MASTER'S THESIS

The nuclear orphan receptor Nr4a1 as therapeutic target to treat aggressive lymphoma

submitted by

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Affidavit

I hereby declare that this diploma thesis is my own original work and that I have fully acknowledged by name all those studies and organizations that have contributed to the research for this master's thesis. Due acknowledgement has been made in the text to all other material used. The text document uploaded to Medical University of Graz is identical to the present master 's thesis.

Graz, August 2021 Lea Thüminger eh

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Abstract German

Hintergrund: Lymphome entstehen durch ein Ungleichgewicht von Proliferation und Apoptose, verursacht durch unterschiedliche Mechanismen. Darüber hinaus zeigen diese Zellen häufig eine hohe Fähigkeit, für immun antwortende Zellen wie T-Zellen, Makrophagen und NK-Zellen unsichtbar zu sein. Diese Prozesse werden verursacht durch die erhöhte Expression von Immun-Checkpoints und entzündungshemmende Co-Faktoren. In verschiedenen Studien wurde Nr4a1 als Tumorsuppressor bei akuter myeloischer Leukämie (AML) und ein Transkriptionsfaktor für unterschiedliche Gene, darunter Immun-Checkpoints und immun regulatorische Zytokine identifiziert. Darüber hinaus aktivieren die Agonisten Cytosporone B (Csn-B), n-Amyl, cDIM und THPN den Rezeptor und in weiterer Folge eine Antitumor-Kaskade. Diese Tatsachen benötigen genauere Untersuchungen bezüglich der Regulation der Prozesse und Gene.

Ziel: Das Ziel dieser Arbeit war die immun regulatorischen Eigenschaften von Nr4a1 bezüglich Tumorzell- und Immunzellinteraktionen in aggressiven Mauslymphomen und die Wirkung einer Behandlung mit ausgewählten Agonisten (Csn-B, n-Amyl, cDIM, THPN) zu untersuchen. Daher wurde für spezifische, pro- und anti-inflammatorische Zytokine das Expressionsrate zwischen *EμMyc Nr4a1-/-* und *EμMyc Nr4a1 +/+* Maus-Lymphomzellen verglichen. Zusätzlich wurden *EμMyc Nr4a1-/-* und *EμMyc Nr4a1+/+* Maus-Lymphomzellen mit Agonisten in unterschiedlichen Konzentrationen (10μM, 5μM, 1μM und 5μM und 1μM) behandelt und die Expressionsrate zu verschiedenen Zeitpunkten (nach 1h, 3h, 6h, 24h, 48h) gemessen. Die Expression von Immun-Checkpoints und immun regulatorischen Zytokinen und Enzymen wurde zwischen *EμMyc Nr4a1-/-* und *EμMyc Nr4a1+/+* Maus-Lymphomzellen und der begleitenden Kontrollgruppe verglichen.

Ergebnisse: Die relative Genexpression von immun regulatorischen Zytokinen war in *EμMyc Nr4a1-/-* und *EμMyc Nr4a1 +/+* Maus-Lymphomzellen gleich, was auf eine Hemmung von Nr4a1 in Lymphomen zurückschließen lässt. Nach der Behandlung mit den Agonisten wurden sowohl Aktivierung als auch Reduktion der Expression mehrere Gene beobachtet. Insbesondere die Genexpression mehrerer immun hemmender Faktoren wurde durch aktiviertes Nr4a1 signifikant unterdrückt. Zusammenfassend zeigen diese Daten, dass Nr4a1 die Antitumorantwort fördert,

indem es die Sichtbarkeit für immun antwortende Zellen erhöht. Diese Studie unterstützt die Annahme, dass die Behandlung mit Nr4a1-Agonisten eine prospektive Therapie für Nr4a1-kompetente Lymphome sein könnte.

Abstract English

Background: Dysregulation of proliferation and apoptosis by multiple indirect mechanisms is associated with the development of lymphoma. Additionally, these cells show a high ability to be invisible for immune responding cells, like T cells, macrophages, and NK cells. These processes are caused by the increased expression of immune checkpoints and anti-inflammatory co-factors. Multiple studies have identified Nr4a1 as a tumor suppressor in acute myeloid leukemia (AML) and a transcription factor for many genes, including immune checkpoints and immune-regulatory cytokines. In addition, the agonists Cytosporone B (Csn-B), n-Amyl, cDIM, and THPN activate the receptor and, in further consequence, an anti-tumor cascade. Therefore, more detailed investigations are required on which genes and processes are regulated.

Aim: This project aims to investigate the immunoregulatory properties of *Nr4a1* concerning tumor cell and immune cell interactions in aggressive mouse lymphomas and the effect of treatment with selected agonists (Csn-B, n-Amyl, cDIM, THPN). Therefore, specific pro- and anti-inflammatory cytokine expression levels were compared between *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice lymphoma cells. Additionally, *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* lymphoma cells were treated with agonists using different concentrations (10μM, 5μM, 1μM and 5μM, and 1μM), and the expression level was investigated at different times (after 1h, 3h, 6h, 24h, 48h). The expression of immune checkpoints and immune-regulatory cytokines and enzymes was compared between lymphoma cells from *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice treated with agonists and the attendant control group.

Results: The relative gene expression of immune-regulatory cytokines was the same in *EμMyc Nr4a1-/-* as in *EμMyc Nr4a1+/+* mice lymphoma cells, which can be attributed to possible inhibition of Nr4a1 in lymphomas. After treatment with the agonists, both activation and reduction in expression of several genes were observed. Especially gene expression of multiple immune inhibitory factors was significant suppressed by Nr4a1 activated by agonists. In conclusion, Nr4a1 promotes anti-tumor response by increasing the visibility for immune responding cells. This study supports the assumption that treatment with Nr4a1 agonists might be prospective therapy for *Nr4a1* competent lymphomas.

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Abbreviation

AF-1	ligand-independent activation function-1
AF-2	ligand-dependent activation function 2
AML	acute myeloid leukemia
BCL2	B-cell lymphoma 2 protein
BCL6	B-cell lymphoma 6 protein
BCL-6	proto-oncogene B-cell lymphoma 6 protein
BL	Burkitt's lymphoma
CARD11	Caspase recruitment domain-containing protein 11
CBT	immune checkpoint blockade therapy
Cd112	Cluster of differentiation 112
Cd160	Cluster of differentiation 160
CD79A/CD79B	B-cell antigen receptor complex-associated protein
Cd80	Cluster of differentiation 80
Cd86	Cluster of differentiation 86
cDIM	1,1-Bis (3-indolyl)-1-(p-substituted phenyl) methane
CDKN1A	CDK-Inhibitor 1
Celastrol	3-hydroxy-9β,13α-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid
COO	cell-of-origin
COX2	Cytochrome c oxidase subunit 2/ cytochrome c oxidase polypeptide II
CREBBP	CREB-binding-protein
Csn-B	Ethyl 3,5-dihydroxy-2-(1-oxooctyl)-benzeneacetate (Cytosporone B)
CtlA4	cytotoxic T-lymphocyte-associated Protein 4
CTLS	cytotoxic T-lymphocyte
DBD	DNA-binding domain
DLBCL ABC	diffuse large B-cell lymphoma activated B-cell-like
DLBCL GBC	diffuse large B-cell lymphoma germinal center B-cell-like
DLBCL NOS	diffuse large B-cell lymphomas not otherwise specified
DLBCL	diffuse large B-cell lymphoma

DNA	deoxyribonucleic acid
EBV	Epstein-Barr-Virus
EP300	E1A-binding protein p300
EZH2	methyltransferase enhancer of zeste homolog 2
FACS	fluorescence-activated cell sorting
FBXO11	F-box only protein 11
FISH	fluorescent in situ hybridization
FLIII	follicular lymphoma grade 3
Gal9	Galectin9
GC	germinal center
HBV	Hepatitis B virus
HIV	human immunodeficiency virus
HL	Hodgkin lymphomas
HLA-A	human leukocyte antigens-A
HLA-B	human leukocyte antigens-B
HLA-C	human leukocyte antigens-C
HPRT1	hypoxanthine Phosphoribosyltransferase 1
Hvem	Herpesvirus entry mediator
IC	Immune checkpoints
IDO	Indoleamine-pyrrole 2,3-dioxygenase
Il1	Interleukine 1
Il10	Interleukine 10
Il13	Interleukine 13
Il18	Interleukine 18
Il23	Interleukine 23
Il27	Interleukine 27
Il6	Interleukine 6
IRF4	Interferon Regulatory Factor 4
JAK	Janus kinase (Orig. just another kinase)
Lag3	Lymphocyte-activation gene 3
LBD	ligand-binding domain

LMO2	LIM domain only 2
MCL	mantle cell lymphoma
MEF2B	Myocyte enhancer-binding factor 2B
MHC	major histocompatibility complex
MYC	myelocytomatosis oncogene cellular homolog
MYD88	Myeloid differentiation primary response 88
n-Amyl	2-[3,5-dihydroxy-2-(1-nonanoyl) phenyl] acetate
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of the activated B-cells
NGFI-B	nerve growth factor-induced clone B
NGS	next-generation sequencing
NHL	non-Hodgkin lymphomas
NK	natural killer cells
Nr4a1/2/3	nuclear receptor subfamily 4 group A member 1/2/3
NRBE	NGFI-B response element
NurRE	Nur-responsive element
PCR	polymerase chain reaction
Pd1	Programmed cell death protein 1
Pdl1	Programmed death-ligand 1
Pdl2	Programmed death-ligand 2
POMC	pro-opiomelanocortin
PPIA	peptidylprolyl Isomerase A
PRDM1	PR domain zinc finger protein
PTLD	Posttransplant lymphoproliferative disorders
REL	proto-oncogene c-Rel
RNA	ribonucleic acid
RXR	retinoid X receptor
SPIB	transcription factor Spi-B
STAT	Signal Transducers and Activators of Transcription
TBP	TATA-Box Binding Protein
TGFβ	Transforming growth factor beta
THPN	1-(3,4,5-trihydroxyphenyl)-nonan-1-one

Tigit	T cell immunoreceptor with Ig and ITIM domains
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TNF	Tumor necrosis factor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
Vista	V-domain Ig suppressor of T cell activation
β2M	β2 microglobulin

1 Introduction

1.1 Lymphoma

Malignant lymphoma is a term for a group of blood cancer that affects lymphocytes. Commonly they start in the lymphatic system and spread to other parts of the human body, such as the liver or lungs.

Lymphomagenesis is associated with different risk factors like tobacco use, obesity [1], human immunodeficiency virus (HIV) [2], Hepatitis B virus (HBV) [3], and hereditary genetic variations [4].

WHO registered about 100 types of lymphoma and classified them into two main groups, non-Hodgkin (NHL) and Hodgkin lymphomas (HL), and additionally posttransplant lymphoproliferative disorders (PTLD) and histiocytic and dendritic cell neoplasms [5]. Hodgkin lymphomas constitute only a minority of the tumor and are accompanied by a reactive inflammatory background. Treatments are well-established, and a high probability of survival is predicted [6]. For non-Hodgkin lymphomas, the treatments and cure rates vary. They are separated into mature B-, T- and NK-cell neoplasms. The detailed classification is based on clinical, biological/genomic, and pathological aspects of the lymphomas. The most common types of all lymphomas are aggressive B-cell lymphomas (almost 50%). It is a heterogeneous group with a high ability of transformation through different pathways. The subgroup is divided into numerous mature B-cell neoplasms, including Burkitt's lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma grade 3 (FL III), and mantle cell lymphoma (MCL) [5]. B-cell lymphomas exhibit different cell-of-origin as they derive from various stages of B-cell differentiation (Figure 1) [7].

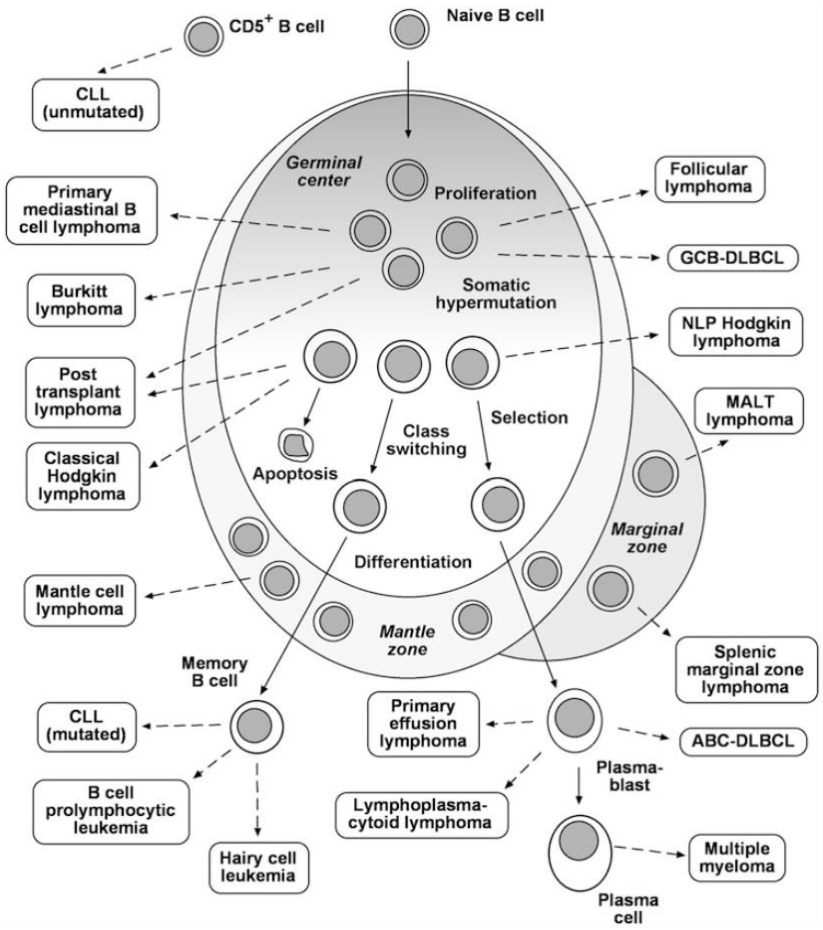

Figure 1: *cell-of-origin of different types of B-cell lymphoma, derived from Seifert et al. [7]*

Since toxic adverse effects are associated with traditional chemotherapy, targeted immunotherapy became more popular in clinical trials for treating lymphomas with encouraging results [8]. Therefore, classification is essential to define the malignancy and to choose the optimal treatment strategy. After immunohistological tests, lymphoma is examined and characterized to make an accurate diagnosis, with several tests like fluorescent in situ hybridization (FISH), polymerase chain reaction (PCR), and next-generation sequencing (NGS). With these steps, tumor markers can be identified and enable targeted therapy [9].

