The role of thyroid hormones in the immune control of cytomegalovirus infection

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University of Rijeka

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Medical Studies in English

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GRADUATION THESIS

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List of abbreviations and acronyms

CMV	:	cytomegalovirus
MCMV	:	mouse cytomegalovirus
MHC	:	major histocompatibility complex
EBV	:	Epstein-Barr virus
T_3	:	triiodothyronine
DNA	:	deoxyribonucleic acid
PFU	:	plaque-forming unit
VZV	:	varicella zoster virus
T_4	:	thyroxine
Daxx	:	death domain–associated protein
HHV	:	human herpes virus
TSH	:	thyroid-stimulating hormone
NB	:	nuclear body
RNA	:	ribonucleic acid
CRP	:	C-reactive protein
HSV	:	herpes simplex virus
KSHV	:	Kaposi’s sarcoma-associated virus
mRNA	:	messenger RNA
PML	:	promyelocytic leukemia protein
IFN	:	interferon
IFI16	:	interferon inducible protein 16
HLA	:	human leukocyte antigen
GrB	:	granzyme B

1 Introduction

1.1 Human Herpes Viruses (HHVs)

Human herpes viruses (HHVs) are part of the Herpesviridae, a large family of DNA. All herpes viruses share a common characteristic: the ability to establish latent infections by evading the immune response of the host. Of the hundreds of herpesviruses known, the eight human herpes viruses (HHVs) which are endemic to humans are classified into three main subfamilies α, β and γ [1–3].

Virus	Subfamily	Sites of Latency
Alphaherpesvirinae	Herpes simplex virus 1 (HHV-1)	Sensory & cranial nerve ganglia
	Herpes simplex virus 2 (HHV-2)	Sensory & cranial nerve ganglia
	Varicella-zoster virus (HHV-3)	Sensory & cranial nerve ganglia
Betaherpesvirinae	Cytomegalovirus (HHV-5)	Monocytes, macrophages, $CD34^+$ cells
	HHV-6	Monocytes, macrophages, $CD34^+$ cells
	HHV-7	$CD4^+$ cells
Gammaherpesvirinae	Epstein-Barr virus (HHV-4)	Memory B cells
	KSHV (HHV-8)	B cells

Table 1: Modified from Elsevier, Inc. Introduction to herpesviridae. In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases. 9th ed. Philadelphia, 2020

The *Alphaherpesvirinae* subfamily has a fast replication cycle, a broad range of hosts and establishes latency in ganglion. The *Betaherpesvirinae* have a slow replication cycle, a more limited range of hosts and persist in monocytes. The two viruses from the *Gammaherpesvirinae* have a very restricted host range, oncogenic potential and persist in lymphoid cells [1–3].

Additionally, some rare zoonotic infections have been observed. Cercopitheceine herpesvirus 1 (Herpes B virus) of the *Alphaherpesvirinae* subfamily, a simian virus that infects macaque monkeys can cause fatal encephalitis in humans [1,4]. Another one is Murid herpesvirus 68 of the γ subfamily mainly found in laboratory personnel handling infected mice, but is asymptomatic [5].

1.2 Cytomegalovirus (CMV)

Cytomegalovirus (CMV) is a large herpesvirus, measuring 220 nm in diameter, and belonging to the Betaherpesvirinae subfamily. It is the most complex herpesvirus, encoding over 150 proteins within its approximately 235 kbp genome. Human cytomegalovirus (HCMV) is one among a variety of species-specific cytomegaloviruses [6].

While HCMV is commonly acquired throughout life and can cause HMV mononucleosis, most infections in immunocompetent individuals remain asymptomatic. Symptoms of CMV mononucleosis include fever, malaise, and myalgias that can last for weeks. Compared to Epstein-Barr virus-induced mononucleosis pharyngitis is less common and severe [7,8].

Laboratory abnormalities may include hemolytic anemia, thrombocytopenia, reactive lymphocytosis, and elevated aminotransferases. Similar findings have been observed in children with acute HCMV infection, but the occurrence of symptomatic HCMV in healthy children is very rare. Even in asymptomatic cases, the virus can be shed in saliva, urine, and vaginal secretions for weeks to months [8,9].

HCMV infection is associated with disease syndromes in organ transplant recipients, including chronic rejection and graft failure [10,11]. It also poses a significant risk as an opportunistic infection in immunocompromised individuals, contributing to morbidity and mortality [12]. Various T-lymphocyte depleting and non-depleting antibodies are frequently utilized for inducing immunosuppression and managing allograft rejection. However, it has been observed that these antibodies can differentially increase the susceptibility to human cytomegalovirus (HCMV) infection following transplantation. These antibodies have been found to confer varying risks for HCMV infection and disease during the post-transplantation period [13–15].

Recent evidence has linked HCMV infections to various diseases, among others, coronary artery disease and inflammatory bowel disease, which are chronic inflammatory conditions, as well as immune senescence in the elderly, multiple sclerosis, and human cancers [16–19]. While the association between HCMV infection and these diseases is not always causative, the high prevalence of HCMV infection (over 60% in the US and over 95% in many regions worldwide) underscores its potential impact on population health [20].

1.2.1 Persistence in the host

Herpesviruses, including HCMV, establish lifelong infections in the host by employing various replication strategies and host cell response regulation mechanisms. The mechanisms of HCMV persistence, though less understood compared to other herpesviruses, likely involve a combination of chronic low-level productive infection and true latency, characterized by limited viral gene expression and absence of genome replication. HCMV persistence has been observed in different tissues such as salivary glands, breasts, intestines, and prostate, but it remains uncertain if the viral DNA detected in these tissues can generate an infectious virus upon reactivation [21,22].

In $CD34^+$ myeloid mononuclear cells, a well-studied reservoir for latent infection, reactivation occurs upon differentiation into $CD14^+$ tissue macrophages, facilitating dissemination of infectious virus into organ tissue [23]. A recent study has provided evidence that mouse cytomegalovirus (MCMV) persists in PDGFRα-positive fibroblastic cells over an extended period and across multiple organs. These cells exhibit comparable or even higher levels of viral genome loads compared to the previously recognized sites of MCMV latency [24]. The establishment and regulation of latency is poorly understood but involves modifications of cellular chromatin, alterations in host cell metabolism, and expression of immunomodulatory cytokines [25,26].

1.2.2 Host response

CMV has a wide range of host cell targets, with primary infection in healthy individuals beginning in mucosal epithelium and spreading to monocytic cells, mainly $CD34^+$ cells and monocytes, establishing latency [27]. While cell immune surveillance in latently infected cells is presumed to be limited, potential T-cell control over latently infected cells has been shown by T-cell responses to latency-associated proteins [28]. Productive infection can be initiated when virus-infected monocytes differentiate into macrophages. Different cell types are responsible for dissemination and for replication. Systemic spread is facilitated by hematopoietic and endothelial cell infection, virus replication by smooth muscle cells and fibroblasts. The first response is by the innate immune system trying to slow down viral replication and dissemination and priming the adaptive immune response through upregulation of costimulatory molecules and inflammatory cytokines like IFN type I [29].

Innate immune system activation involves the recruitment of antigen-presenting cells (APCs), phagocytes, and NK cells [29,30].

The cytokine environment created by NK cells is believed to promote the maturation of adaptive immunity, particularly T cells [31]. The immune evasion strategies employed by CMV to inhibit NK cell activation further emphasize the significance of NK cells in the innate response to CMV [32].

