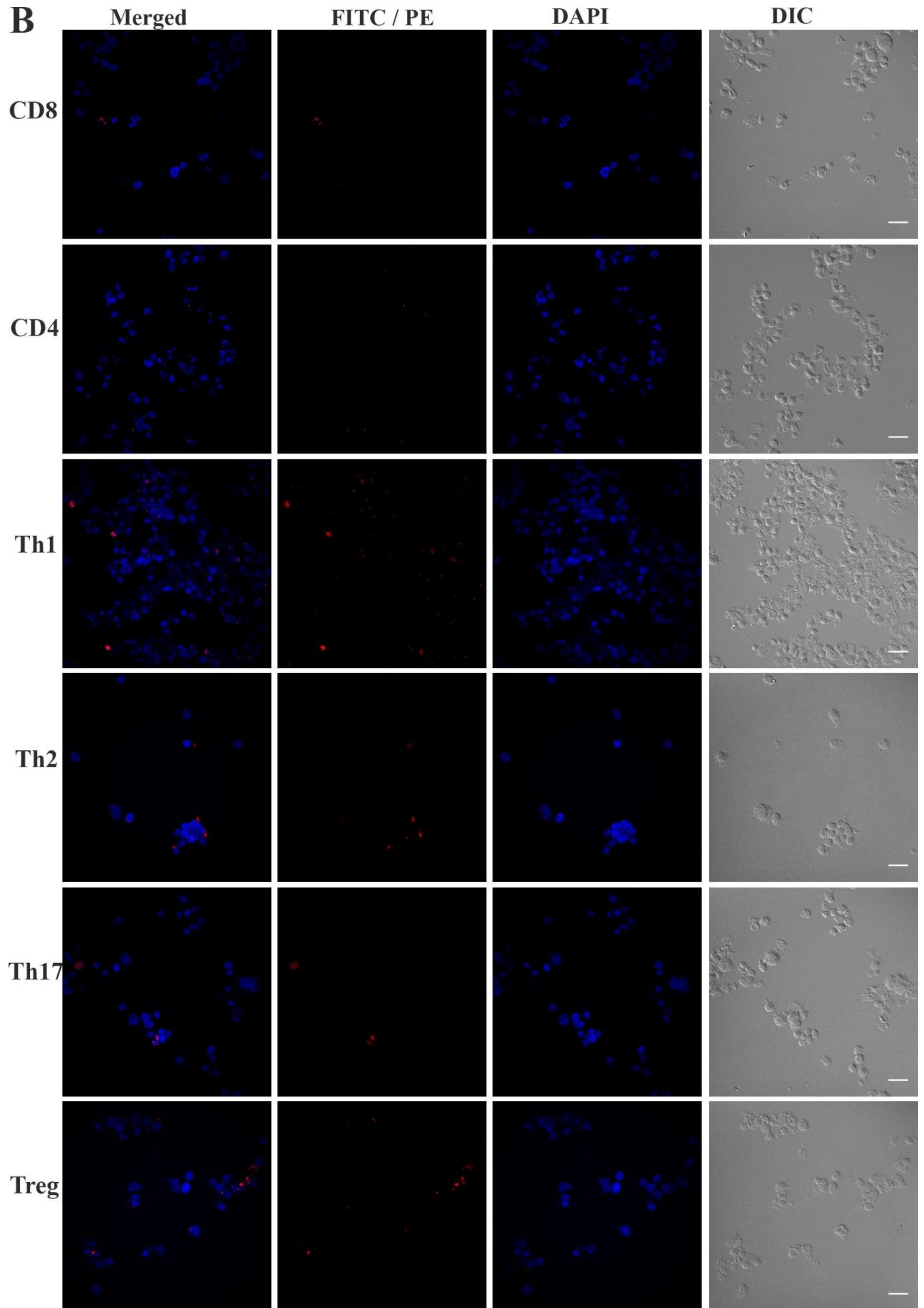


Figure 5. **The percentages of differentiated T cells from 7 d time point of 2nd co-culture assay.** All except CD8 T cell subtypes were differentiated in the NS1 induced ApoBods sample, whereas the staurosporine ApoBods sample had CD4, Th1, Th2 and Th17 cells.

Figure 6 demonstrates the representative confocal images of each stain of NS1 ApoBods –stimulated co-cultured samples at 24 h (A), 72 h (B) and 7 d (C) time points, respectively. The CD4 stain was FITC (green) while the rest were PE (red). As the fixing and mounting of the cells happened after the FC analysis, some of the samples had few cells left in them. However, there was a CD –signal seen in all stains except CD8 at 24 h time point (Figure 6A), demonstrating the differentiation of T cell subsets.