1.2 Diffuse large B-cell lymphoma

In the western world, 95% of diagnosed lymphomas are of B-cell origin [10], and the most frequent subtype is DLBCL. Together with BL, it accounts for about 80% of aggressive B-cell non-Hodgkin lymphoma [11]. DBLCLs are the most aggressive B-

cell lymphomas with high heterogeneous morphologic, genetic (alteration), immunophenotypic, and distinct clinical malignancies. Although a durable remission of more than 50% is achieved, about 30% of the patients do not respond to traditional chemotherapy [12]. Because of the different treatment responses within the subtypes, prognostic markers are essential in identifying the optimal medical treatment [9]. These days, the typical therapy includes treatment with rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (R-CHOP) and achieves a cure rate of 60-70% [13].

WHO classified DLBCLs not otherwise specified (NOS) according to their cell-of-origin (COO) into germinal center B-cell-like (GBC) and activated B-cell-like (ABC) [9]. While GBC DLBCLs derive from germinal center (GC) light zone B-cells, ABC DLBCLs develop later during the early stages of cell differentiation in the cell plasma [11]. The classification is identified by lymphoma cells' gene expression profile analyses and characterizes different clinical outcomes and prognoses, as ABC DLBCLs are more aggressive with lower treatment response and cure rate than GBC DLBCLs [14].

1.2.1 Genetic background

B-cells are activated by contact with antigens in an accessible form and undergo a complex pattern of migration which is subject to multiple different pathways [15]. The most common genetic events for DLBCL are epigenetic modifications via loss-of-function mutation in *acetyltransferase CREB-binding-protein (CREBBP)* or *E1A-binding protein p300 (EP300)* ~40% and *histone methyltransferase MLL2* in ~30%. Thus, inactivation of *EP300* and *CREBBP* inhibits the gene expression of *tumor suppressor protein p53* and activates gene expression of *proto-oncogene B-cell lymphoma 6 protein (BCL-6)*. BCL6 suppresses cell cycle arrest as well as the detection and repair of DNA damage and generates lymphomagenesis. A dysregulation can be affected by multiple indirect mechanisms like gain-of-function mutation in *Myocyte enhancer-binding factor 2B (MEF2B)*. Another regulator of BCL6 is F-box only protein 11 (FBXO11) that usually causes ubiquitylation and degradation of BCL6. In healthy and neoplastic cells, BLC6 plays an essential role in suppressing the proliferation factor MYC and the anti-apoptotic factor BCL2, preventing uncontrolled proliferation in the germinal center and maintaining a pro-

apoptotic state. Deregulation of this pathway contributes to pathogenesis with a poor prognosis [14].

Inhibited *PR domain zinc finger protein (PRDM1)* gene expression leads to a blockade of terminal differentiation. In 30% of all ABC DLBCL cases, *PRDM1* is repressed by BCL6. Additionally, *PRDM1* expression can be inhibited by a complex of gain-of-function mutated transcription factor *Spi-B (SPIB)* and *Interferon Regulatory Factor 4 (IRF4)*. Another event associated with the pathogenesis of ABC DLBCL is the constitutive activation of the nuclear factor 'kappa-light-chain-enhancer' of the activated B-cells (NF-κB) pathway. NF-κB can be activated by multiple genetic effects like gain-of-function mutation in *B-cell antigen receptor complex-associated protein (CD79A/CD79B)* -in 20% of cases- or *Caspase recruitment domain-containing protein 11 (CARD11)* -in 10% of cases- or *Myeloid differentiation primary response 88 (MYD88)* and loss-of-function mutation in *Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3) -in 30% of cases.* This deregulation protects the tumor cells from apoptosis and promotes proliferation [14]. In addition, mutations in other genes like *TRAF2* and *REL* (NF-κB Subunit) were found in ABC DLBCLs [13].

Although the evolution of GCB DLBCL is poorly understood, some mutations are associated with pathogenesis. For example, in 30% of all GBC DLBCL cases, cell migration is deregulated by the inactivation of the Gα13 signaling pathway resulting in the early release of GC cells in the lymph and blood circulation. Furthermore, in 21% of all cases, the methyltransferase *enhancer of zeste homolog 2 (EZH2)* carries a gain-of-function mutation. Since EZH2 acts as a gene suppressor, overexpression will inhibit tumor suppressor genes like *CDK-Inhibitor 1 (CDKN1A)*. As another proto-oncogene, myelocytomatosis oncogene cellular homolog (MYC) has an essential role in proliferation, metabolism, telomere maintenance, and re-entry into the dark zone from the light zone. Therefore, simultaneous MYC and BCL2 translocation (so-called double-hit) result in a poor prognosis [14]. In addition, several other genes like *CD10, LMO2*, and *REL* can be overexpressed in GBC BLBCLs [13].

1.3 Immune regulation in lymphoma

1.3.1 Immune escape in DLBCL

The major histocompatibility complex (MHC) is an endogenous antigen on the surface of body cells, and it is necessary for cytotoxic T-lymphocyte (CTLS) detection. Over 60% of DLBCL cases carry a lack of MHC class I complex. Inactivation of gene expression of *β2M, HLA-A, HLA-B*, and *HLA-C*, and defect transport of β2M and HA-1 inhibit the immune response. HLA-1 is necessary for a correct gene expression and transport of CD58 for a working immune response by NK-cells [14].

1.3.2 Immune checkpoints

Immune stimuli interact with T-cells and initiate T-cell proliferation and activation. Conversely, negative feedback of immune checkpoints binding to their co-inhibitory molecules dampens T-cell activation by producing inhibitory signals leading to a lower immune response against cancer cells [16].

PD1 surface receptor on macrophages, dendritic cells, and T-cells leads to an inhibition of immune response after binding with PDL1, which is often seen in DLBCL and tumor microenvironment and associated with poor prognosis and high-risk group [13]. Other studies demonstrated that the PD1-PDL1-axis significantly contributes to tumor progression in a DLBCL subgroup [17] and associated PD1/PDL1 interaction with adverse prognostic effects in a large DLBCL cohort [18]. Similar interactions were detected with other immune checkpoints and co-inhibitors like CTLA4-Cd80/Cd86, TIM3-Gal9, PD1-PDL1/PDL2, TIGIT-Cd112, Cd80-PDL2, Cd160-HVEM, Lag3-?, and VISTA (Figure 2) [16,19,20].

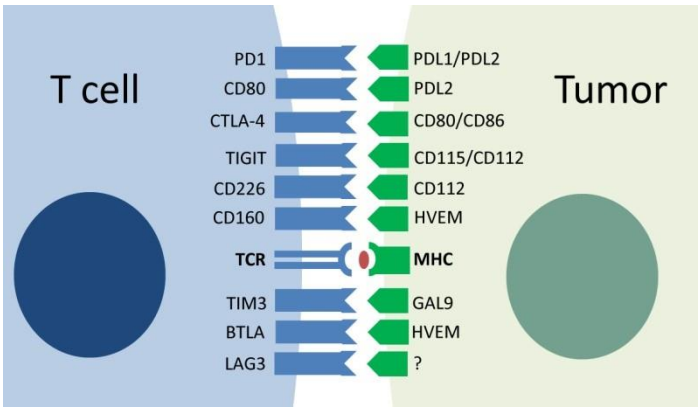

***Figure 2**: Interaction between T-cells and tumor cells via cell surface receptors and ligands, adapted from Cogdill et al. [16]*

1.3.3 Genetic mechanisms in immune escape in noninflamed lymphomas

In DBCLs, deregulations of MYC, BCL6, and BCL2 are associated with a high proliferation rate, relatively devoid of infiltrating T-cells, low transcription in pro-inflammatory genes, and immune escape. A gain-of-function mutation in the suppressor gene *EZH2* is associated with downregulation in *HLA* and inflammatory cytokines expression in several cancers, making tumor cells invisible for immune responding cells [21].

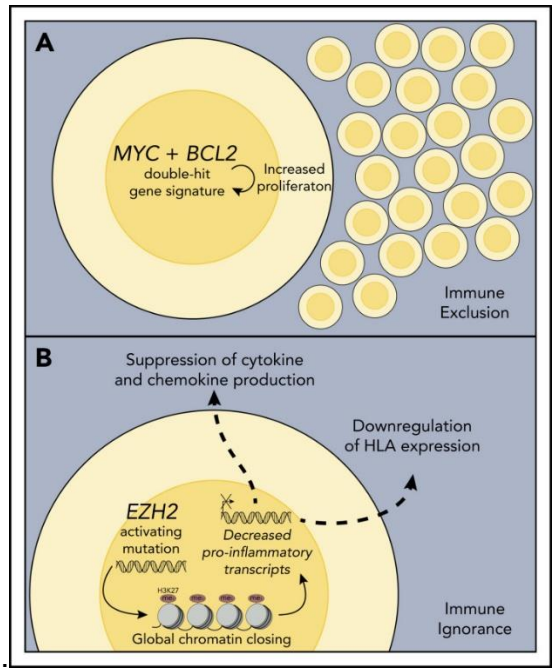

Figure 3: *regulatory mechanisms in noninflamed lymphoma environments A: Myc and BCL2 double-hit gene signature leads to a high proliferation rate B: overexpression of EZH2 inhibits immune response, derived from Kline et al. [21]*

Moreover, PDL1 and PDL2 are overexpressed by JAK/STAT amplification or by EBV infection in Hodgkin B-cell lymphoma [22]. In almost 20% of patients with DLBCL, PDL1 but not PDL2 is overexpressed due to genetic alterations affecting the 9p24 [23,24].

1.3.4 Cytokines and enzymes

Secretion of anti-inflammatory cytokines inhibits T-cell activation and their effector function directly by the immune inhibitory functions [25–27]. Then again, immune checkpoints can reduce the activity of intracellular signaling pathways and downregulate pro-inflammatory cytokines (Table 1) [28].

***Table 1**: factors categorized in anti-inflammatory/immune suppressive and pro-inflammatory/immune activating function*

immune suppressive function		immune activating function	
cytokine	**effect**	**cytokine**	**effect**
Il10	inhibition of cytotoxic effects of T-cells Supporting the activity of dendritic cells and macrophages [29–37]	TNFα	inflammatory immune reactions T-cell activation T-cell survival higher tumor progression specific T-cell anti-tumor immunity [38–47]
Il13	stimulating factors for antigen-presenting capacities Promotion the macrophage activation [48–50]	Il18	CD8+ tumor-infiltration T-cells (TILs) activation and/or NK immune responses [51,52]
Il27	induced expression of immune inhibitory molecules (Il10, PDL1, LAG-3, CTLA4, and TIGIT) [53–56]	Il1	T-cell activation Proliferation of B-cells [57–63]
TGFβ	protect the cancer cells against inflammatory processes force stroma cells to develop into tumor cells [64]	Il6	inhibition of Tregs differentiation Stimulation of Tfh differentiation Production of IL-21 Promoting of B cell differentiation of B-cells into IgA secreting plasma cells Involvement in CD28 - independent T cell activation [65–68]
		Il23	regulation of tumor progression and cancerogenesis [69,70]
enzyme	**effect**	**enzyme**	**effect**
COX2	promotes apoptotic resistance, proliferation, angiogenesis, inflammation, invasion, and metastasis [71,72]	IDO	single or co-targets to inhibit the facilitation of immunosuppressive tumor microenvironment [73,74]

1.3.5 Therapy

Effective cancer therapy with less undesirable side effects might be the novel immune checkpoint blockade therapy (CBT). Clinical studies have shown promising

results in therapeutical antibodies preventing the connection between these inhibitory receptors and their co-inhibitory ligands [21]. Blockage of the PD1 pathway was associated with high response rates seen in heavily pre-treated Hodgkin B-cell lymphoma patients [75]. However, heavily pre-treated DLBCL patients with advanced-stage DLBCL being refractory to chemotherapy or relapse after initial show low response rates through PD1 or CTLA4 blockade [76–79]. Additionally, CBT is not appropriate for different types of cancer like ABC DLBCL, despite their high rate of markers. Therefore, studies on this topic are essential to identify additional markers and immune checkpoints as therapeutic targets [21].