Primary CMV infection is controlled by adaptive immunity, forcing CMV into latency, which is contrasted by the uncontrolled infection in immunocompromised individuals [33].

The presence of CMV-specific T cells appears to be vitally important in regulating CMV viral replication and safeguarding against disease development. However, they are unable to completely eliminate the virus or prevent transmission. These CMV-specific T cells are broadly targeted, and they make up approximately 10% of the memory compartments in both CD4+ and CD8+ T cells in the peripheral blood of individuals exposed to CMV [34].

Approximately one week after the peak of CMV replication, there is an observable presence of circulating CD4+ T cells that specifically target the virus. These T cells exhibit a characteristic synthesis of T helper cell (Th) 1 cytokines, including IFN-γ and TNF-α. Subsequently, within the peripheral blood the emergence of CMV-specific CD8+ T cells can be observed. This sequential appearance of immune cells demonstrates the coordinated response against CMV infection, with CD4+ T cells initiating the immune reaction followed by the subsequent involvement of CD8+ T cells [35,36].

Th1 cytokines, particularly IFN-γ, appear to play a significant role in both early and late virus-specific T cells that accumulate during CMV latency. The continuous and sustained upregulation of IFN-γ genes enables CD8+ effector-type T cells to rapidly release cytokines upon detection of the virus. This mechanism is crucial in suppressing viral replication during the reactivation of latent CMV [37].

1.2.3 Immune evasion by CMV

The species specificity of CMVs is attributed to numerous orthologous viral genes that modulate host cell responses to infection. These genes often share functional similarities and are sometimes organized in conserved blocks across CMV genomes. It is believed that these

viral genes have coevolved with their respective hosts, facilitating CMV persistence while imposing strict species specificity, thereby hindering maintenance in intermediate hosts [38].

Among the functions involved in the optimization of HCMV infection are inhibition of apoptosis, necroptosis, and pyroptosis usually triggered by viral infection, manipulation of the cell cycle optimizing replication of viral DNA, suppression of cellular stress responses (e.g. the unfolded protein response), modifying host cell metabolism, and evasion of host innate and adaptive immune responses [39,40].

The innate immune response is modified by both immediate and early genes in various ways. Inflammatory responses driven by nuclear factor-κB are limited. The expected silencing of initial viral gene transcription by death domain–associated protein (Daxx) of the promyelocytic leukemia protein (PML) nuclear body (NB) is prevented and type I interferon (IFN) gene expression is limited by inhibition of two proteins from different DNA sensor pathways, cytosolic cyclic GMP-AMP synthase and nuclear interferon inducible protein 16 (IFI16) [41,42]. Viral effector functions, including virus-encoded microRNAs, tegument proteins, and immediate-early gene products, inhibit early host cell responses [43].

Other immune evasion mechanisms include downregulating cell surface expression of NK cell-activating ligands, expressing viral proteins that mimic major histocompatibility complex (MHC) molecules, and expressing inhibitory NK ligands such as human leukocyte antigen (HLA)-E [44–46].

Direct evidence supporting an important role of HCMV immune evasion functions that target NK cell activity in HCMV infections comes from studies in animal models, including studies in rhesus macaques that have clearly demonstrated that in the absence of virus-encoded NK cell immune evasion functions, CMV infection was dramatically attenuated [47].

Mechanisms that degrade class I and II MHC molecules and disrupt peptide loading of class II MHC molecules have been found to significantly inhibit the recognition of HCMV-infected cells by $CD4^+$ and $CD8^+$ T lymphocytes [45,48,49].

1.2.4 Pathogenesis in immunocompromised individuals

HCMV infection poses a longstanding challenge in allograft recipients, leading to various end-organ complications such as hepatitis, bone marrow dysfunction, colitis, pneumonitis, and, less frequently, central nervous system (CNS) involvement including encephalitis.

Similarly, disseminated HCMV infection with a comparable range of end-organ complications was prevalent among HIV/AIDS patients before antiretroviral treatment options became available [12].

Among individuals with HIV/AIDS, HCMV retinitis is the leading cause of blindness [50]. In patients with disseminated HCMV infection, active viral replication can be detected in peripheral blood, often in buffy coat leukocytes. Histopathological examination reveals characteristic nuclear inclusions, known as owl's eye inclusions, which are surrounded by a clear halo. Newer studies utilizing polymerase chain reaction (PCR) for accurate quantification of HCMV viremia have shown that higher viral loads are associated with the presence of owl's eye inclusions in tissue sections. This suggests a direct correlation between the detection of histopathological findings and the level of HCMV replication [51].

Studies revealed that effective control of HCMV replication and spread relies on the timely development of virus-specific T cell responses. Correlations were observed between T cell responses and the risk of invasive HCMV disease. Importantly, the significance of virus-specific T cell responses was demonstrated by the transfer of ex vivo expanded HCMV-specific $CD8^+$ T lymphocytes into patient groups with an increased likelihood for HCMV infection [52,53]. In HIV/AIDS patients before widespread use of newer treatment options, like highly active antiretroviral agents, risk of invasive HCMV infections was closely linked to the decline of HCMV-specific T-lymphocyte responses [54,55].

1.2.5 Possible pathogenesis of diseases independent of virus replication

Due to its persistent nature, a possible association between chronic HCMV infection and other health conditions should be taken into consideration. However, conclusive evidence of a causality between chronic disease and HCMV is still missing. The virus's high prevalence in the population, exceeding 80% in some groups, has made it challenging to conduct epidemiological studies with sufficient statistical power. Mechanisms other than lytic virus replication are poorly understood and the presence of viral components in tissues, genetic variations among viral strains, and the variability of viral gene expression within the host pose complexities in understanding HCMV's contribution to chronic disease.

Nonetheless, studies in transplant recipients and normal hosts have strongly implicated HCMV as a co-factor in chronic inflammatory processes, particularly vascular disease. Animal models have identified plausible mechanisms for HCMV's potential role in chronic

vascular disease. Epidemiological studies suggest that HCMV may serve as a co-factor in certain human malignancies, potentially promoting tumor invasion or proliferation through various mechanisms [1,56].

1.2.6 Transmission routes and risk factors

HCMV infection is prevalent worldwide, affecting a significant portion of the global population, particularly during early childhood. More than 50% of the population in the United States carries HCMV, and the prevalence of infection varies based on racial and socioeconomic factors, with infection rates progressively rise during adulthood, exhibiting higher rates of infection during adolescence [20].

In adults, HCMV is commonly acquired through exposure to infected individual's saliva, urine, and genital secretions, including semen. Intimate contact, including sexual activity, plays a significant role in HCMV transmission, particularly in populations with high rates of sexually transmitted infections. Therefore HCMV can be considered a sexually transmitted infection in adults [57].

Exposure in young children is a significant risk factor for HCMV infection. Children can acquire HCMV through various means, such as intrauterine, during childbirth, exposure to breast milk containing HCMV, and contact with other infected children. Intrauterine infection leading to congenital HCMV infection occurs in approximately 1 in 200 births in the United States and potentially as frequently as 1 in 100 live births in certain regions worldwide [58,59]. Transmitted through breast milk from seropositive women ranges from 50% to 70%, depending on the duration of breastfeeding [60].