The images from the 72 h and 7 d time points all exhibited T cell differentiation, even the CD8 stains. Moreover, Th1, Th2 and Treg cell stains all elucidated rather strong signals from the last two time points. Meanwhile, the Th17 stains from each time point illustrated slightly lower signal intensities. Nonetheless, a CD –signal can be clearly detected from the samples, confirming the FC results and providing evidence for the proper functioning of the co-culturing protocol.


A
Merged
FITC / PE
DAPI
DIC
CD8
CD4
Th1
Th2
Th17
Treg


B
Merged
FITC / PE
DAPI
DIC
CD8
CD4
Th1
Th2
Th17
Treg

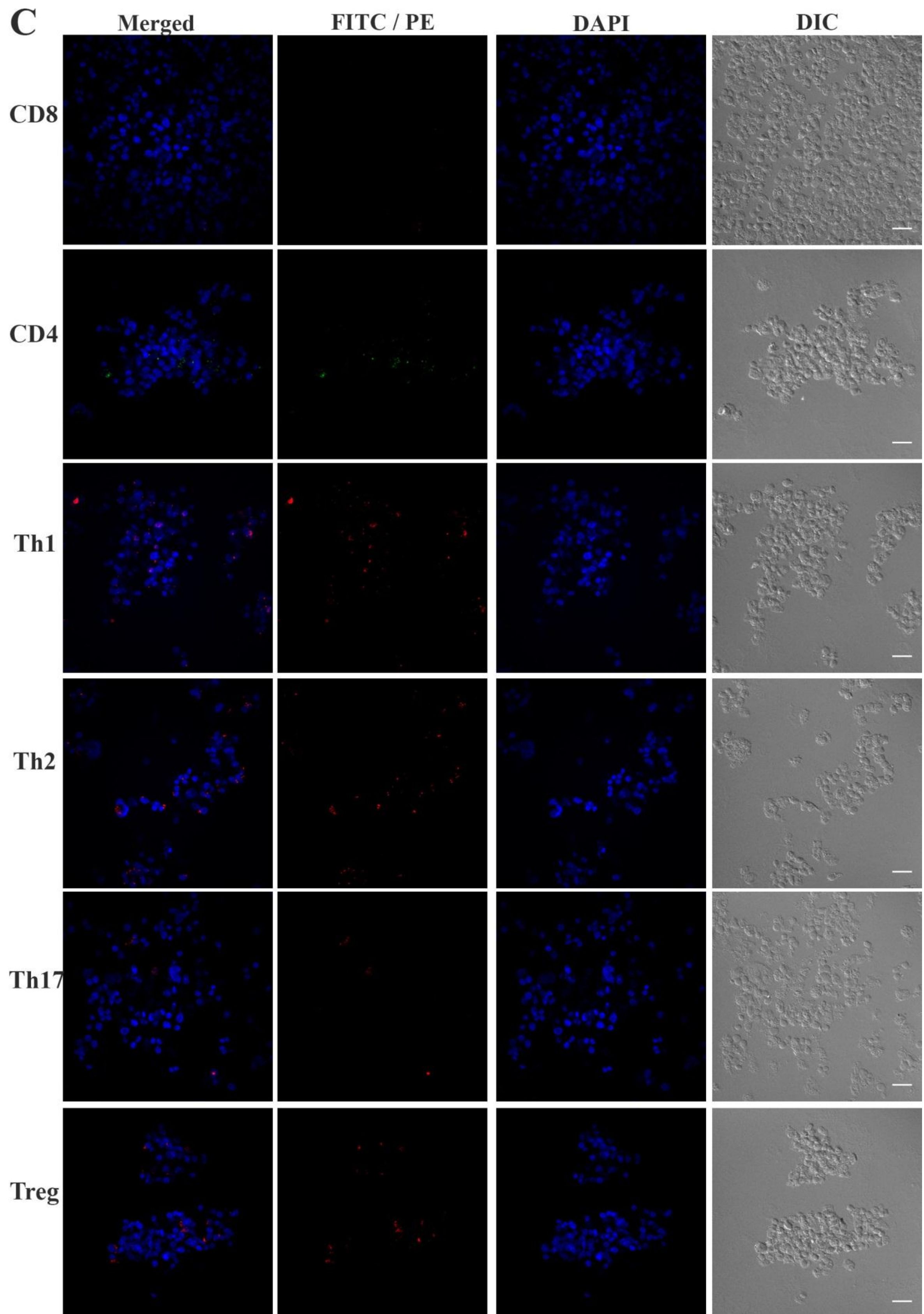


Figure 6. **Second co-culture assay of DCs and T cells.** Confocal images of NS1 ApoBod samples from 24 h (A), 72 h (B), and 7 d (C) time points are presented. Differentiated T cells can be detected at each time point.

Merged images of DAPI (blue) and PE/FITC (red/green), as well as, separate images of each stain are represented. DIC (grey) images exemplify the morphology of the cells. Scale bars are 20 µm.