1.4 Nr4a

The orphan nuclear receptor family (Nr4a) has three Members Nr4a1, Nr4a2, and Nr4a3 (also referred to as Nur77/NGFI-B and Nurr1, and NOR-1). As nuclear receptors, they are transcription factors that regulate the expression of target genes in blood cells and tissues like skeletal muscle, adipose tissue, heart, kidney, liver, brain, and T-cells, respectively [80]. Multiple physical stimuli (like magnetic fields, mechanical agitation (causing fluid shear stress), and membrane depolarization) and physiological stimuli (like fatty acids, stress, prostaglandins, growth factors, calcium, inflammatory cytokines, peptide hormones, and neurotransmitters) affect the Nr4a receptors [81,82].

1.4.1 Nr4as in tumorgenesis

Nr4a subfamily is involved in various pathways regulating apoptosis, proliferation, inflammation, metabolism, DNA repair and is associated with diseases like diabetes, Alzheimer's disease, cancer, and cardiovascular disease [81,83]. Studies exhibit a primarily pro-oncogenic function of Nr4a receptors as they show a higher representation of *Nr4a* RNA in cancer cells. For example, *Nr4a1* is more expressed in breast cancer and lung cancer cells [84,85], and a higher expression of *Nr4a2* was found in breast, bladder, colon, and gastric cancer than in normal tissue. Additionally, drug resistance and low patient survival increase with a higher expression [86–89].

In contrast other studies have shown that the downregulation of Nr4a1 and Nr4a3 affects the genesis of aggressive lymphoma [90–92], and loss

of *NR4A1* and *NR4A3* was found in leukemic blasts from human AML patients [93]. Gene expression studies with *Nr4a* knockout mice have confirmed *Nr4a1* and *Nr4a3* as tumor suppressors in AML [94]. Tumor suppressive function studies in aggressive lymphoma showed a significant reduction of both - *NR4A1* and *NR4A3* - in B-CLL (in 71% of tested cases), follicular lymphoma (70%), and DLBCL (74%), which was associated with poor cancer-specific survival [91]. Further experiments demonstrated that both receptors regulate a similar pattern of genes in aggressive lymphoma [92].

1.4.2 Structure

The molecular structure of all Nr4a family members shows several functional domains. The N-terminal domains interact with other transcription factors and contain the ligand-independent activation function-1 (AF-1). In the center, there is the highly conserved (more than 90%) DNA-binding domain (DBD) followed by a hinge region (D) and the less conserved (about 60%) ligand-binding domain (LBD). The following C-terminus contains the ligand-dependent activation function 2 (AF-2). The members of the Nr4a family vary in length and amino acid composition (Figure 4) [95,96].

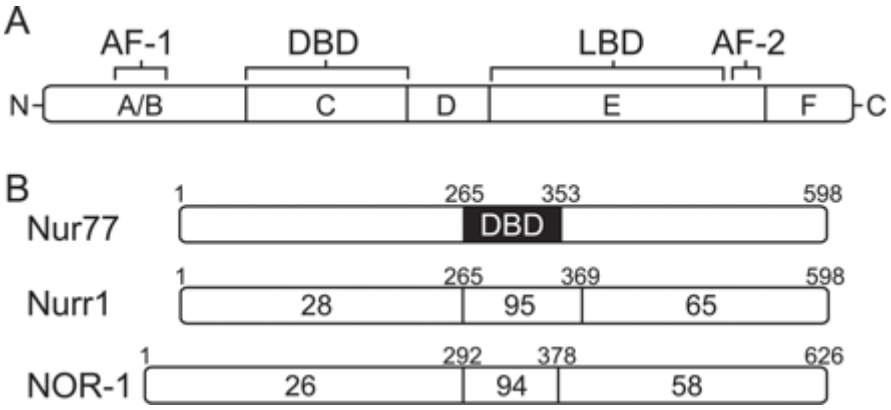

***Figure 4**: functional structure of Nr4a A: schema of the Nr4a family B: the different number of amino acids in the members of the subfamily, derived from Martínez-González et al.* [96]

1.4.3 Nr4a as a transcription factor

A transcription factor detects a specific nucleotide sequence of the DNA and binds with its DBD. The transcription can be activated or inhibited depending on coefficients like the ligand, the regulated gene, and the receptor itself [97].

Nr4a receptors bind at different sequences in the DNA to activate gene expression. Nr4a binds as a monomer to the NGFI-B response element 5'-A/TAAAGGTCA-3' (NRBE) [98]. It can bind as a homodimer or a heterodimer to the Nur-responsive element AAA(G/A)(C/T)A (NurRE) what is found in pro-opiomelanocortin (POMC) gene promoter [99,100]. Nr4a1 and Nr4a2 can build a heterodimer with retinoid X receptor (RXR) and bind to a specific sequence called DR5 [101].

1.4.4 Nr4a1 and immunoregulatory genes

Deutsch et al. declared results of studies with lymphoma models and patients that manifest strong evidence that establishes *Nr4a1* as tumor-suppressor in aggressive lymphomas, possibly through regulating immune evasion by regulating immune checkpoints. Furthermore, common tumor *Nr4a1* expression inhibits practical T-cell function via the enhanced expression and activity of immune checkpoints receptors and ligands, which contributes to the inhibition of immunosurveillance [92,102,103].

1.5 Agonists

Agonists are ligands that bind to receptors in their inactive state and activate them. Thus, the receptor initiates a signaling pathway.

The nuclear receptors NR4A1, NR4A2, and NR4A3, respond to multiple cellular stressors. Since no endogenous ligands were found yet, synthetically created and from external sources, isolated molecules were identified as agonists that regulate the function of Nr4a1 under pathological conditions in several studies [104–107].

Ethyl 3,5-dihydroxy-2-(1-oxooctyl)-benzeneacetate (Cytosporone B; Csn-B) as an agonist increases Nr4a1-dependent gene expression. Additionally, it induced *Nr4a1* gene expression and translocation, which causes apoptosis [104]. The structurally related compound 2-[3,5-dihydroxy-2-(1-nonanoyl) phenyl] acetate (n-Amyl) seems to possess lower binding energy as it behaves as an agonist of Nr4a1 but with higher

anti-tumor activity [106]. Another derivate of Csn-B is 1-(3,4,5-trihydroxyphenyl)-nonan-1-one (THPN). It induces autophagy via increased Nr4a1 level in the cytoplasm. 1,1-Bis (3-indolyl)-1-(p-substituted phenyl) methane (cDIM) has multiple subtypes that bind to Nr4a1 in various types of cancer and activate several signaling pathways, including those that affect anti-tumor activity. Another agonist, 3-hydroxy-9β,13α-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (Celastrol), shows anti-inflammatory activities under inflammatory conditions. Binding with Nr4a1 inhibits inflammation and induces autophagy [104].

1.6 Aim primary tumors

This project aims to investigate the immunoregulatory properties of *Nr4a1* concerning tumor-immune cell interactions in aggressive mouse lymphomas. Therefore, functionally examined the role as a transcriptional factor for cytokines associated with inflammatory processes. The expression of these genes was compared between lymphoma from *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice.

1.7 Aim treatment

The second aim of the project was to identify the effect of interaction between selected agonists and Nr4a1. Therefore, we determined the expression levels of genes implicated in immune regulation and immune evasion of tumor cells as described by Cogdill et al. [16] and added cytokines. The expression of these genes was compared between lymphoma from *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice treated with agonists and the attendant control group.

2 Materials and Methods

2.1 Sample

The research group established murin lymphoma cell lines derived from *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* lymphomas. They were cultured in medium IMDM/DMEM (1:1) containing 10% FBS, 1% A/A, and 1% β-mercaptoethanol in an incubator at 37°C with 5% CO2. The cells were split three times a week to keep them in the concentration of 1x106/ml.

2.2 Treatment

Treatments were performed with the Nr4a1 agonists Csn-B, n-Amyl, cDIM, THPN, and Celastrol. The substances were solved in DMSO.

The cultured murine lymphoma cell lines (*EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+*) were treated with different concentrations of Nr4a1 agonists. They were added in a concentration of 10μM, 5μM, 1μM. Treatments with THPN and Celastrol resulted in the induction of apoptosis. Therefore, these agonists were added in a lower concentration of 5μM, 1μM, and 0.5μM. DMSO treated lymphoma cells cell served as control.

The cell pellets for RNA were harvested after 1 hour, 3 hours, 6 hours, 24 hours, and 48 hours of treatment by centrifugation (>10 000 x g, 1 minute) and stored at -20°C. After that, the RNA extraction and cDNA synthesis were carried out.

After 48 hours, an annexin V staining was performed for detecting apoptotic cells to evaluate the toxicity of the substance.

2.2.1 Primer

To determine the gene expression of selected genes, primers were bought from Microsynth and Eurofine Genomics (Table 2). They were checked by real-time qPCR on a serial dilution of cDNA extracted from a spleen of a healthy *EμMyc Nr4a1-/-* mouse plus a genomic DNA. dH2O served as a negative control. In addition, the housekeeping genes *β-Actin*, *hypoxanthine Phosphoribosyltransferase*

1 (*HPRT1*), and *peptidylprolyl Isomerase A* (*PPIA*) were used as an endogenous reference to normalize the qPCR data.

Table 2: *Primer bought from Microsynth and Eurofine Genomics*

Genes	**Forward**	**reverse**
mm_Il27	5'-GGA CCA GCT CAG AGA GGG A-3'	5'-ATT TGA ATC CTG CAG CCA GC-3'
mm_Il10	5'-TAA CTG CAC CCA CTT CCC AG-3'	5'-TGT TGT CCA GCT GGT CCT TT-3'
mm_Il6	5'-AGT TCC TCT CTG CAA GTA AGT GA-3'	5'-AGC ATC AGT CCC AAG AAG GC-3'
mm_Il18Bp	5'-CCA TGA GAC ACT GCT GGA CA-3'	5'-ACA AAA GCA GGA CCC ACC AA-3'
mm_Il13	5'-TGA GGA GCT GAG CAA CAT CAC ACA-3'	5'-TGC GGT TAC AGA GGC CAT GCA ATA-3'
mm_Il1rnTX	5'-GCT CAT TGC TGG GTA CTT ACA A-3'	5'-CCA GAC TTG GCA CAA GAC AGG-3'
mm_Il23	5'-ATG CTG GAT TGC AGA GCA GTA-3'	5'-ACG GGG CAC ATT ATT TTT AGT CT-3'
mm_TNF	5'-GGA ACT GGC AGA AGA GGC AC-3'	5'-GGG TCT GGG CCA TAG AAC TG-3'
mm_TGFβ	5'-TGT GGA GCA ACA TGT GGA ACT-3'	5'-GCA GTG AGC GCT GAA TCG AA-3'
mm_COX2	5'-TAC CAG TCT CTC AAT GAG TAC C-3'	5'-TGG TAG GCT GTG GAT CTT GCA CAT TG-3'
mm_Lag3	5'-CTG GGA CTG CTT TGG GAA G-3'	5'-GGT TGA TGT TGC CAG ATA ACC C-3'
mm_Cd160	5'-CCA TCC TGC TGG CAA TTG TG-3'	5'-CCC TCC TTT CTG GGA TGC TG-3'
mm_CTLA4	5'-GCT TCC TAG ATT ACC CCT TCT GC-3'	5'-CGG GCA TGG TTC TGG ATC A-3'
mm_Vista	5'-GGA ACC CTG CTC CTT GCT ATT-3'	5'-TTG TAG ATG GTC ACA TCG TGC-3'
mm_IDO	5'-AGG GGA TGA CGA TGT TCA-3'	5'-TCC TTT TTC TTC CAG TTT GCC-3'
mm_TigitR	5'-CCA CAG CAG GCA CGA TAG ATA-3'	5'-CAT GCC ACC CCA GGT CAA C-3'
mm_Pdl1	5'-GCT CCA AAG GACTTG TAC GTG-3'	5'- TGA TCT GAA GGG CAG CAT TTC-3'
mm_Pdl2	5'-CTG CCG ATA CTG ACC CTG AGC-3'	5'- GCG GTC AAA ATC GCA CTC C-3'
mm_Cd112	5'-TGC TAC TGC TCC TGC TTC AG-3'	5'-GTA ACT CCA CGG TGC CTC C-3'
mm_Gal9	5'-CTC TTC AGT GCC CAG TCT CC-3'	5'-TCT TGG TAG TCC CCT GGA GG-3'
mm_Cd80	5'-ACC CCC AAC ATA ACT GAG TCT -3'	5'-TTC CAA CCA AGA GAA GCG AGG-3'
mm_Cd86	5'-TGT TTC CGT GGA GAC GCA AG-3'	5'-TTG AGC CTT TGT AAA TGG GCA-3'
mm_Hvem	5'-CAG GCC CCT ACA GAC AAC AC- 3'	5'-ACT CGT CTC CCA CAA GGA ACT-3'

2.3 Annexin V staining

Pellets were harvested from 200µl treated cell lines by centrifugation (5 minutes, 300g) and resuspended in 200µl Annexin V binding buffer. The cells were stained with 2,5 µl Annexin V + 2,5 µl 7-AAD. The measurements have been carried out using FACS (fluorescence-activated cell sorting).