Infected infants can act as reservoirs for the transmission of the virus, particularly in settings that promote close contact with an infected child. These settings include interactions with parents, caregivers, family members, individuals living in crowded conditions with an infected infant, as well as other young children and caregivers in group care facilities [61].

HCMV can be acquired in hospitalized patients through blood product exposure and organ transplantation from infected donors. However, nosocomial transmission was uncommon even before the implementation of universal precautions in healthcare facilities [62].

The transmission of HCMV through blood products has been well-documented, and various strategies have been explored to prevent such transmission. One widely accepted approach is

to use blood products from donors who are seronegative for HCMV. However, since HCMV seroprevalence is high in donor populations, this approach poses challenges in maintaining an adequate supply of blood products. In high-risk recipient populations, such as premature infants and individuals undergoing allogeneic hematopoietic stem cell transplantation, the use of leukocyte-reduced blood from HCMV-seronegative donors has been recommended [63]. However, some researchers argue that the use of unselected leukocyte-reduced blood products is sufficient [64,65].

In a recent survey on transfusion practices, it was found that the majority (over 90%) of transfusion services adhere to the standard practice of using leukocyte-reduced blood products. However, there was less consistency in the use of leukocyte-depleted products from HCMV seronegative donors for high-risk populations [66].

1.3 Thyroid Hormones

1.3.1 Overview of thyroid hormones

The thyroid hormones regulate metabolism, growth, and various bodily functions. The thyroid gland, anterior pituitary gland, and hypothalamus form the hypothalamic-pituitary-thyroid axis, a self-regulatory circuit. The main hormones produced by the thyroid gland are thyroxine (T_4) and triiodothyronine (T_3). Homeostasis is maintained by the combined action of the anterior pituitary thyroid-stimulating hormone (TSH) and the hypothalamic thyrotropin-releasing hormone (TRH). Hypothyroidism results in symptoms like weight gain, cold intolerance, fatigue, bradycardia and constipation. On the other hand, hyperthyroidism causes muscle weakness, fine tremor, diarrhea, heat intolerance and weight loss [67–69].

The small intestine absorbs the trace element iodine, which is crucial to produce T_3 and T_4. The main sources of iodine are vegetables, seaweed, seafood and iodized table salt. Reduced synthesis of thyroid hormones can be an effect of iodine deficiency following inadequate iodine intake. This can result in hypothyroidism, myxedema coma, goiter and cretinism [67,70–72].

Most thyroid hormones are bound, with only a fraction (0.2%) being unbound and active. Transporter proteins include thyroxine-binding globulin (TBG), transthyretin, and albumin. T_3 and T_4 can detach from their binding proteins to enter cells through diffusion or carrier-mediated transport. Receptors for T_3 are pre-bound to DNA in the nucleus. T_3 or T_4 binding to

nuclear receptors activates transcription factors, leading to gene expression and cell-specific responses. Excretion of TH happens through the bile, after degradation in the liver [67,69,73].

Thyroid receptors, which can bind to both T_3 and T_4, have a much higher affinity for T_3. As a result, T_4 is more inactive and converted to T_3 by or inactive reverse T_3 (rT_3) by the action of three different types deiodinases. Liver, kidneys, thyroid glands and muscles have deiodinases type I (DIO1) and type II (DIO2) which convert T_4 into active T_3, while type III (DIO3), which converts T_4 to rT_3, can be found in the CNS and the placenta [67,69,73].

While influence of thyroid hormones on homeostasis are well established, evidence of their major role in immune response is widely accepted, but only slowly emerging.

1.3.2 Influence of thyroid hormones on immune cell development and function

Thyroid hormones exert significant influences on immune cell populations, contributing to the maintenance of immune homeostasis and the defense against pathogens. T_4 and T_3, are known to affect immune cell differentiation, maturation, and activity. They act through binding to thyroid hormone receptors present in immune cells, thereby modulating gene expression and signaling pathways involved in immune responses [74]. Thyroid hormones also influence the maturation and function of these immune cell subsets [75,76].

Furthermore, thyroid hormones influence the function and activity of immune cells. They regulate the production and release of cytokines, chemokines, and other immune mediators, thereby shaping the immune response. Thyroid hormones have been reported to enhance nitric oxide production as well as phagocytic capacity of macrophages and in NK cells an increase in cytotoxic activity has been observed. They also modulate the synthesis of pro-inflammatory and anti-inflammatory cytokines, affecting the balance between immune activation and regulation [75,77–79].

The precise action on NK cells is currently debated and requires additional investigation. *In vitro* studies have suggested that exogenous T_3 can enhance natural killer (NK) cell activity in older individuals with low serum T_3 levels. On the other hand, hyperthyroidism caused by Graves' disease may restrict NK cell activation. Similarly, treating aged mice with T_4 systemically has been shown to enhance NK cell activity and sensitivity to IFNγ, whereas hyperthyroid mice demonstrate impaired NK cytotoxic function [75,80–83].

Dysregulation of thyroid hormone levels, such as hypo- or hyperthyroidism, can disrupt immune cell development and function, leading to immune system imbalances and increased susceptibility to infections or autoimmune disorders. Conversely, alterations in immune cell activity and cytokine production can also influence thyroid hormone synthesis and metabolism [77,84–86].

CD4+ and CD8+ T cells, the main subsets of T lymphocytes, which are part of the adaptive immune system, orchestrate its cellular immune response [1,87].

Our current understanding of the mechanisms involved in local TH activity within T lymphocytes is limited. It has been reported that thyroid hormone receptors are expressed in T cells at both mRNA and protein levels. Additionally, the process of TH deiodination has been observed in human lymphocytes, although the expression or activity of deiodinases specifically in T cells remains unexplored. Furthermore, there is a lack of analysis regarding the transport of TH into T cells [75,88,89].

2 Methods

2.1 Materials

2.1.1 Cell culture media

Complete Dulbecco's Modified Eagle Medium (DMEM)

DMEM medium (Pan Biotech, GmbH, Aidenbach, Germany), 3-10% fetal calf serum (FCS, Pan Biotech, GmbH, Aidenbach, Germany), 10 mM HEPES (pH 7.2), 2 mM L-glutamine, 105 U/L Penicillin, 0.1 g/L Streptomycin.

Roswell Park Memorial Institute Medium (RPMI)

RPMI medium (Pan Biotech, GmbH, Aidenbach, Germany), 3-10% fetal calf serum (FCS, Pan Biotech, GmbH, Aidenbach, Germany), 10 mM HEPES (pH 7.2), 2 mM L- glutamine, 105 U/L Penicillin, 0.1 g/L Streptomycin, 5 x 10,5 M 2-merkaptoetanol.

2.1.2 Buffers and other media

Methylcellulose medium:

DMEM, 2.2% methylcellulose, 10% FCS.

Medium for flow cytometry:

PBS, 1% bovine serum albumin (BSA), 0,1% NaN3, 1 mM EDTA

Buffer for erythrocyte lysis:

140 mM ammonium chloride (NH4Cl), 2.7 mM potassium chloride (KCl), 1.5 mM potassium dihydrogen phosphate (KH2PO4), 6.5 mM disodium hydrogen phosphate (Na2HPO4), 0.7 mM calcium chloride (CaCl2). For 1x buffer, dilute with H2O.