Some of the T cell subtypes indicated a 0 % of differentiation at various time points. However, this could be attributed to the subtracted background values from the final percentages illustrated in the Figures (3, 4, and 5) above. Therefore, the confocal images correlated to the FC results, since a CD –signal could be observed at each time point even in the samples that demonstrated 0 % differentiation with the FC analysis. Hence, based on the FC and confocal microscopy image analyses, a functional DC and T cell co-culture protocol was established, which resulted in an immune reaction that manifested in differentiated T cell subtypes.

4.4 Cytokine production analysis

In order to verify the T cell stimulation and differentiation, the cytokine production from the NS1 ApoBods –stimulated samples from 24 h and 72 h time points (2nd co-culture) were tested. As a negative control, the non-stimulated cell samples from the same time points were used. The background was removed, and the resultant values were normalized in accordance with the positive controls of each membrane. A paired student's t-test was used to detect any significance in the cytokine production between the samples from the same time points, as well as, between the same samples from varying time points. A *p*-value <0.05 was considered significant. Figure 7 below demonstrates the 12 cytokines/chemokines from the 36 tested ones that produced higher relative signal intensity than 0.01.

As can be seen from Figure 7, NS1 ApoBods –stimulated cells produced more anti-inflammatory cytokines IL-1ra (A), IL-10 (B) and IL-13 (C) at 72 h than at 24 h. Additionally, GM-CSF (D), CCL5 (G), CXCL10 (I), CCL1 (K) and sICAM-1 (L) were produced more, later in the co-culture. Meanwhile, IL-6 (E), MIF (F), CCL3 (H) and CXCL8 (J) were produced more at 24 h.