2.4 Assessment of cell growth

To investigate the effect of Celastrol on lymphoma cell growth, the cell growth under its treatment was determined using the EZ4U assay (Biomedica). The cell culture was again treated with different concentrations (10µM, 5µM, 1µM, 0.5µM, 0.1µM,0.05µM, 0.01µM,0.005µM,0.001µM, 0.0005µM, 0.0001µM) of Celastrol solved in DMSO and 10µM DMSO as a negative control. After 72 hours, the toxicity was tested using the EZ4U (MTS-based) assays according to the manufacturer's protocol. Four determinations were made for each sample. The wavelength for measurement was 492 nm and 620nm as the reference wavelength.

2.5 RNA extraction and cDNA synthesis

RNA was extracted from the lymphoma tissue with the Tissue Total RNA Purification Mini Kit (Favor Prep™) according to the manufactor's protocol. Total RNA was stored at-80°C. The removal of genomic DNA from RNA preparation was done using the RNase-free DNase I (1U) and 10x reaction Buffer with MgC2 from Thermo Scientific™. The cDNA was generated by the RevertAid RT Reverse Transcription Kit (Thermo Scientific™) according to the manufacturer's instructions.

2.6 Real-time PCR

For real-time qPCR detection, the Luna® Universal qPCR Master Mix (New England Biolabs) was added with diluted cDNA and Primers (Table 3). For each sample, duplicates were performed. In addition, negative control with dH2O instead of cDNA was included for each gene.

The real-time PCR was run in a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) using the SYBR® channel. The protocol was designed by the research group (Table 4).

***Table 3**: Luna® Universal (Neb) qPCR Master Mix Protocol*

cDNA	1:10 dH_2O	4µl
Primer	1:20 (Forward + reverse) + dH_2O	1µl
Luna Mastermix		5µl
total		**10µl**

***Table 4**: Protocol qPCR for Luna Mastermix separated into different steps*

	Temperature (°C)	**Time**	**Cycles**
Step 1 PCR initial activation step	95°C	1 min	1
Step 2 DNA denaturation	95°C	15 sec	45
Step 3 Combined annealing/extension +Plate read	60°C	30 sec	
Step 4 Melt curve analysis	95°C	5 min	1
Step 5 Melt curve analyses	Melt curve 65°C to 95°C	5 sec	1
END			

2.7 Statistic

Differences in gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method.

For primary tumors, the statistical analyses and the graphs were performed using the Graphpad Prism 5. Mann-Whitney U-Test evaluated the p-value to show the statistical difference between the expressions of the genes in lymphoma of *EµMyc Nr4a1-/-* and *EµMyc Nr4a1+/+* mice. The p-value $p \leq 0.05$ indicates a significant difference.

For treatment, the graphs and the statistical analysis (the Student's T-test) were performed with Microsoft Excel 365. Again, the p-value of $p \leq 0.05$ indicates a significant difference.

3 Results

3.1 Primary tumor

To investigate the immunoregulatory properties of *Nr4a1* in the antitumor immune response in aggressive lymphomas, the expression of immune regulatory cytokines was compared between lymphomata from *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice.

No significant difference was detected between *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice in *Il27, Il23, Il10, Il1rnTX, Il18BP* nor in *TNF* or *TGFβ* ($p > 0.05$)., as shown in (Figure 5).

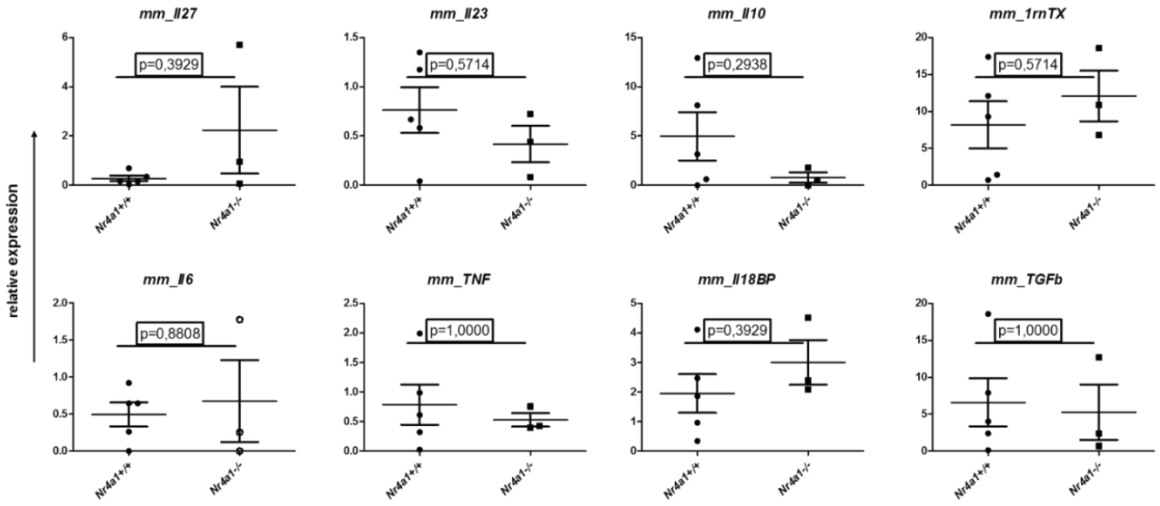

Figure 5: *Expression of* Il27, Il23, Il10, Il1rnTX, Il18BP, TNF, and TGFβ *in Nr4a1 -/- and Nr4a1 +/+ lymphomas. Each dot represents one EμMyc Nr4a1 +/+ lymphoma sample and each square one EμMyc Nr4a1 -/- lymphoma sample*

3.2 Treatment

To evaluate whether Nr4a1 agonist can modulate immune regulatory gene, mice lymphoma cells were treated with Csn-B, n-Amyl, cDIM, and THPN, followed by gene expression analysis. Each treatment was performed on *EμMyc Nr4a1+/+* and *EμMyc Nr4a1-/-* lymphoma cell lines to demonstrate which effects on gene

expression are *Nr4a1*-dependent. The investigated genes can be subdivided into the following classes and are described more in detail below.

3.2.1 Cytosporone B (Csn-B) treatment

In *EμMyc Nr4a1+/+* lymphoma cells, Csn-B induced *Nr4a1* expression in a dose-dependent manner after 3h of treatment compared to DMSO controls. The treatment caused a 5-fold upregulation with a concentration of 10μM ($p=0.026$) and a 4-fold upregulation at a concentration of 0.5μM ($p=0.004$) (Figure 6). As expected, the treatment did not induce *Nr4a1* expression in the *EμMyc Nr4a1-/-* mice lymphoma cells.

Csn-B treatment did not affect *Nr4a2* nor *Nr4a3* expression in a significant manner, as shown in (Figure 6).

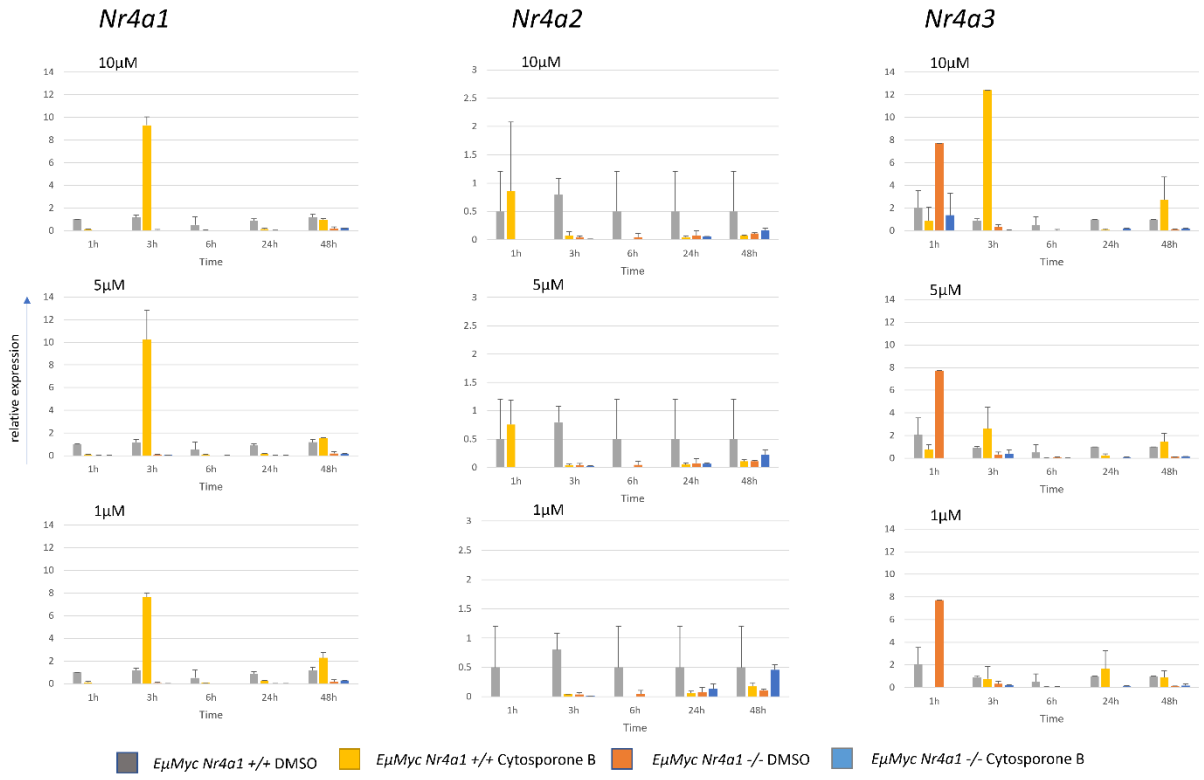

Figure 6: *Relative gene expression of* Nr4a *receptors after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a significant upregulation of* Nr4a1 *3h after treatment can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.1.1 Immunoregulatory genes

Csn-B treatment caused downregulation of *TNF* (0.08-fold after 24h 10µM p=0,006; 5µM p=0,023; 1µM p=0,021), *TGFβ* (0.3-fold after 3h 10µM p=0.019; 5µM p=0.029; 1µM p=0.031), and no regulation of *IDO* in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TNF* (6-fold after 48h 10µM p=0.046; 5µM p=0.033), *TGFβ* (3-fold after 1h 5µM p=0.009), *IDO* (2.8-fold after 1h 5µM p=0.038, 25-fold after 6h 10µM p=0.037) (Figure 7). This data indicates that reducing gene expression of pro-inflammatory cytokine *TNF* and anti-inflammatory cytokine *TGFβ* by Csn-B treatment might be Nr4a1-dependent. Regulation of pro-inflammatory enzyme *IDO* might not be Nr4a1-dependent.

Csn-B treatment did not influence the expression levels of the anti-inflammatory enzyme *COX2.* Therefore, this data is whether shown nor interpreted in more detail.

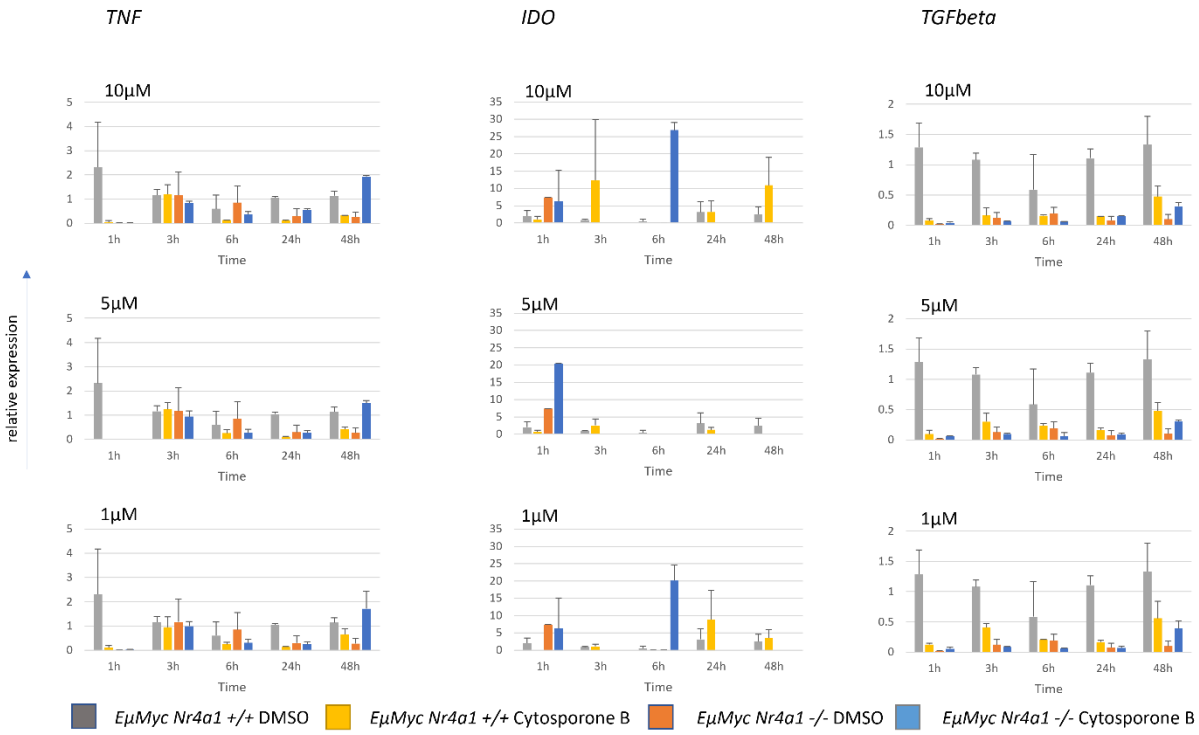

Figure 7: *Relative gene expression of immune regulatory genes after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* TNF *(after 24h) and* TGFβ *(after 3h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.1.2 Immune checkpoints (IC) and the co-inhibitory receptors

Csn-B treatment exhibited no effects on the immune checkpoint *CTLA4* and its ligands *Cd80 and Cd86* in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Cd80* (24-fold after 1h 10µM p=0.007) and *Cd86* (1.4-fold after 48h 10µM p=0.032), but no effects were seen for *CTLA4* (Figure 8). This data indicates that regulation of *CTLA4*/*Cd80*/*Cd86* by Csn-B treatment might not be Nr4a1-dependent.