2.1.3 MHC I molecule tetramers

I used the following tetramers for the detection of MCMV-specific $CD8^+$ T lymphocytes:

- H-2Kb MCMV, m139, APC - TWYGFCLL (419-426)
- H-2Kb MCMV, m57, APC - SCLEFWQRV (816-824).

The tetramers were synthesized at the NIH Tetramer Core Facility (Emory University, Atlanta, USA).

2.1.4 Laboratory mice

Specific pathogen-free (SPF) conditions were used in breeding all laboratory mice at the Laboratory Animal Breeding and Engineering Center of the Faculty of Medicine, University of Rijeka (LAMRI).

Male mice aged 8-12 weeks were bred in individually ventilated cages (IVC system) under a light/dark cycle lasting 12 hours each, water and food accessible as desired, and maintained at an average temperature of 23°C and 55% humidity. The mice used were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and of wild-type C57BL/6J.

Throughout all experiments involving the use of laboratory animals, I followed professional guidelines, ethical norms, and regulations for working with experimental animals (Regulation on the Protection of Animals Used for Scientific Purposes, NN 55/2013, harmonized with DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL, Animal Protection Act (NN 135/06, 37/13, and 102/17), and the Ethical Code of the Faculty of Medicine, University of Rijeka. Additionally, all research was approved by the Ministry of Agriculture, Veterinary Administration. Prior to conducting the experiments, I established the endpoints to ensure the welfare of the animals. Throughout the research, I adhered to the principles of the 3Rs (Reduction, Refinement, and Replacement). I determined the minimum number of animals required to achieve statistically significant results while minimizing animal usage (power of the study) [90].

2.1.5 Viruses

I intravenously infected mice with $2x10^5$ PFU of MCMV (derived from a bacterial artificial chromosome, BAC) pSM3fr-MCK-2fl (full length, clone 3.3). Studies have demonstrated that this clone exhibits biological equivalence to the Smith strain of MCMV. (VR-1399; ATCC, Manassas, Virginia, USA) [90].

2.1.6 Mono- and polyclonal antibodies

- Rat anti-mouse monoclonal antibody specific for CD4 labeled with PE-Cyanine 7 (clone GK1.5), IgG2b, eBioscience, San Diego, USA.

- Anti-mouse monoclonal antibody specific for CD3 derived from Armenian hamster, labeled with PE-Cyanine 5.5, PE-Cyanine 7, or FITC (clone 145-2C11), IgG, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for CD127 labeled with PE (clone A7R34), IgG2a, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for Gr1 labeled with PE-Cyanine 5.5 (clone RB6-8C5), IgG2b, eBioscience, San Diego, USA.
- Mouse anti-mouse monoclonal antibody specific for NK1.1 labeled with FITC, APC, A780, PE-Cyanine 5.5, or PE-Cyanine 7 (clone PK136), IgG2a, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for F4/80 labeled with PE (clone BM8), IgG2a, eBioscience, San Diego, USA.
- Mouse anti-mouse monoclonal antibody specific for CD62L labeled with PE-Cyanine 7 (clone MEL-14), IgG2a, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for Granzyme B labeled with PE (clone NGZB), IgG2a, eBioscience, San Diego, USA.
- DNA intercalator (Fixable Viability Dye) eFluorTM 780, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for IFN-γ labeled with FITC, PE, or APC (clone XMG1.2), IgG2a, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for TNF labeled with PE-Cyanine 7 (clone MP6-XT22), IgG1, eBioscience, San Diego, USA.
- Rat anti-mouse monoclonal antibody specific for CD8b labeled with FITC, PE, APC, Alexa 780, PE-Cyanine 5.5, or PE-Cyanine 7 (clones 53-6.7), IgG1a, eBioscience, San Diego, USA.

2.1.7 Medications

- Euthyrox 100μg (active substance: levothyroxine), Merck d.o.o., Zagreb, Croatia
- Letrox 100μg (active substance: levothyroxine), Berlin-Chemie AG, Berlin, Germany
- Athyrazol 10mg (active substance: thiamazole), JGL d.d., Rijeka, Hrvatska

2.2 Methods

2.2.1 Design of the experiment and *in vivo* treatment

Two experimental series were conducted, each comprising multiple groups of mice. Each group consisted of five mice. Harvesting of the first series of mice took place three days after infection, while the second series was harvested on the seventh day post-infection.

The experimental groups consisted of a control group, an MCMV-infected group, an MCMV-infected group subjected to administration of levothyroxine on a daily basis, and an MCMV-infected group subjected to administration of thiamazole, also daily.

Levothyroxine administration began one day prior to infection using Euthyrox for the first series and Letrox for the second series. The medication was dissolved in water to achieve a concentration of 20 micrograms of levothyroxine per milliliter. Each mouse consumed approximately 5 ml of the solution daily, resulting in an intake of approximately 100 micrograms of levothyroxine per mouse.

Thiamazole administration also began one day before infection and followed a similar administration method. Athyrazol was dissolved in water with a dilution of 200 µg/mL, resulting in an dosage of 1 mg per mouse per day. Due to a wide range of variation or the absence of detectable virus, several findings from this group were excluded and not considered for analysis in this particular series.

Moving forward, the infected group will be referred to as Group 1, the infected group receiving levothyroxine will be referred to as Group 2, the infected group receiving thiamazole will be referred to as Group 3, and the control group will retain its original designation.

2.2.2 Isolation of immune cells from different organs

After euthanizing the animals in accordance with professional standards, I isolated the spleen for cell isolation.

After isolation, I carefully passed the spleen through a mesh using 3% DMEM. The cell suspension was then centrifuged at 500 g for 3 minutes. The pellet was resuspended in 3 ml of red blood cell lysis buffer. After 5 minutes of incubation on ice, I added 10 ml of 3% DMEM

to prevent further cell lysis. The suspension was then centrifuged for 5 minutes at 500 g. The pellet was resuspended in 5% RPMI, and the cells were counted before further analysis [90].

2.2.3 Cell counting

I determined the cell count by using a Neubauer counting chamber. I mixed 200 µl of trypan blue (a dye for dead cells) with 25 µl of the cell suspension. Using a micropipette, I filled the chamber beneath the cover glass with the cell suspension. I counted the cells in 2 opposing quadrants (each quadrant consists of 16 smaller squares). The total cell count was obtained by multiplying the average value from the 2 counted quadrants by a correction factor of 90,000 (correcting for dilution (9x), chamber depth (10x), and volume (1000x)) to obtain the cell concentration per milliliter [90].

2.2.4 Analysis of immune cells by flowcytometry

For phenotypic analysis of cells labeled with fluorescently labeled antibodies, I used flow cytometry. I prepared the cell suspension from the spleen according to the previously described protocols [91]. For surface marker analysis, I diluted the labeled antibody in FACS medium (30 µl) to which I added a specific antibody for FcRII/III (CD16/32, clone 2.4G2) to prevent nonspecific antibody binding. After a 30-minute incubation at +4°C, I washed the cells with FACS medium and centrifuged them for 4 minutes at 300 g. Then, I resuspended them in 150 µl of FACS medium. I differentiated between live and dead cells using eFluor® 780 dye or propidium iodide (PI). For intracellular staining, I used a cell fixation and permeabilization kit according to the manufacturer's instructions (BD Biosciences, San Jose, USA). I obtained the results of the phenotypic analysis of cells using the FACSVerse flow cytometer (BD Bioscience, San Jose, USA) and analyzed them using FlowJo software (FlowJo LLC, Ashland, Oregon, USA) [90].