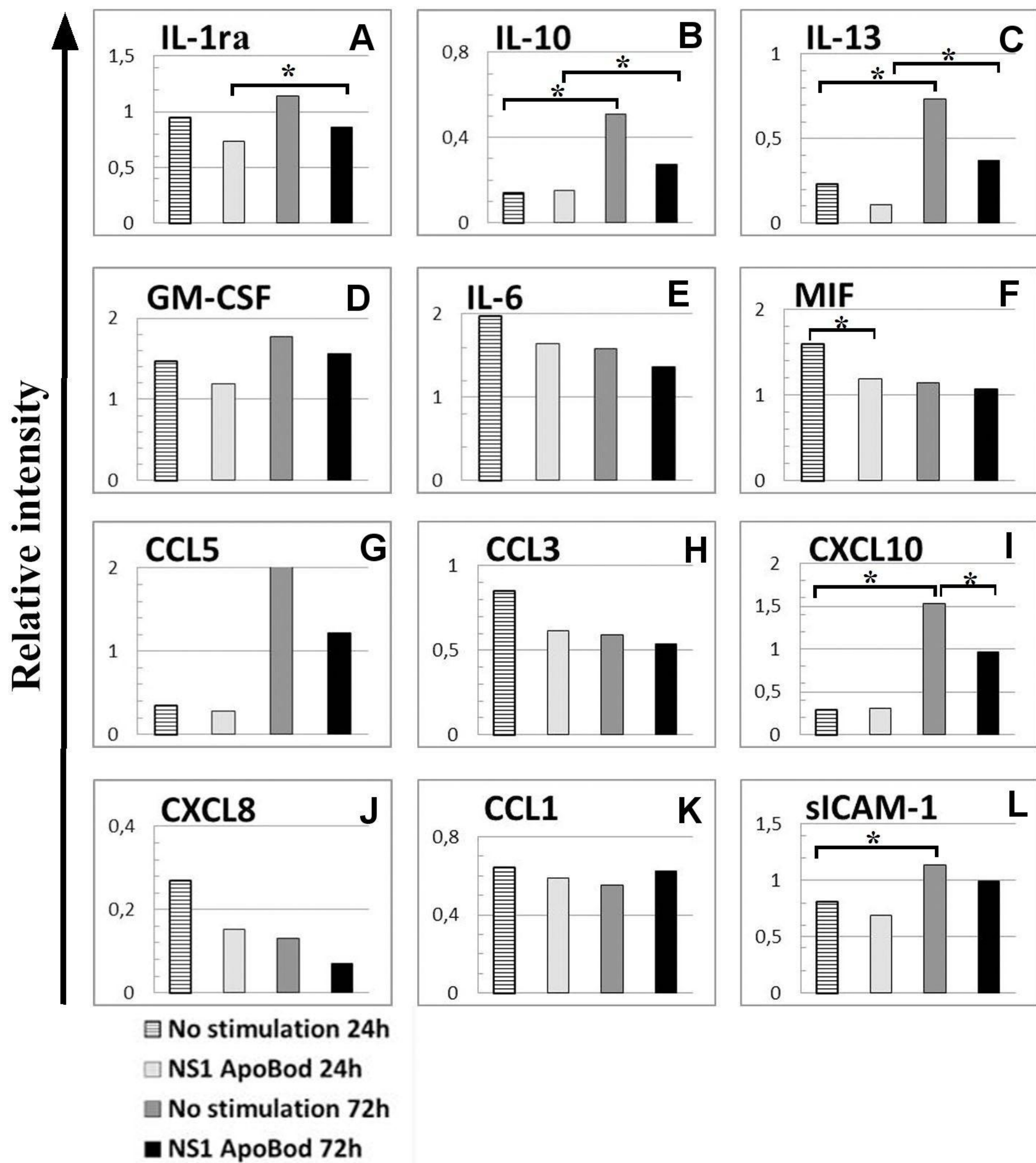


Figure 7. **Cytokine/chemokine production from non-stimulated and NS1 ApoBods -stimulated cells from 24 h and 72 h time points**. A commercial kit was used to analyze the cytokine and chemokine production by co-cultured cells either from the NS1 ApoBods -stimulated or the non-stimulated cells from two time points (24 h and 72 h) of the second co-culture assay. 12 cytokines/chemokines had a relative signal intensity higher than 0.01 and are exemplified here. The y-axis illustrates the relative intensity of signal from each cytokine/chemokine produced in each sample. The background has been subtracted, and the values normalized against the positive controls in the respective membranes. A student's t-test was done both between the different samples from the same time points and the same samples from different time points. Significant values of $p < 0.05$ are illustrated (*). A) IL-1ra, B) IL-10, C) IL-13, D) GM-CSF, E) IL-6, F) MIF, G) CCL5, H) CCL3, I) CXCL10, J) CXCL8, K) CCL1, L) sICAM-1.

There was significance in MIF production between the non-stimulated and NS1 ApoBods –stimulated samples from 24 h and CXCL10 production from 72 h. Yet, mostly there were significant differences between the same samples at different time points as can be seen in IL-1ra (7A), IL-10 (7B) and IL-13 (7C) production by NS1 ApoBods –stimulated cells and sICAM-1 (L), IL-10, IL-13 and CXCL10 (I) production by the non-stimulated cells.

Curiously the signals from the non-stimulated cell samples from a particular time point were higher than their counterpart (NS1 ApoBods samples of the same time point) in all except IL-10 (7B), CXCL10 (7I) and CCL1 (7K). However, as there was some signal found in the FC analyses (considered as background), as well, the discovery of cytokine production here was expected. Moreover, as the number of differentiated T cells in the non-stimulated samples was low, the effect of the produced cytokines has stayed minimal. Hence, the results found here were continued to be considered as valid.

In addition, there were traces of other cytokines being produced by both non-stimulated and NS1 ApoBod –stimulated cell samples from both time points that had very low signal. These included C5/C5a (0.01), CD40L (0.01), IL-1β (0.01), IL-5 (0.01-0.03), IL-16 (0.01-0.02), IL-17 (0.01-0.06), IL-23 (0.01), CCL2 (0.01-0.02), PAI-1 (0.01), CXCL1 (0.01-0.03), CXCL12 (0.01), TNF-α (0.02) and sTREAM-1 (0.01). Mostly, the signals were from 24 h NS1 ApoBods –stimulated or 72 h non-stimulated samples. As the test was performed only once, these low signaling cytokines/chemokines were regarded with apprehension.

Overall, the cytokines produced correlated with the FC analyses, since Th2 and Treg cell –related cytokines IL-13 and IL-10, respectively, were produced. Moreover, the low signal cytokines IL-17 and IL-23 indicated the presence of Th17 cells, as well. However, no signature cytokines for the T cell subtypes were detected. Yet, the expression of proinflammatory cytokines and chemokines produced evidence of a developing immune reaction. Therefore, the cytokine analysis provided further evidence of a reliable establishment of a co-culture protocol, and an inflammatory immune reaction caused by the NS1 induced ApoBods.

5 DISCUSSION

5.1 A successful set-up of DC maturation and DC / T cell co-culture protocols

The immune system fights against pathogens with a subtle interplay between the innate and adaptive immunity. DCs function as a bridge between the two by engulfing, processing and presenting antigens to T cells (for review see Banchereau and Steinman, 1998). Naïve T cells then differentiate into subsets of T helper, cytotoxic and memory T cells based on the stimulus received from an antigen presenting cell. In this study, novel co-culture protocol was set up in order to examine the interactions of T cells and MUTZ-3 dendritic cells after NS1 induced ApoBods –stimulation. Flow cytometry, confocal microscopy, and cytokine production were used in the analysis of the co-cultured cells.