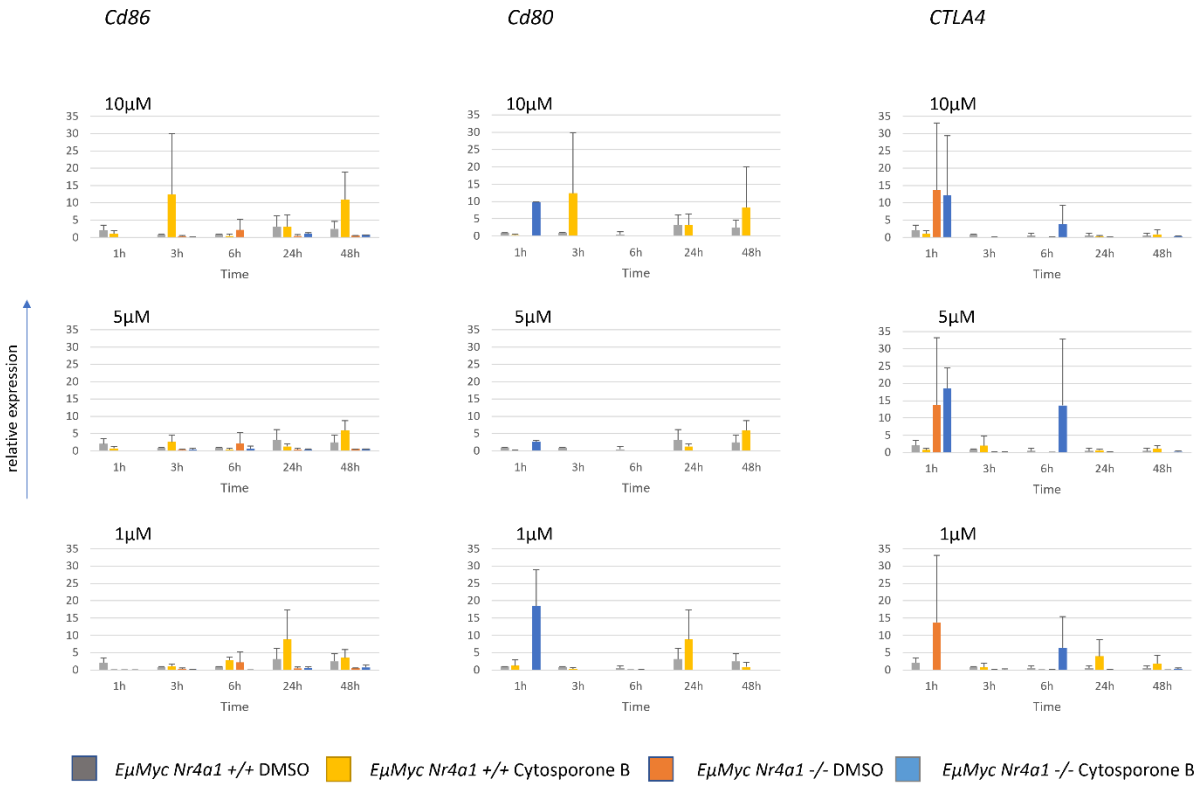

Figure 8: *Relative gene expression of immune checkpoint* CTLA4 *and its ligands* Cd80/Cd86 *after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; no Nr4a1-depending regulation can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

Csn-B treatment exhibited no effects on the immune checkpoints *TigitR* and *Cd160* or its ligands *Cd112* and *Hvem* in *EµMyc Nr4a1-/-* lymphoma cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TigitR* (4.3-fold after 1h 10µM p=0.012; 5µM p=0.034; 1µM p=0.011) and its ligand *Cd112* (1.4-fold after 48h 10µM p=0.032), and upregulation of *Cd160* (1.4-fold after 48h 10µM p=0.032) and its ligand *Hvem* even earlier (6-fold after 1h 10µM p= 0.036; 1µM p=0.025; 2.8-fold after 6h 10µM p=0.029; 3.7-fold after 48h 10µM p=0.046) (Figure 9). This data indicates that induction of *TigitR/Cd112* and *Cd160/Hvem* by Csn-B treatment might not be Nr4a1-dependent.

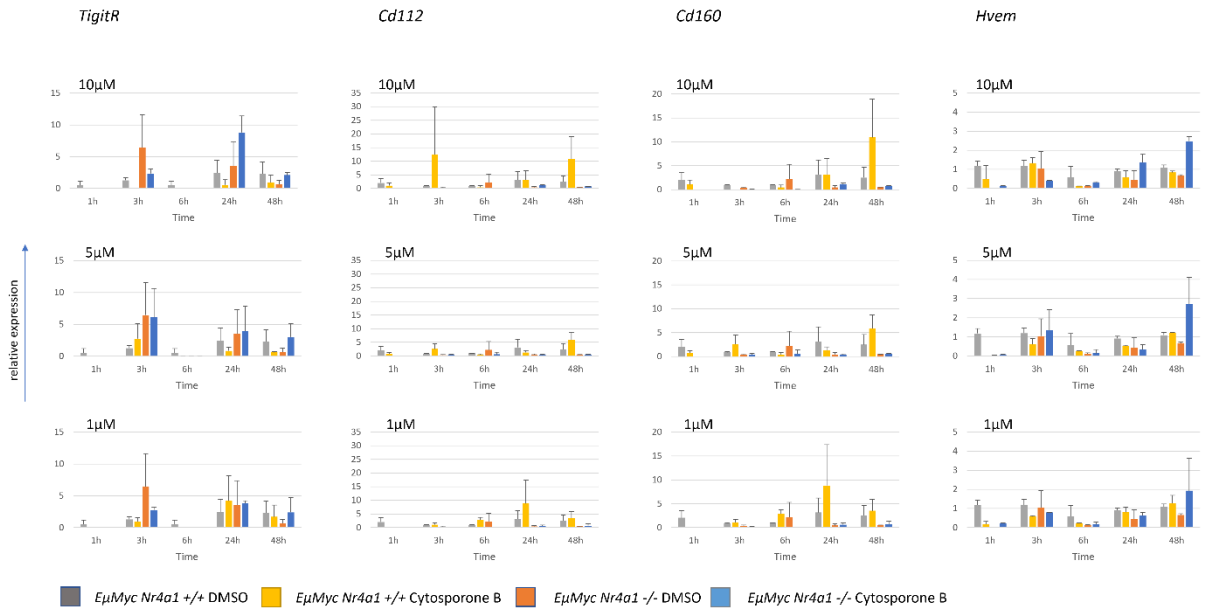

Figure 9: *Relative gene expression of immune checkpoints* TigitR/Cd112 *and* Cd160/Hvem *after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; no Nr4a1-depending regulation can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

Csn-B treatment did not affect *Pdl2* and *Lag3* in *EµMyc Nr4a1+/+* cell lines. However, in the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Pdl2* (1.5-fold after 48h 10µM p=0.032) and downregulation of *Lag3* (0-fold after 48h p=0.012) (Figure 10). This data indicates that induction of *Pdl2* and *Lag3* by Csn-B treatment might not be Nr4a1-regulated.

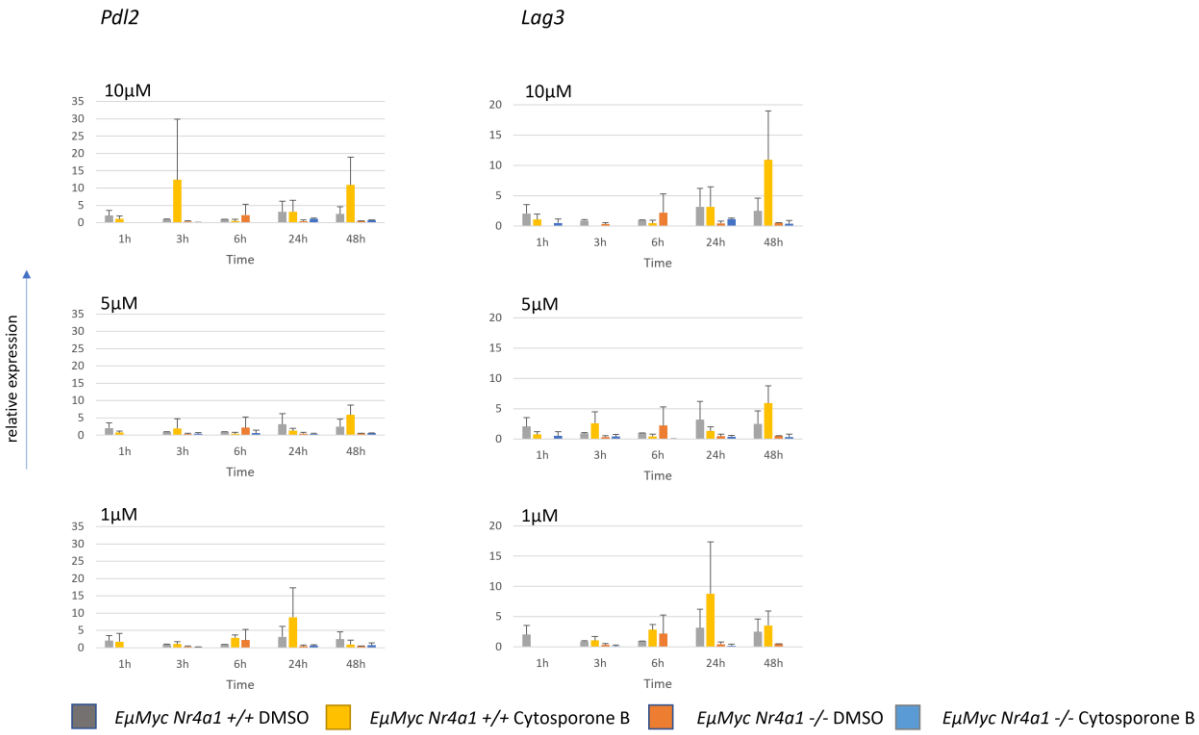

Figure 10: *Relative gene expression of immune checkpoints* Pdl2 *and* Lag3 *after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; no Nr4a1-depending regulation can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

For the Csn-B treatment, no effects were observed on the gene expression of *Gal9* and the immune checkpoint *Pdl1*. Therefore, this data is whether shown nor interpreted in more detail.

3.2.1.3 Interleukines

Csn-B treatment caused no regulation of the gene expression of *Il13 in EµMyc Nr4a1+/+* lymphoma cell lines. Interestingly, an upregulation can be seen in *Il27* (50-fold after 6h 1µM p=0.049) and downregulation in *Il10* (0.003-fold after 1h 1µM p=0.002). In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Il13* (197-fold after 1h 5µM p=0.025) but *Il27* and *Il10* were not affected (Figure 11). This data indicates that induction of the anti-inflammatory *Il27* and reduction of *Il10* by Csn-B might be Nr4a1-dependent. On the other hand, the reduction of *Il13* by Csn-B might not be Nr4a1-dependent.