2.2.5 Stimulation of cells *ex-vivo*

For ex vivo analysis of cytokine production by $CD8^+$, $CD4^+$ and γδ T lymphocytes as well as NK cells, I isolated lymphocytes from the spleen of the mice. I resuspended the lymphocytes in 10% RPMI medium supplemented with Brefeldin A (eBioscience, San Diego, USA) at a 1/1000 dilution and stimulated them with 1 µg of either PMA/ionomycin, m57 or m139

peptides that bind to MHC I molecules for 4 hours at 37°C and 5% CO2. After 4 hours, I surface stained the cells and then fixed and permeabilized them using a kit according to the manufacturer's instructions (BD Biosciences, San Jose, USA). Following fixation, I intracellularly stained the cells with antibodies diluted in PermWash buffer (BD Biosciences, San Jose, USA). After 30 minutes, I washed the cells with FACS medium and analyzed them by flow cytometry [90].

2.2.6 Determining the viral titer in the liver of infected animals

After infecting mice with MCMV, I stored the livers in Eppendorf tubes containing 2 ml of complete DMEM media supplemented with 3% FCS, and then froze them at -20°C until the viral titers were determined. The viral titer in the liver was determined using a virus plaque assay on MEF cells derived from BALB/cJ mice. The pre-prepared MEF cells were thawed and cultured in Petri dishes four days prior to the virus plaque assay. The day before conducting the virus plaque assay, I seeded the MEF cells in a 48-well plate, with a density per well of 5 x 104 cells. On the day of titration, I thawed and homogenized the organs of interest in 2 ml of 3% DMEM using a tissue homogenizer and metal beads. The homogenate was then diluted 200, 2000, 20,000, and 200,000 times using 3% DMEM. I removed the excess media from the wells containing MEF cells and added 100 µl of the homogenate at the respective dilutions to each well. Each dilution was plated in duplicate. The plates were incubated at 37°C with 5% CO2 for 30 minutes, followed by centrifugation at 300 g for 30 minutes. I then incubated the plates again at 37°C with 5% CO2 for an additional 30 minutes before adding approximately 0.5 ml of methylcellulose media to each well. The prepared plates were incubated for 3-4 days at 37°C with 5% CO2, and the resulting plaques, representing virus plaques, were counted using an inverted microscope. One virus plaque corresponds to one infectious unit of the virus (plaque-forming unit, PFU). The number of infectious viral particles in the liver was calculated based on the number of virus plaques at the corresponding dilutions (viral titer). The lower limit of detection was 100 PFU per liver [90].

2.2.7 Statistical analysis of data

Statistical analysis of the data was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, California, USA). The choice of the appropriate test depended on the

number and distribution of the subjects (Mann-Whitney test or Student's t-test for comparing two groups of animals, and ANOVA test with Bonferroni correction for comparing multiple groups). A significance level of $p < 0.05$ was considered statistically significant for comparing results between two groups, while a significance level of $p < 0.01$ was considered statistically significant for comparing results among three or more groups of animals [90].

3 Aims and Objectives

By investigating the influence of thyroid hormones on the immune response in CMV infections, this study aims to provide valuable insights into the complex interplay between endocrine and immune systems during viral infections. It aims to determine the influence of thyroid hormones on antiviral response following CMV infection. Its general aim is to analyze if thyroid hormones have an effect on immune response during viral infection, namely after infection with CMV.

The first objective is to investigate the influence of TH on some innate lymphocytes, mainly NK cells and γδ T cells which are believed to serve as a link connecting the innate and adaptive responses of the immune system.

The second objective is to investigate the influence of TH on the adaptive immune response, mainly $CD8^+$ and $CD4^+$ T cells as well as on leukocytes ($CD45^+$) in general.

The third objective is to see if possible changes in the severity of the immune response lead to changes in viral dissemination.

We want to achieve this by analyzing immune cell subsets and functional markers through flow cytometry to examine the impact of thyroid hormone levels on immune cell activation.

4 Results

4.1 Influence of thyroid hormones on innate lymphocytes

To investigate the influence of thyroid hormones on antiviral immunity, animals were infected with MCMV and simultaneously treated with thiamazole and levothyroxine through drinking water. To evaluate the influence of hormones on the function of innate lymphocytes, specifically NK cells (see Figure 1) and γδ T cells (see Figure 2), animals were euthanized three days after infection.

Levothyroxine is a synthetic form of T_4 that undergoes peripheral conversion to T_3, the biologically active metabolite, as well as rT_3, the biologically inactive metabolite. Thiamazole, on the other hand, inhibits the synthesis of thyroid hormone by disrupting the incorporation of iodine into tyrosyl residues of thyroglobulin.

Figure 1 illustrates the differential expression of GrB, TNF and IFNγ. While no significant differences were observed for GrB (Figure 1A and TNF (Figure 1B), there was a statistically significant difference in IFNγ expression (Figure 1C). These findings indicate that thyroid hormones had a limited impact on the function of NK cells.

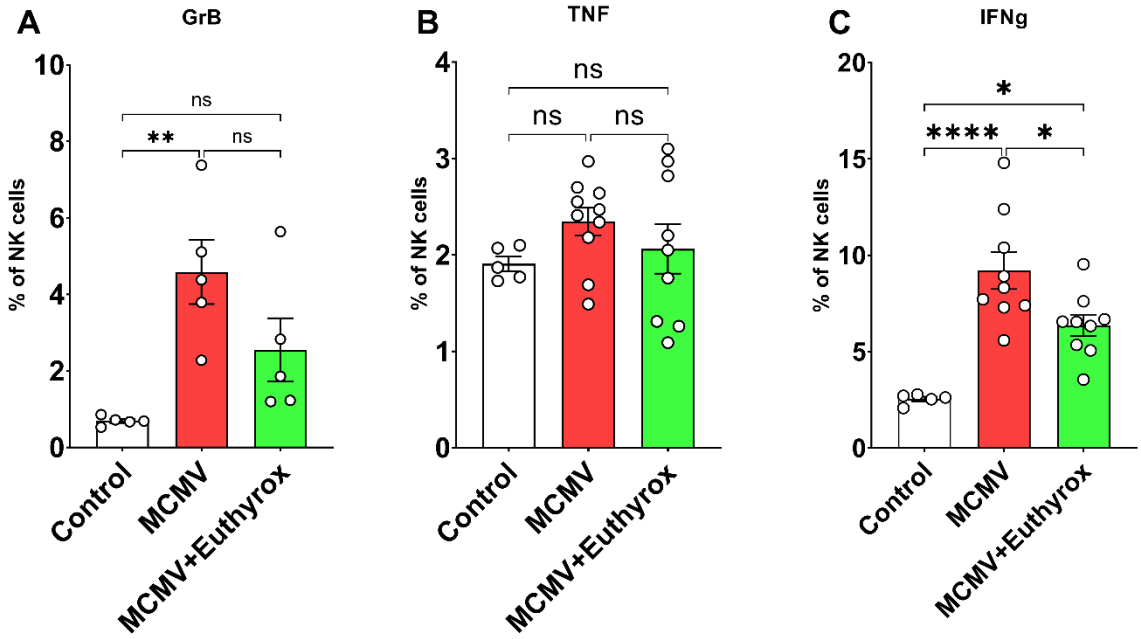

Figure 1: *Influence of TH on NK cell activity.* ***A:*** *Percentage of NK cells producing GrB.* ***B:*** *Percentage of NK cells producing TNF.* ***C:*** *Percentage of NK cells producing IFNγ*

Regarding γδ T cells (Figure 2), the only noteworthy difference observed was an increase in TNF expression between the control group and infected mice (Figure 2A). Since the

levothyroxine receiving group did not exhibit significant deviations from either the control group or Group 1, the influence of levothyroxine on these cells remains inconclusive. IFNγ however did not differ with statistical significance between any group of mice (Figure 2B).