Before the co-culture assays could begin, the DCs had to be appropriately matured. Here a >10% maturation of DCs was gained, which was less than expected (Masterson *et al.*, 2002). However, DCs are prominent APCs, and moreover, MUTZ-3 cells are known to be efficient T cell stimulators (Larsson *et al.*, 2006). Still, the protocol could be adjusted, for instance, by providing the cells extra three to four days (Albert *et al.*, 1998) with monocyte conditioned media (Reddy *et al.*, 1997) for full maturation after the initial seven-day pre-maturation period. However, the cells might not uptake any further stimulus afterward, since DCs' phagocytic abilities lessen after full maturation (for review see Banchereau and Steinman, 1998). Therefore, the yield of matured DC with the current maturation protocol was acceptable, and the co-culture experiments were initiated.

The first co-culture assay involved duplicate experiments (Figure 2). A 1:4 ratio of DCs and T cells was chosen based on previous studies (Fleeton *et al.*, 2004; Larsson *et al.*, 2006), and remembering that the DC maturation percentage was lower than expected (Masterson *et al.*, 2002) the ratio was considered suitable. A seven-day co-culture procedure has been used with DCs and T cells (Sauter *et al.*, 2000; Larsson *et al.*, 2002). Therefore, the third time point (7 d) was added to the second assay. However, the very initial immune reactions were of interest here and since, the cytokine profile of the cells was to be examined, as well, the first two time points (24 h and 72 h) were chosen to be investigated in both of the assays. The time points and the cell ratio seemed well suited as in each time point T cell subsets were being differentiated (Figures 3, 4, and 5), and

inflammatory cytokines were produced (Figure 7). Yet, it would be interesting to examine the effect of various cell ratios on the development of an immune reaction, since cell ratios of 1:30 and even 1:100 have been previously used (Sauter *et al.*, 2000). Moreover, additional time points, such as 14 d (Herr *et al.*, 2000), could be included to examine further the possibility of chronicity of the immune reaction.

Six different extracellular markers for T cell subsets were analyzed with FC. Yet, the extracellular marker examination does not always suffice for determining T cell differentiation, because different subset can have some of the same extracellular markers (for review see Evans and Jenner, 2013). Therefore, a transcription factor analysis should be included in the experiment. For instance, investigation of the master regulators for each cell type (Table 1), hence, a more thorough assessment of differentiated T cell subtypes would be gathered. Nevertheless, examination of both extracellular markers and cytokine production provides valid information of the cells being differentiated (for review see Zhu *et al.*, 2010), and hence, would suffice for the scope of this study.

Staurosporine ApoBods have demonstrated to contain some self-antigens (Thammasri *et al.*, 2013), which can lead to immune stimulation. However, the reaction should not cause a stronger or as strong a reaction as NS1 ApoBods –stimulation. Hence, the higher percentages of stimulated Th2 cells from 72 h in both of the assays (Figures 2A and 4) were intriguing. Two possibilities were thought of being the cause for this: either the protocol needed more adjustments for that particular stain (CD194) or something had happened in the ApoBods production phase, which had resulted in very high stimulation of Th2 cells. Still, as the reactivity seemed to be terminating by the final time point (7 d), the staurosporine ApoBods were continued to be considered as a good null immune reaction control.

Initially, the first co-culture assay resulted in inconsistencies with suspiciously high T cell differentiation for Th1, Th2 and Treg cells at the first 72 h time point (Figure 2A). Moreover, the confocal images of the non-stimulated sample illustrated very high signal (Figure 2B). Unspecific binding of the antibodies was suspected to be the cause. Therefore, a detergent (Tween® 20) was added to the washing solution; antibody concentration was decreased and overall the staining procedure was adjusted (Table 2) to better remove any excess antibody. Hence, even though the second co-culture experiment was only done

once, the results were considered more reliable than in the first assay, because the negative controls were always lower than the tested samples. Therefore, it was concluded that a successful DC and T cell co-culture protocol was established.

5.2 B19V NS1 ApoBods induce an immune reaction *in vitro*

The immune cells of the innate and adaptive immunities work coherently in trying to rid the body of foreign intruders. B19V infections affect people of all ages around the world, usually seasonally (Kerr *et al.*, 1999). Therefore, the possible immune reaction caused by B19V NS1 induced ApoBods was examined here by stimulating DCs with NS1 ApoBods and co-culturing the stimulated DCs with T cells. As a result, an inflammatory immune reaction being formed was discovered. Apoptosis or programmed cell death is a normally occurring event. Apoptotic cells need to be efficiently cleared to maintain tissue homeostasis and avoid inflammation (Kerr *et al.*, 1972). Moreover, apoptotic cells have various signals, which alert the appropriate phagocytic cells of the need to be phagocytized (for review see Poon *et al.*, 2014). Therefore, ApoBods should not induce an immune reaction. Here however, the NS1 induced ApoBods initiated an immune reaction where a variety of T cell subtypes were differentiated.