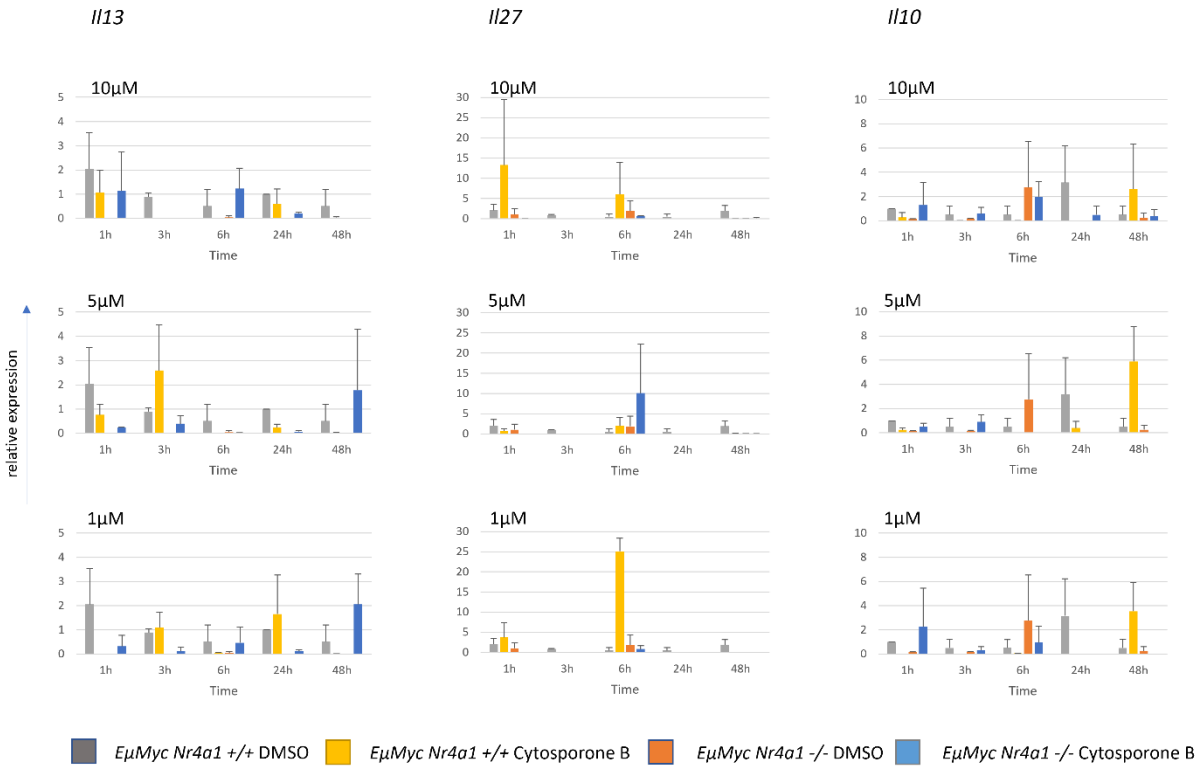

Figure 11: *Relative gene expression of anti-inflammatory interleukines after treatment with different concentrations of Csn-B and DMSO control at different spots in time* in EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending induction of* Il27 *(after 6h) and* Il10 *(after 1h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

Csn-B treatment caused downregulation of *Il18Bp* (0.06-fold after 24h 1µM p=0,022) and *Il23 (*0.02-fold after 3h 10µM p=0.013; 1µM p=0.028; 0.002-fold after 48h 1µM p=0.002), and upregulation of *Il6* (9-fold after 48h 5µM p=0.043) *in EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused no effects on *Il18Bp* and *Il23* expression, and downregulation of *Il6* (0-fold after 48h 10µM p=0.012; 5µM 0.012) (Figure 12). This data indicates that reducing the pro-inflammatory interleukine *Il18Bp* and *Il23* and inducing *Il6 by Csn-B* might be Nr4a1-dependent.

For the Csn-B treatment, no effects were observed on the gene expression of *Il1*. Therefore, this data is whether shown nor interpreted in more detail.

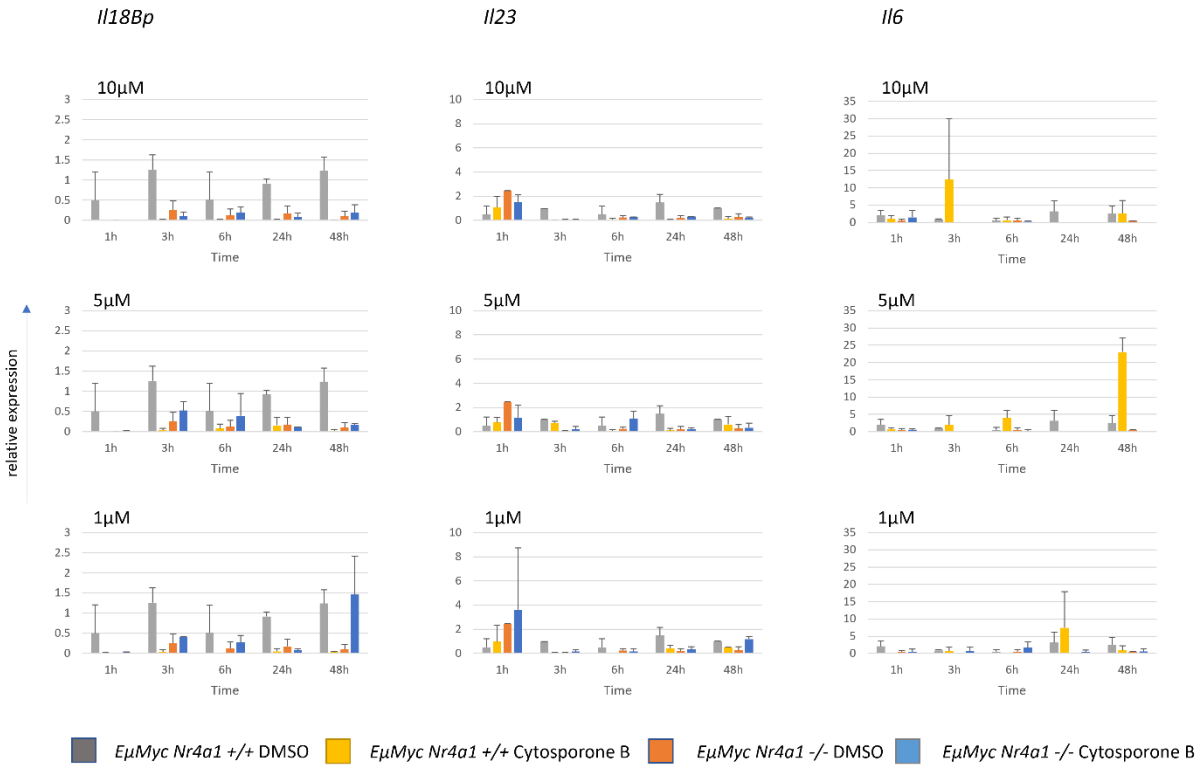

Figure 12: *Relative gene expression of pro-inflammatory interleukines after treatment with different concentrations of Csn-B and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Il18Bp *(after 24h) and* Il23 *(after 3h) and induction of* Il6 *(after 48h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.2 n-Amyl treatment

N-Amyl treatment induced *Nr4a1* expression (5-fold) in a 10µM dose after 3h of treatment compared to DMSO controls (p<0,016) in *EµMyc Nr4a1+/+* lymphoma cells. However, a downregulation was visible after 6h of treatment. As expected, the treatment did not induce *Nr4a1* expression in the *EµMyc Nr4a1-/-* mice lymphomata.

N-Amyl treatment induced a downregulation of *Nr4a3* (0.17-fold after 24h 1µM p=0.038) in *EµMyc Nr4a1+/+* lymphoma cells, whereas *Nr4a2* was not affected. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Nr4a2* (2.9-fold after 48h 1µM p=0.01) wheras *Nr4a3* was not affected (Figure 13). This data indicates that regulation of gene expression of *Nr4a2* by n-Amyl treatment might not

be Nr4a1-dependent. However, a Nr4a1-dependent pathway might regulate gene expression of *Nr4a3* by n-Amyl.

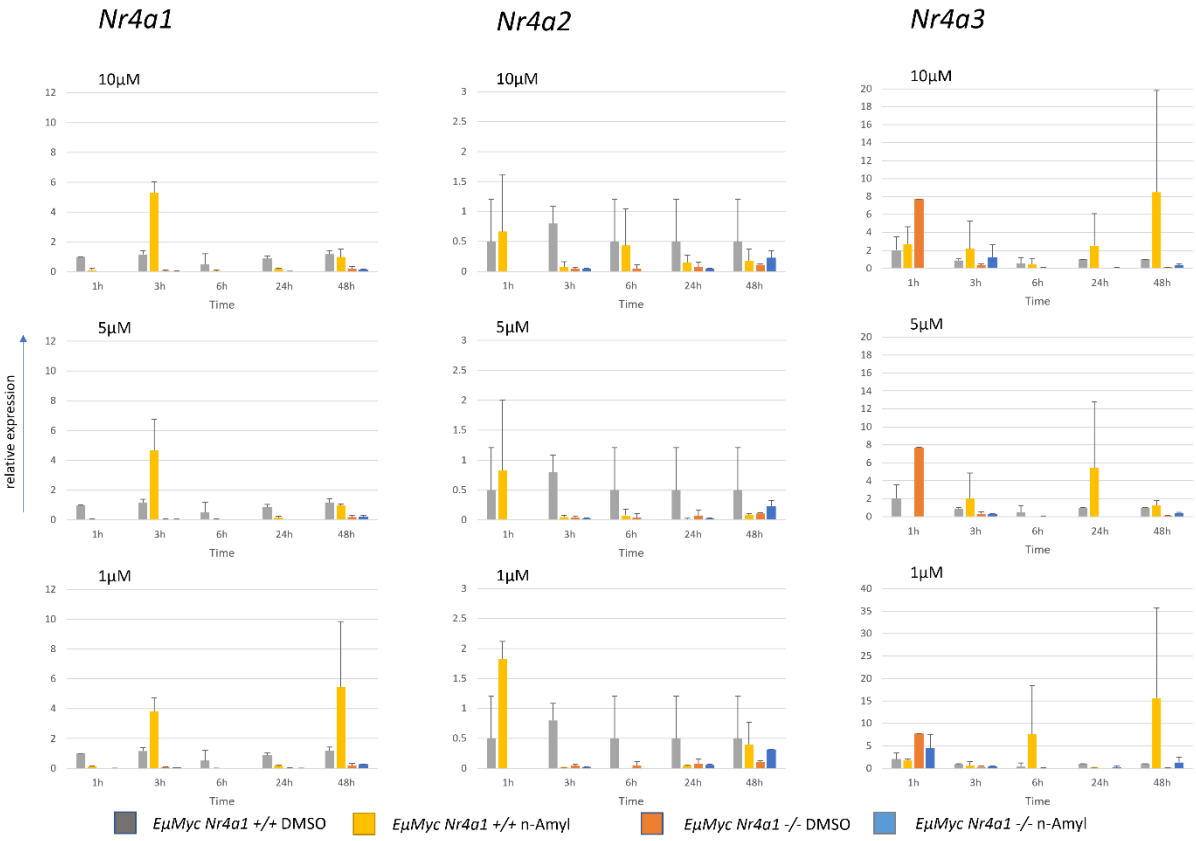

Figure 13: *Relative gene expression of* Nr4a *receptors after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a significant upregulation of* Nr4a1 *3h after treatment and downregulation of* Nr4a3 *24h after treatment can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.2.1 Immune regulatory genes

N-Amyl treatment caused downregulation of *IDO* (0.4-fold after 1h 5µM p=0,02), *TNF* (0.2-fold after 24h 10µM p=0.009; 5µM p=0.006; 1µM p=0.006) *a*nd *TGFβ* (0.3-fold after 3h 10µM p=0.02; 5µM p=0,034) in *EµMyc Nr4a1+/+* lymphoma cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *IDO* (366-fold after 6h 10µM p=0.049), *TNF* (4-fold after 1h 10µM p=0.026; 37-

fold 48h 10µM p=0.008; 5µM p=0.022) and *TGFβ* (19.5-fold after 1h 10µM p=0.027; 5µM p=0.03; 1µM p=0.049) (Figure 14). This data indicates that reducing gene expression of pro-inflammatory enzyme *IDO* and anti-inflammatory cytokine *TGFβ* by n-Amyl treatment might be Nr4a1-dependent. Upregulation of pro-inflammatory cytokine *TNF* might not be Nr4a1-dependent.

N-Amyl treatment did not influence the expression levels of the anti-inflammatory enzyme *COX2.* Therefore, this data is whether shown nor interpreted in more detail.