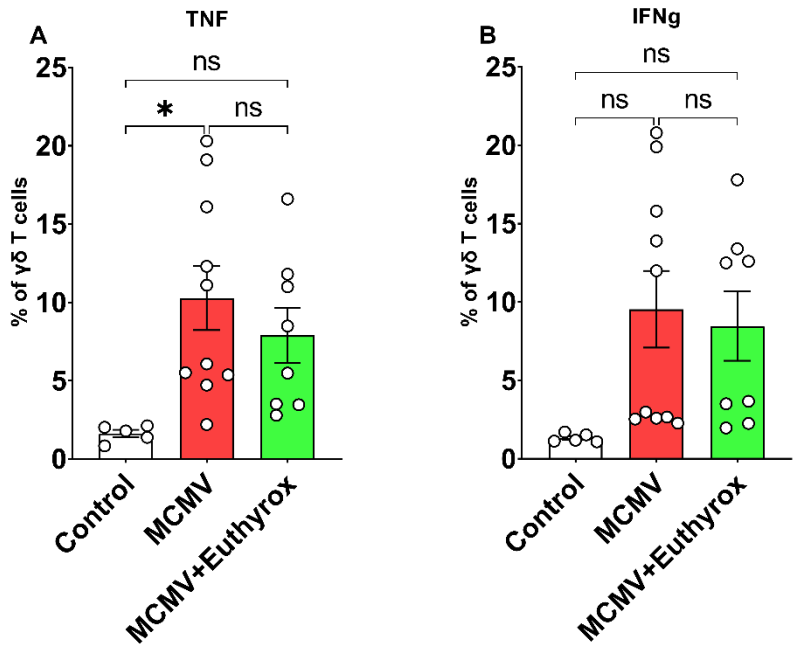

***Figure 2**: Influence of TH on γδ T cell activity. **A:** Percentage of γδ T cells producing TNF. **B:** Percentage of γδ T cells producing IFNγ.*

4.2 Influence of TH on adaptive lymphocytes

To investigate the impact thyroid hormones have on the function of T cells, animals were infected with MCMV in parallel with treatment with letrox and thiamazole. Seven days post infection the mice were analyzed.

First, we evaluated the total count of lymphocytes in the spleen (Figure 3). We observed a significant increase in lymphocyte numbers in the infected group and the infected group receiving thiamazole, while the infected group receiving levothyroxine did not exhibit a significant increase compared to the control group. However, the disparity among the three groups did not reach statistical significance so conclusions about the exact influence of levothyroxine on the number of lymphocytes are hard to draw.

Next, we examined the percentage of $CD8^+$ cells in the spleen (Figure 4). We observed a notable increase in all groups compared to the control group; however, there were no significant differences observed between the groups. No influence of TH was observed.

Subsequently, we investigated the impact on the distribution of $CD45^+$ lymphocyte subsets in the spleen (Figure 5). We observed a significant reduction in B cells between the control group and all other groups. However, no changes were observed between the groups themselves, and there were no alterations in the other subsets of $CD45^+$ lymphocytes. TH did not appear to influence these subsets.

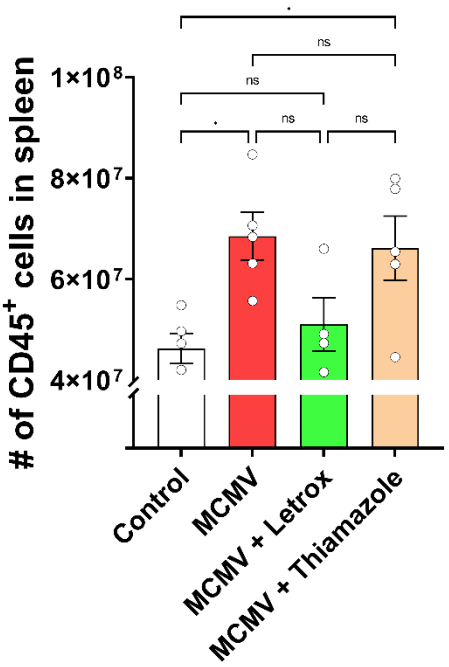

Figure 3: *Influence of levothyroxine and thiamazole on the number of lymphocytes in the spleen in the context of CMV infection.*

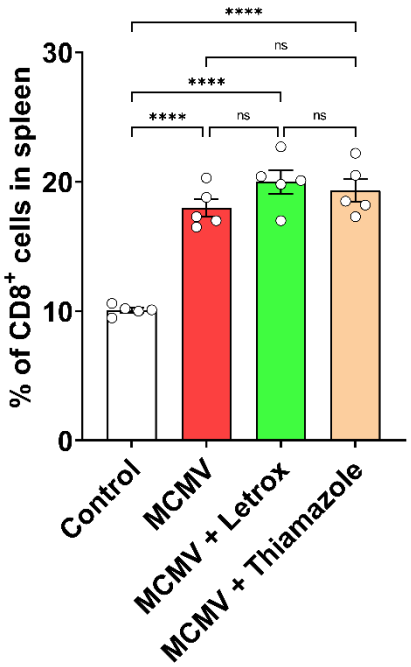

Figure 4: *Influence of levothyroxine and thiamazole on the percentage of $CD8^+$ cells in the spleen in the context of CMV infection.*

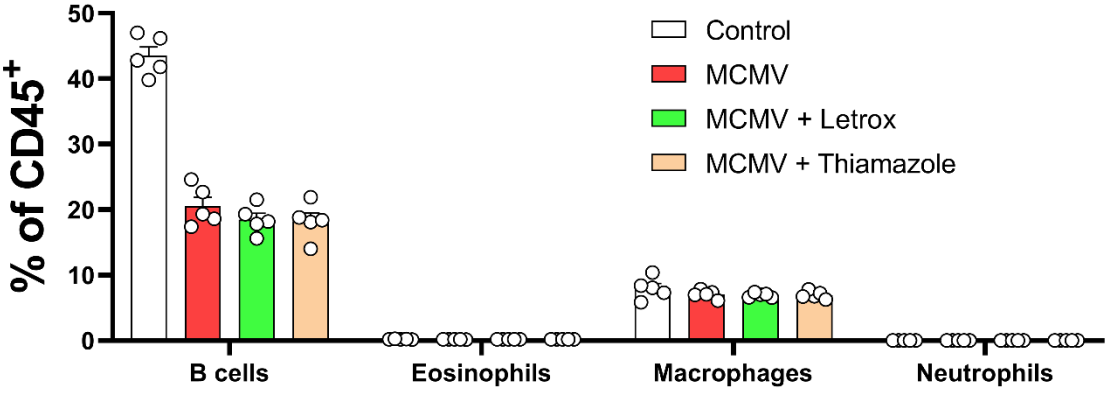

Figure 5*: Influence of levothyroxine and thiamazole on the subsets of $CD45^+$ lymphocytes.*