Moreover, DCs can present antigens contained inside ApoBods whereas macrophages lack that ability (Albert *et al.*, 1998). Furthermore, it has been demonstrated that apoptotic cells do not mature DCs, and thus, help to maintain tolerance (Sauter *et al.*, 2000; Steinman *et al.*, 2000). Using DCs and T cells for the investigation of NS1 ApoBods induced the initiation of an immune reaction. DCs have been demonstrated to uptake antigens from virus infected apoptotic cells and cross-presenting these antigens to cytotoxic T lymphocytes (Albert *et al.*, 1998; Herr *et al.*, 2000; Larsson *et al.*, 2002; Fleeton *et al.*, 2004). Cross-priming involves the presentation of exogenous antigens via MHC class I molecules (for review see Carbone *et al.*, 1998). Moreover, the cross-presenting ability of DCs has been used in stimulating an immune response against pathogens and tumors (Herr *et al.*, 2000; Xing *et al.*, 2009), thus, providing an important mechanism for medical usage. However, the first investigation of the immune reaction instigated by B19V NS1 ApoBods was studied here. Moreover, instead of discovering cytotoxic T cells (CD8+ cells), a variety of CD4+ Th cells were detected. Hence, no cross-priming occurred with B19V NS1 induced ApoBod –stimulation.

As mentioned previously, the cytokine profile of different T cells led to the discovery of a variety of Th subsets (for reviews see Abbas *et al.*, 1996 and Zhu *et al.*, 2010). Hence, the cytokine production from NS1 ApoBod –stimulated cells was analyzed in addition to the extracellular markers analysis. The FC analysis demonstrated the differentiation of all examined T cell subsets (Figures 3, 4, and 5). Moreover, the results of the cytokine analysis illustrated the production of both pro- and anti-inflammatory cytokines (Figure 7). Firstly, the cytokine analysis corroborated the FC analysis in that several Th2 cell –related cytokines were found: IL-13, IL-10, GM-CSF, and IL-5 (for reviews see Commins *et al.*, 2009 and Luckheeram *et al.*, 2011). Additionally, IL-6, produced by APCs, is a known promoter of Th2 cells and a suppressor of Th1 cell lineage (Luckheeram *et al.*, 2011). Moreover, the Th2 –related chemokines CXCL10 and CCL2 (Culley *et al.,*2006), and MIF (Bacher *et al.*, 1996) were detected, as well. Hence, even if IL-2 and IL-4, the cytokines required for Th2 differentiation were not visible in this analysis, the cytokines and chemokines mentioned above did point to Th2 differentiation. Secondly, natural Tregs are known to require TGF-β for differentiation, yet IL-10 is needed for induced Tregs (for review see Commins *et al.*, 2009). Hence, an indication of immune suppression was, also, visible. Thirdly, the complete lack of Th1 –related cytokines IFN-γ, IL-2, IL-12 and TNF-α (for review see Zhu *et al.*, 2010) contradicted with the FC results. However, as the FC analysis indicated, there were no Th1 cells at 24 h and only 0.76 % at 72 h while nearly 2.5 % had differentiated by 7 d. Therefore, analyzing the supernatant from 7 d might have presented Th1 –related cytokines.

Surprisingly, there was more signal coming from the non-stimulated samples than the NS1 ApoBods samples. The high signal could have been because activated cells express more receptors than non-activated cells (Commins *et al.*, 2009), which would lead to more binding and therefore, less free cytokines in the supernatant. However, as the cytokine analysis was only done once, as well, the results need to be examined with apprehension. Therefore, to gain more reliability, the experiment should be adjusted as follows. Firstly, a second experiment should be performed, and secondly, an mRNA analysis should be incorporated into the assay. After all, the mRNA analysis would verify which cytokines

were being transcribed even if the proteins were not showing in the supernatants (Culley *et al.*, 2006).

However, as important as the production of some cytokines was, even more curious was the complete absence of others. The lack of IL-2 and IL-4, for instance, raised suspicions about the capability of T cell proliferation and even some cases the differentiation into a variety of Th subsets. Many of the signature cytokines were missing, as can be seen when comparing Table 1 and Figure 7. Still, the presence of GM-CSF indicated T cell differentiation, as it is secreted by a variety of T cells after activation (Hamilton, 2002; Commins *et al.*, 2009). Furthermore, a new subset of Th cells called ThGM was recently proposed based on the cells ability to produce high amounts of GM-CSF and IL-3 with low production of IFN-γ and IL-17 (Codarri *et al.*, 2011; Zhang *et al.*, 2013). Moreover, Zhang and colleagues (2013) noticed that the ThGM cells differentiated in the absence of IL-4, IL-12, and IFN-γ. Hence, based on the cytokine profile gathered here, ThGM cells might have been differentiated. Unfortunately the cytokine kit (R&D Systems) lacked IL-3 as one of the cytokines being analyzed, hence, providing only speculations about the possibility of ThGM differentiation.