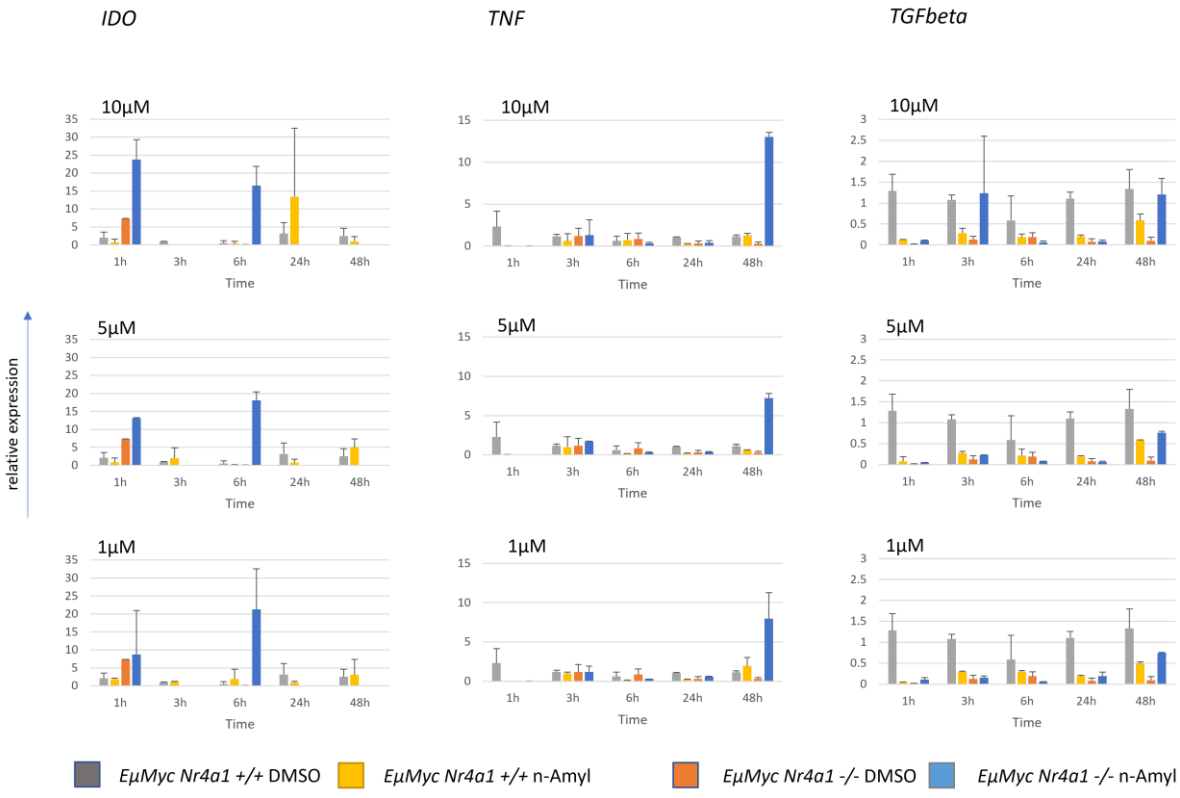

Figure 14: *Relative gene expression of immune regulatory genes after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* IDO *(after 1h) and* TGFβ *(after 3h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.2.2 Immune checkpoints (IC) and the co-inhibitory receptors

N-Amyl treatment did not affect of *CTLA4* and *Hvem*, but it caused downregulation of *Cd86* (0.04-fold after 6h 10µM p=0.026) and *Cd160* (0.4-fold after 6h 10µM

p=0.026*)* in *EμMyc Nr4a1+/+* lymphoma cell lines. In the *EμMyc Nr4a1-/-* setting, the treatment caused upregulation of *CTLA4* (106-fold after 48h 10μM p=0.044) and *Hvem* (7.5-fold after 1h 1μM p=0.031) and no regulation of *Cd86 and Cd160* (Figure 15).

In addition, the treatment caused upregulation of *TigitR* (2.5-fold after 1h 5μM p=0.012) and downregulation of its ligand *Cd112* (0.02-fold after 6h 10μM p= 0.026) in *EμMyc Nr4a1+/+* lymphoma cell lines. In the *EμMyc Nr4a1-/-* setting, the treatment caused no regulation of *TigitR* and *Cd112* (Figure 16). This data indicates that the induction of gene expression of immune checkpoint *CTLA4* by n-Amyl treatment might not be Nr4a1-dependent, but the reduction of its ligand *Cd86* might be Nr4a1-dependent. The reduction of immune checkpoint *Cd160* by n-Amyl treatment might also be Nr4a1-dependent, but not its ligand *Hvem*. The upregulation of *TigitR* and the downregulation of *Cd112* by n-Amyl treatment might be Nr4a1-dependent.

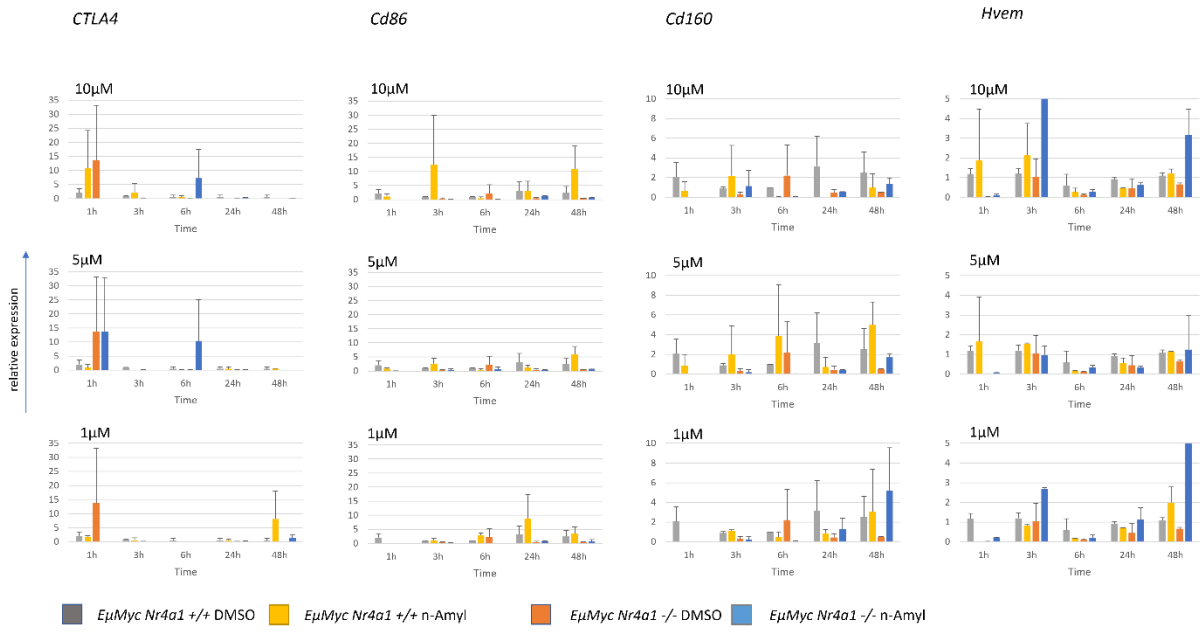

Figure 15: *Relative gene expression of immune checkpoint* CTLA4/Cd80 *and* Cd160/Hvem *after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EμMyc Nr4a1-/- *and* EμMyc Nr4a1+/+ mice *lymphoma cells; a Nr4a1-depending reduction of* Cd86 *(after 6h) and* Cd160 *(after 6h) can be seen in* EμMyc Nr4a1+/+ *mice lymphoma cells*

N-Amyl treatment caused downregulation of the immune checkpoints *Pdl1 (*0.4-fold after 3h 1µM p=0.018), *Pdl2* (0.04-fold after 6h 10µM p=0.026; 1µM p=0.023) and *Lag3* (0.04-fold after 6h 10µM p=0.026) , whereas *Vista expression was not affected* in *EµMyc Nr4a1+/+* lymphoma cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused no regulation of *Pdl1* and *Pdl2*, downregulation of *Lag3* (0-fold after 48h 10µM p=0.012; 1µM p=0.012) and upregulation of *Vista* (15-fold after 6h 10µM p=0.008; 5µM p=0.008) (Figure 16; Figure 17). This data indicates that the reduction of *Pdl1* and *Pdl2* by n-Amyl treatment might be Nr4a1-regulated. Regulation of *Lag3* and *Vista* by n-Amyl treatment might not be Nr4a1-dependent.

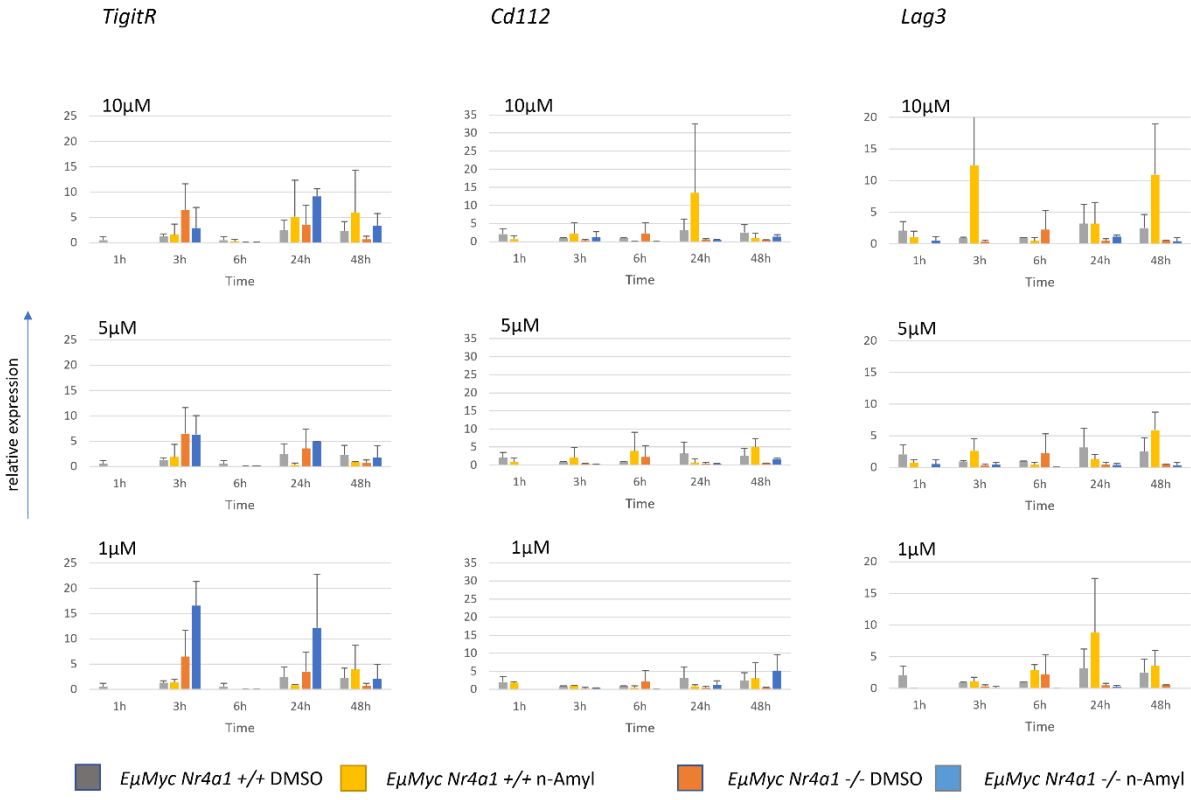

Figure 16: *Relative gene expression of immune checkpoints* TigitR/Cd112 *and* Lag3 *after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Cd112 *(after 6h) and upregulation of* TigitR *(after 1h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

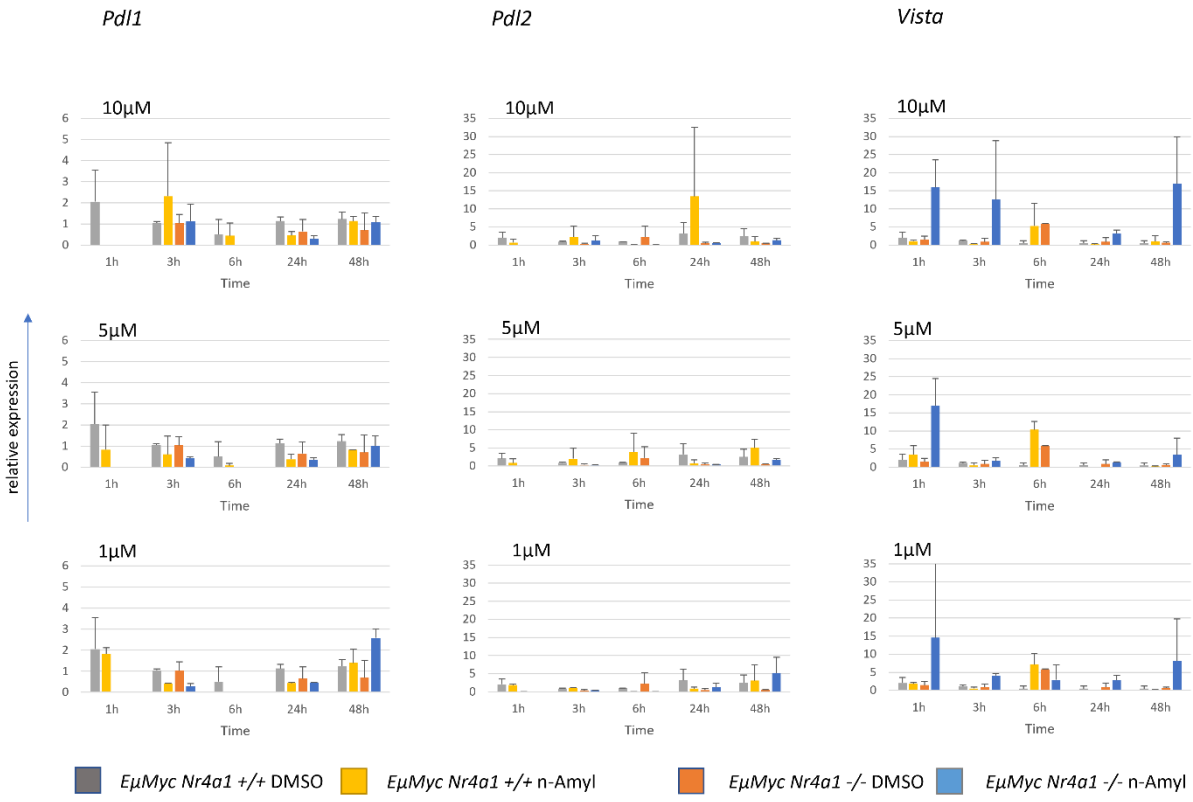

Figure 17: *Relative gene expression of immune checkpoints* Pdl1, Pdl2*, and* Vista *after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Pdl1 *(after 3h) and* Pdl2 *(after 6h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

For n-Amyl treatment, no effects were observed on *Galectin-9* (*Gal9*) gene expression and the ligand of *CTLA4*, *Cd80*. Therefore, this data is whether shown nor interpreted in more detail.