We aimed to assess the impact on the percentage of $CD4^+$ (Figure 6A) and $CD8^+$ (Figure 6B) T cells expressing IFNγ and TNF, respectively. To that end we stimulated them with PMA and

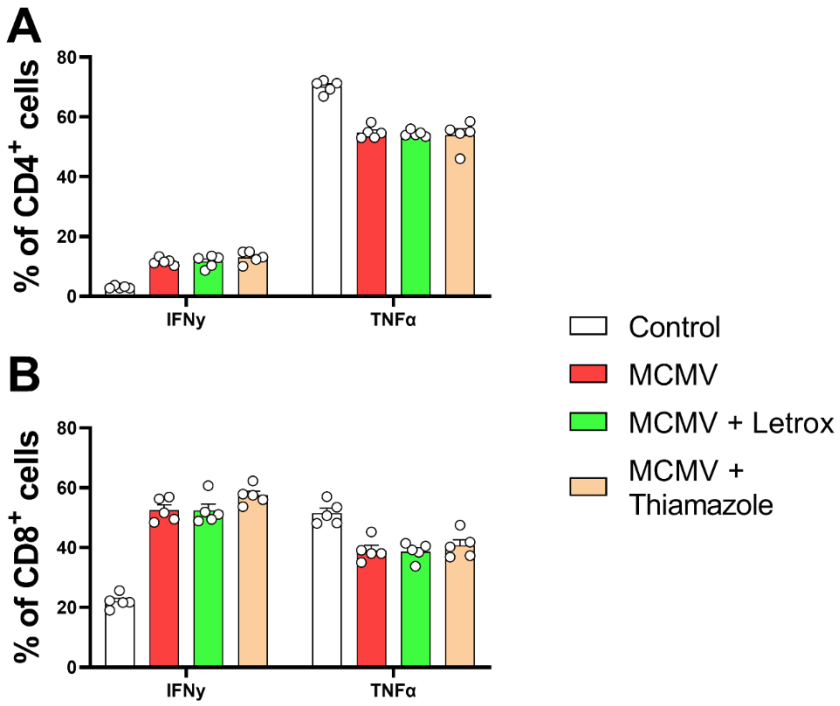

Figure 6: *Cytokine expression of PMA ionomycin stimulated $CD8^+$ and $CD4^+$ cells.* ***A:*** *Percentage of $CD4^+$ cells expressing IFNγ and TNF.* ***B:*** *Percentage of $CD8^+$ cells expressing IFNγ and TNF*

Ionomycin and were able to observe an increase in IFNγ expression between the control group and the other groups, while TNF expression showed a decrease from the control group to the other groups. However, within each group, no statistically significant changes were observed, so no TH influence was detectable.

We then stimulated $CD8^+$ T cells with viral epitopes m57 (Figure 7) and m139 respectively (Figure 8).

In the m57-stimulated cells (Figure 7A, B), we observed a significant increase in cytokine expression in levothyroxine receiving mice compared to simply infected once. However, thiamazole did not demonstrate any notable influence, as there were no significant differences observed between infected and infected, thiamazole receiving groups. We conclude that TH did influence cytokine expression in m57 stimulated $CD8^+$ cells.

Upon stimulation with the viral epitope m139 (Figure 8A, B), the changes in the expression of IFNγ and TNF exhibited varying levels of significance. Notably, while there was no disparity in IFNγ expression (Figure 8A) between the infected group and the group receiving

levothyroxine, the difference in TNF expression (Figure 8B) between these two groups was statistically significant. However, thiamazole treatment resulted in decreased expression of both IFNγ and TNF compared to both infected and levothyroxine receiving groups. Here we can conclude that levothyroxine increased expression of TNF while thiamazole decreased the expression of both IFNγ and TNF in m139 stimulated $CD8^+$ cells.