Numerous proinflammatory cytokines and chemokines, as well as, anti-inflammatory cytokines were produced by cells stimulated with NS1 ApoBods. Quite possibly a battle between immune reaction and suppression was taking place, given that anti-inflammatory cytokines IL-1ra, IL-10 and IL-13 illustrated rather high levels of signal alongside with proinflammatory cytokines. IL-1ra is known as an antagonist for the proinflammatory cytokine IL-1, and it is produced 100-fold more during activation, thus, competing for the IL-1 receptor and suppressing inflammatory signaling (Arend, 2002). Moreover, IL-13 is known to be produced early in infection, and it functions in downregulating proinflammatory cytokines (de Vries, 1998). IL-10, on the other hand, can suppress the immune reaction in several ways: firstly, through inactivating DCs and macrophages (O´Garra and Vieira, 2007), secondly, by downregulating class II MHC molecules on monocytes (de Wall Malefyt *et al.*, 1991), and thirdly, by stimulating Tregs (Commins *et al.*, 2009). Additionally, IL-10 has been shown to induce apoptosis in DCs, which has resulted in lessened stimulation of T cells (Cella *et al.*, 1997). Furthermore, immature DCs do not mature after ApoBod engulfment and can still stimulate the formation of IL-10

producing T cells (Dhodapkar *et al.*, 2001). When taking all this into consideration, a conclusion that an immune reaction was caused by the NS1 ApoBods can be drawn. Clearly the immune cells were responding, since proinflammatory cytokines were being produced, and Th cells were being differentiated; and yet simultaneously, controlling the response was being attempted, as can be seen from the production of anti-inflammatory cytokines and Treg cell differentiation.

5.3 Initiation of autoimmunity by B19V NS1 ApoBods

Tolerance must be achieved for the immune system to avoid attacking self and averting autoimmunity. The exact cause of a particular autoimmune disease can be difficult to pinpoint, however, genes, the immune reaction, and environmental factors are recognized to be involved in the development of autoimmunity (Ermann and Fathman, 2001). Furthermore, autoantibodies and/or autoreactive immune cells are an indicator of autoimmune disease. Human parvovirus B19 has been implicated in autoimmune diseases such as RA and SLE, even though the exact means of initiation are currently unknown (Lunardi *et al.*, 2008). The NS1 protein is proven to be cytotoxic to the host by increasing proinflammatory cytokine production and causing DNA damage and even apoptosis (Kerr, 2015). Furthermore, both NS1 DNA and self-antigens were demonstrated to be included in NS1 induced ApoBods (Poole *et al.*, 2011; Thammasri *et al.*, 2013). In this study, the possible relation of NS1 protein to the development of autoimmune disease was hypothesized with the differentiation of autoimmune related Th1 and Th17 cells. The hypothesis was confirmed since there was a steady growth in Th1 and Th17 cells during a week-long co-culture procedure as analyzed by flow cytometry (Figures 3, 4, and 5). However, as there was a lack of signature cytokines for both cell subsets seen in the cytokine analysis, a confirmed involvement of NS1 protein in the induction of autoimmunity cannot be fully proven, yet, although indicators of it was observed. Moreover, the polarization of different Th cell subtypes is more prominent in chronic states, and in the case of *in vitro* studies, in repeated stimulation experiments (Abbas *et al.*, 1996; Lafaille, 1998). Hence, the protocol here should be adjusted by either lengthening the time points or providing additional stimulation, preferentially by carrying out both.

B19V involvement in RA and SLE autoimmune diseases has been studied for numerous years without any conclusive evidence of the exact mechanism by which the virus initiates

either of the illnesses (for reviews see Lehmann *et al.*, 2003 and Lunardi *et al.*, 2008). B19V is thought to promote autoimmunity through molecular mimicry, by the induction of antiphospholipid antibodies or through NS1 stimulated expression of TNF-α and IL-6 (for reviews see Lehmann *et al.*, 2003 and Lunardi *et al.*, 2008). Additionally, Th17 –related cytokines (IL-17, IL-6, IL-1β, and TNF-α) were recently discovered more in SLE patients with B19V infection than patients without the infections (Chen *et al.*, 2014). There were no signs of TNF-α or IL-1β production from the NS1 ApoBods –sample (the low signal of TNF-α described in Section 4.4 came from the non-stimulated –sample). Instead, a high signal from IL-6 and a low signal of IL-17 were obtained, as a result of NS1 stimulation. Thus, IL-6 and Th17 cell differentiation might play a significant role in B19V –related autoimmunity.