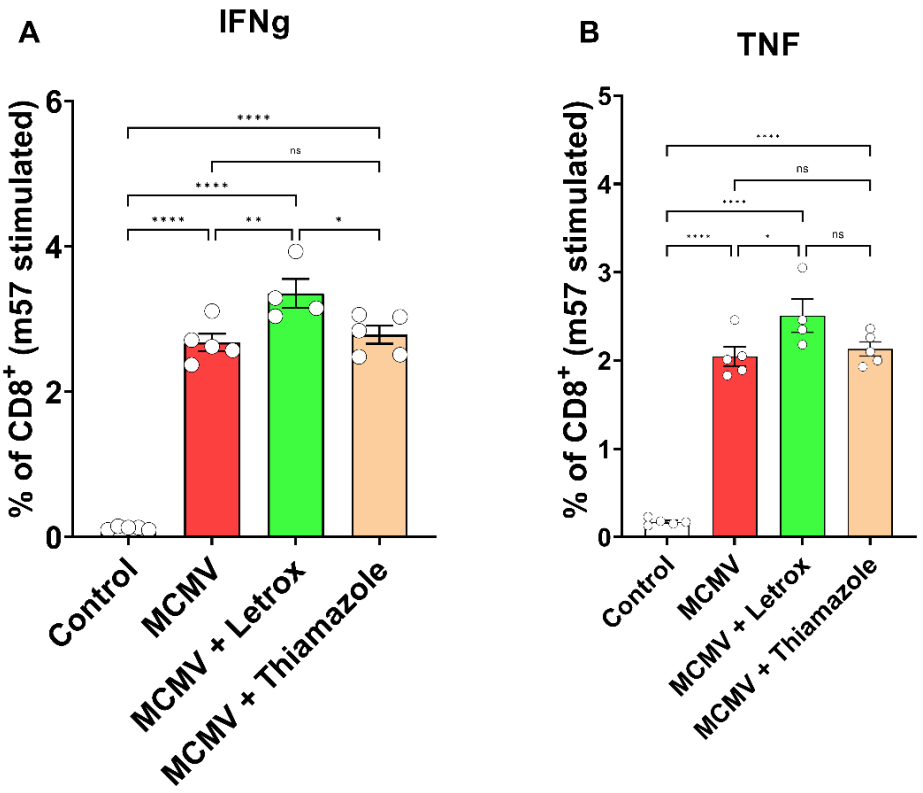

***Figure** 7: Cytokine expression of m57 stimulated CD8⁺ cells. **A:** IFNγ expression; **B:** TNF expression*

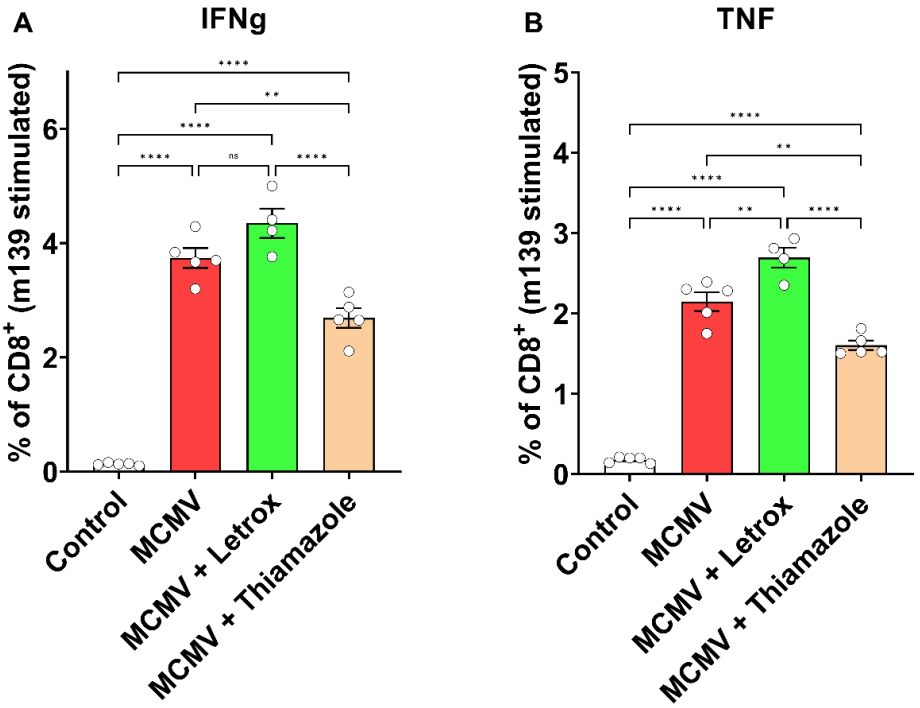

***Figure 8:** Cytokine expression of m139 stimulated CD8⁺ cells.*

Lastly, we wanted to observe the number of plaque forming units (PFU) in the liver (Figure 9A, B). To that end we counted the PFUs of mice sacrificed three days (Figure 9A) post infection and of mice sacrificed 7 days (Figure 9B) post infection. Here we could not find any significant changes in PFUs between the groups. TH did not show influence on the amount of PFUs in the liver.

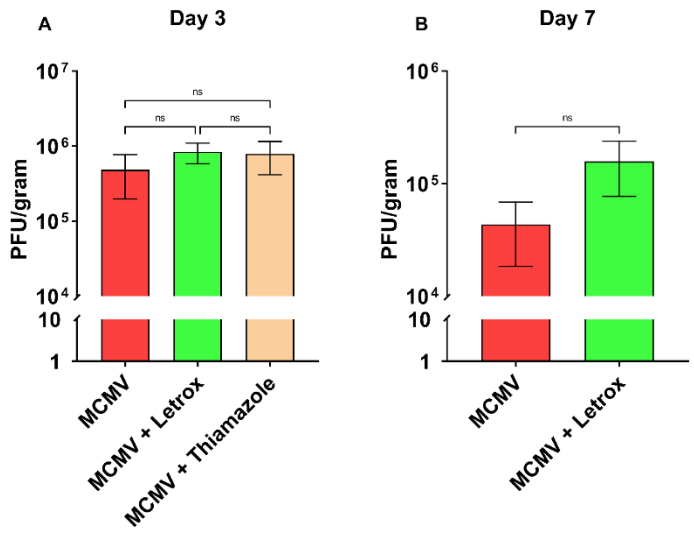

Figure 9: *Plaque forming units per gram in the liver of CMV infected mice.* ***A:*** *PFU/gram 3 days post infection.* ***B:*** *PFU/gram 7 days post infection.*

5 Discussion

The goal of this experiments was to investigate the impact of thyroid hormones on antiviral immunity during MCMV infection.

Our first objective was to investigate the impact of TH on innate immune cells. Here we observed no significant differences between infected mice and infected mice receiving Levothyroxine at three days post-infection, except for IFNγ-producing NK cells. However, considering the spread of our data from this series, we believe the difference to be insignificant. The moderate, immune response decreasing influence we observed was contrary to our initial expectations that TH would lead to an increased immune response [92,93], as a higher percentage of NK and γδ T cells in infected mice expressed IFNγ, TNFα, or GrB compared to the levothyroxine receiving group. Those findings may be more in line with previous observations in hyperthyroid mice [80,82]. Nevertheless, these differences did not reach statistical significance and future experiments need to be done to better define the role of thyroid hormones on immune cell function.

Our second objective was to investigate the impact of TH on adaptive immune cells. Here we saw significant variations in cytokine production on the seventh day post infection among the different groups. In cells stimulated with m139, levothyroxine appeared to enhance the TNF production but not the IFNγ, which might be explained by the IFN production reducing influence of m139 proposed by Puhach et. Al [94], while thiamazole seemed to diminish the response for both IFNγ and TNF. Conversely, cells stimulated with m57 did not exhibit a notable response to thiamazole but did respond to levothyroxine. These findings are contrary to a previously proposed negative impact of TH on T cell immune response [95]. However, these changes were relatively minor, and due to the high variability in our data, drawing definitive conclusions becomes challenging. Overall we saw an increase in T cell response to infection under the influence of TH.

While we were not able to confirm the *in vitro* proposed increase in B cell proliferation under TH stimulation *in vivo* [96], further research into B cell differentiation under TH stimulation needs to be undertaken.

For our third objective, we checked the amount of PFUs between the different groups, but did not find statistically relevant differences between them, showing that the positive influence of TH on the immune response does not show clinical relevance at this point as the virus appeared to reach and lyse tissue independent of TH administration.

The effect of thiamazole was less consistent than expected. We assume this has to do with the mechanism of action of thiamazole. While thiamazole inhibits the synthesis of TH, the thyroid gland is filled with colloid and can release preproduced TH for some time. Future studies could avoid this effect by starting the thiamazole treatment for a longer period before infection, or by gathering results at a later point.

Overall varying degrees of influence on the immune response were observed, so further investigation into this topic might be of scientific and possibly clinical interest.

6 Conclusion

The goal of this study was to investigate the impact of thyroid hormones on antiviral immunity during MCMV infection, with a focus on NK cell and T cell activity.

While we did find statistically significant changes in the immune response related to TH action, these changes were relatively minor and did not seem to impact disease dissemination.

We did have a significant spread in our data which makes conclusions difficult. This could be mitigated by using more mice per group in future studies.

More studies about the specific actions of TH on the immune system should to be conducted, as our understanding about their interplay is still limited.

It might be worthwhile to repeat a similar study and observe the effects of cytokines on the immune response when treated with TH or with thiamazole, or in the context of CMV a more long term study might be of interest in researching possible TH action on CMV latency.

7 Summary

Recent research indicates that the interaction between the immune and endocrine systems plays a vital role in protecting tissues during pathogen encounters. Immune and endocrine cells are frequently found together in endocrine tissues, where they interact and influence each other's responsiveness. Furthermore, even when these cell types are located in separate areas, endocrine cells can receive signals from peripheral immune cells or vice versa. This immuno-endocrine interaction has been observed to collaborate in various organs to enhance antiviral immunity. In this study, we specifically investigate the impact of thyroid hormones on immune cell function. To that end, we infected groups of mice in parallel with MCMV and treated some with levothyroxine and some with thiamazole.

We observed an impact of TH on cells of the innate and adaptive immune system, but it did not influence the overall dissemination of the virus to distant tissues. While there is no clear clinical application at the moment the exact mechanisms at play need to be studied further.

Keywords:

thyroid hormones, CMV

8 Curriculum Vitae

Ivan Modric was born on the 8th of May in Duisburg, Germany. After finishing high school in 2010 and a misspent youth including unfinished studies in physics and informatics he started studying medicine in Rijeka, Croatia in October 2017.

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