Th2 cells have usually been implicated in allergic and asthmatic inflammatory reactions (for review see Romagnani *et al.*, 1996). However, Th2 cells with increased IL-4 and reduced IFN-γ production have been discovered in RA patients, as well (Haddad *et al.*, 1998). Furthermore, high levels of TNF-α, IL-1β, IL-6, IL-15, IL-13 and GM-CSF were found to be indicators of a more severe arthritis in RA patients, while CXCL10 was upregulated in early RA (Hueber *et al.*, 2006). On the other hand, SLE is marked with the prevalence of autoantibodies (Schiller *et al.*, 2008). Atypical production of IL-6 can result in autoantibody-producing B cells (Kishimoto, 1989). Moreover, it was discovered that B cells were activated through IL-6 receptor signaling in SLE, and high levels of IL-6 can be found in RA patients (Ishihara and Hirano, 2002). Additionally, IL-6 has been identified in SLE pathogenesis (Lourenco and Cava, 2009). Recently, experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, was shown to be induced more by ThGM cells than Th1 or Th17 cells (Codarri *et al.*, 2011; Sheng *et al.*, 2014). Hence, the results gathered here could indicate a different initiation of autoimmunity than thought before, since, some of the most expected cytokines, such as the signature cytokines for each Th subtype, were missing, yet others, also related to autoimmunity, were discovered. Additionally, autoreactive B cells might be involved in the initiation of B19V –related autoimmunity, especially in SLE, since autoantibodies are a known indicator of the disease. Therefore, expanding the experiment to include B cells would shed more light on the matter. Still, the discovery of IL-6, IL-13, and GM-CSF in this study gives a strong indication for the possible initiation of an autoimmune disease.

The concentration of the antigen in T cell stimulation affects the polarization of the differentiation. Low antigen concentrations have been shown to produce Th1 cells while high concentration induces Th2 differentiation (Abbas *et al.*, 1996; Tubo *et al.*, 2013). Hence, the prevalence of Th2 cells in this study indicated strong antigen stimulation. Additionally, Tregs can be activated with low concentrations of self-antigens on DCs, which do not suffice to stimulate self-reacting T cells (Sakaguchi *et al.*, 2008), thus, providing tolerance to self. Disruption of tolerance is ultimately the leading cause of autoimmune diseases. Therefore, a strong antigen signal, as seen here, can lead to DCs not stimulating Tregs, which could result in the break of tolerance and autoimmunity. Furthermore, autoimmune diseases can be the outcome of a normal immune reaction extending to self, as well (Ermann and Fathman, 2001). That could be the case with B19V infection. After all, NS1 induced ApoBods contain both foreign and self-antigens (Thammasri *et al.*, 2013), which could easily confuse the immune system to start treating the self-antigens as foreign too. Moreover, it is known that apoptotic self-antigens are uniquely altered, and many of these autoantigens have been recognized in systemic autoimmune diseases (Navratil *et al.*, 2006). Therefore, as NS1 induces apoptosis that would not normally occur, the autoantigens might have specific alterations in them that direct the immune reaction to self, hence, causing the break of tolerance.

Overall, several indicators of allergic immune reaction were found in this study. This was based on the FC analyzes, where Th2 cells were the dominant T cell subtype being formed, as well as, the cytokine production of 24 h and 72 h time points, which illustrated Th2 –related cytokine production and several chemokines related to allergic immune reactions (CCL5, CCL3 and IL-13 (Rot *et al.*, 1992; de Vries, 1998)). However, there were indicators of differentiation of other T cell subtypes, as well. Therefore, with the scope of this study, only the fact that an immune reaction was induced with NS1 ApoBods can be stated with absolute certainty. Nevertheless, it could be possible that B19V –related autoimmune diseases have several different initiation mechanisms, which might involve the break of tolerance through ApoBods formation and the spreading of the inflammatory immune reaction to self, as well as, Th2 cell –like autoimmune disease initiation and/or ThGM cell involvement. Further studies involving transcriptional factor analysis and cytokine mRNA sequencing are needed for obtaining a deeper understanding of T cell differentiation by NS1 ApoBods and the resultant immune reaction.

CONCLUSIONS

This study aimed to set up a protocol for the purpose of investigating the interactions between dendritic cells and T cells after NS1 ApoBods –stimulation. A procedure was successfully established since T cell differentiation occurred. More optimization is still required, as well as, duplicating the results from the second co-culture assay. However, the implications of NS1 ApoBods induced immune reaction were clear. Th1, Th2, Th17 and Treg subsets were being formed with NS1 ApoBods –stimulation. Furthermore, both pro- and anti-inflammatory cytokines were found from the NS1 ApoBods –stimulated sample supernatants, although, proinflammatory cytokines and chemokines were observed in more variation. Therefore, NS1 ApoBods stimulated an inflammatory reaction in the immune cells. Still, conclusive evidence of the development of autoimmune diseases by B19V NS1 protein requires further studies.

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