3.2.2.3 Interleukines

N-Amyl treatment exhibited no effects on interleukine*Il27 and Il10* expression *in EµMyc Nr4a1+/+* lymphoma cell lines. A downregulation of *Il13 was*(0.2-fold 24h 1µM p=0.038). In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Il27* (4-fold after 48h 1µM p=0.024), *Il10* (48-fold after 1h 10µM p=0.034) and *Il13* (451.5-fold after 1h 10µM p<0.001; 3470.5-fold 48h 10µM p=0.015) and downregulation of *Il10* (0.005-fold after 3h 5µM p=0.036) and *Il13* (0.008-fold after 24h 5µM p=0.032) (Figure 18). This data indicates that

regulation of the anti-inflammatory interleukine *Il27*, *Il10*, and *Il13* might not be Nr4a1-dependent.

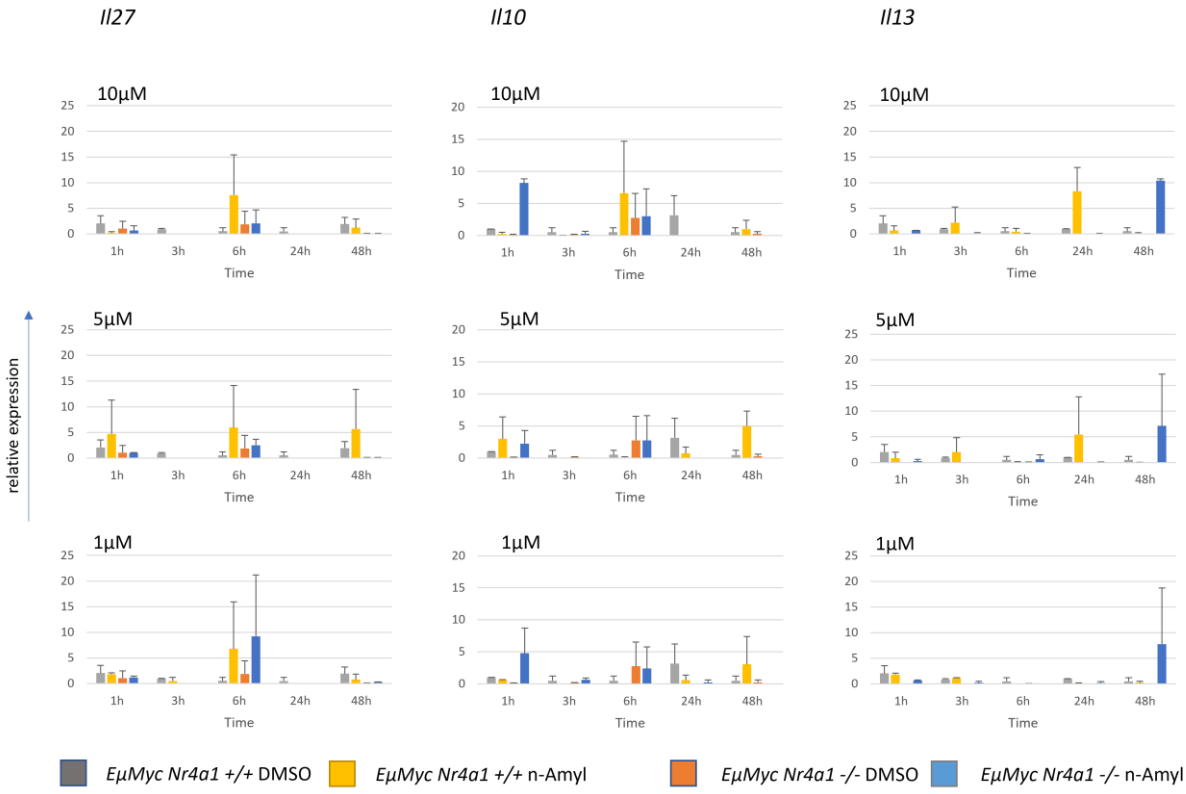

Figure 18: *Relative gene expression of anti-inflammatory interleukines after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; no Nr4a1-depending regulation can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

N-Amyl treatment caused downregulation of *Il18Bp* (0.1-fold after 24h 10µM p=0,029; 1µM p=0.045) and *Il23 (0.1-fold after 3h 10µM p=0.033; 1µM p=0.035; 0.02-fold after 48h 10µM p=0.003),* but had no effects on *Il6* in *EµMyc Nr4a1+/+* lymphoma cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment exhibited no effects on *Il18Bp* and *Il23,* and downregulation of *Il6* (0 -fold after 48h 10µM p=0.012; 5µM 0.012; 1µM 0.012) (Figure 19). This data indicates that reducing the pro-inflammatory interleukine *Il18Bp* and *Il23* by n-Amyl might be Nr4a1-dependent. Regulation of *Il6* might not be Nr4a1-dependent.

For n-Amyl treatment, no effects were observed on the gene expression of *Il1*. Therefore, this data is whether shown nor interpreted in more detail.

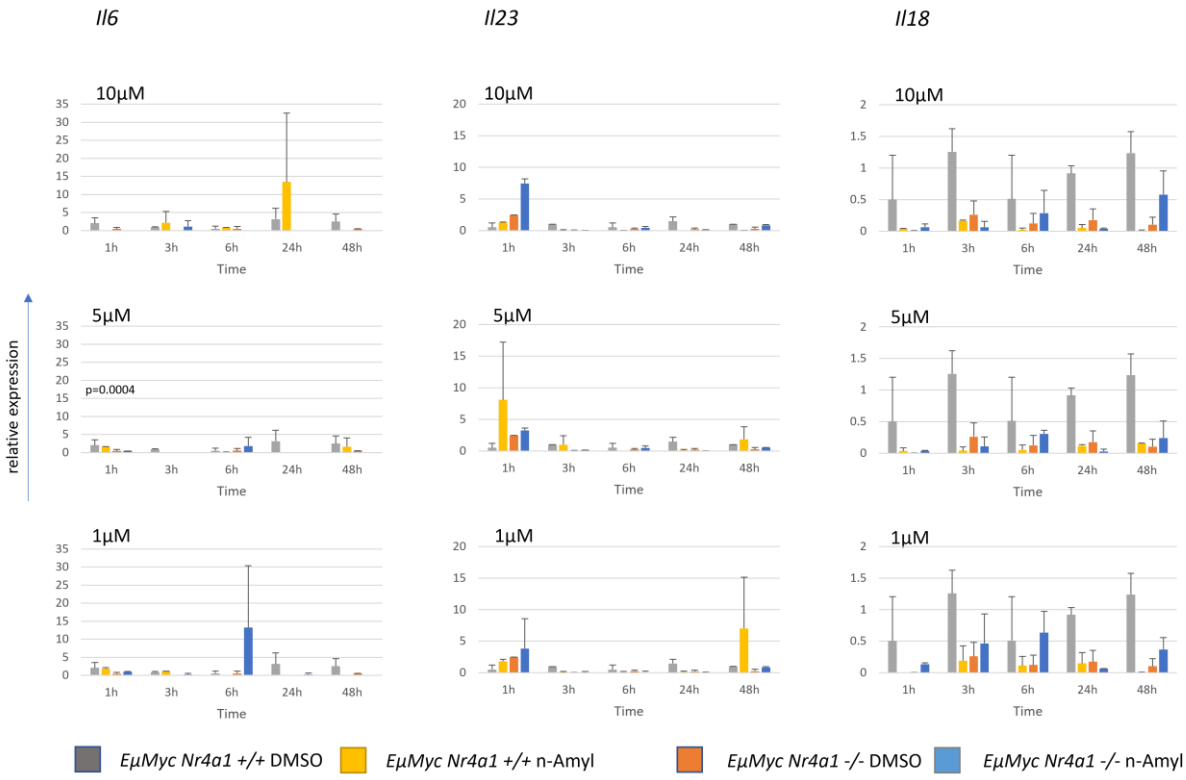

Figure 19: *Relative gene expression of pro-inflammatory interleukines after treatment with different concentrations of n-Amyl and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Il18 *(after 24h) and* Il23 *(after 3h and after 48h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.3 cDim treatment

cDim treatment induced *Nr4a1* expression after 3h of treatment (3.6-fold 10µM p=0.034; 5µM p=0.005) in *EµMyc Nr4a1+/+* lymphoma cells. As expected, the treatment did not induce *Nr4a*1 expression in the *EµMyc Nr4a1-/-* mice lymphomata (Figure 20).

cDim treatment did not induced the *Nr4a2* expression, whereas *Nr4a3* was downregulated (0.17-fold after 24h 1µM p=0.014) in *EµMyc Nr4a1+/+* lymphoma cells. In the *EµMyc Nr4a1-/-* setting, the treatment caused no effects on *Nr4a2* and *Nr4a3* expression (Figure 20). This data indicates that the reduction of gene expression of *Nr4a3* by cDim treatment might be Nr4a1-dependent. However, Nr4a1 might not regulate the gene expression of *Nr4a2.*

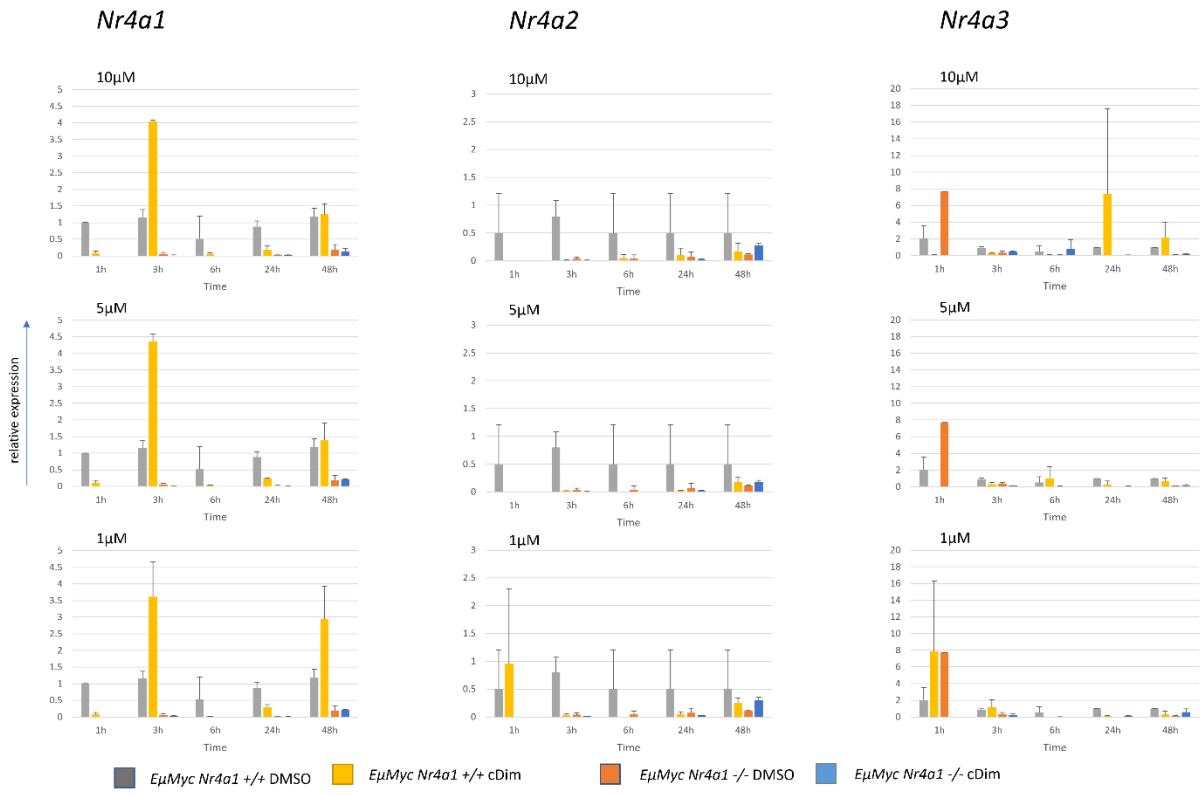

Figure 20: *Relative gene expression of* Nr4a *receptors after treatment with different concentrations of cDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a significant upregulation of* Nr4a1 *3h after treatment and downregulation of* Nr4a3 *24h after treatment can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.3.1 Immunoregulatory genes

cDim treatment caused downregulation of *TNF* (0.15-fold after 24h 10µM p=0,013; 5µM p=0,021; 1µM p=0,006), *TGFβ* (0.17-fold after 3h 5µM p=0.035), and no regulation of *IDO* in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TNF* (7-fold after 1h 10µM p=0.017; 5µM p=0.048), *TGFβ* (3-fold after 1h 10µM p=0.022), *IDO* (2.3-fold after 1h 10µM p=0,023; 5µM p=0,012; 1µM p=0,042) (Figure 21). This data indicates that reducing gene expression of pro-inflammatory cytokine *TNF* and anti-inflammatory cytokine *TGFβ* by cDim treatment might be Nr4a1-dependent. Regulation of pro-inflammatory enzyme *IDO* might not be Nr4a1-dependent.

cDim treatment did not influence the expression levels of the anti-inflammatory enzyme *COX2.* Therefore, this data is whether shown nor interpreted in more detail.

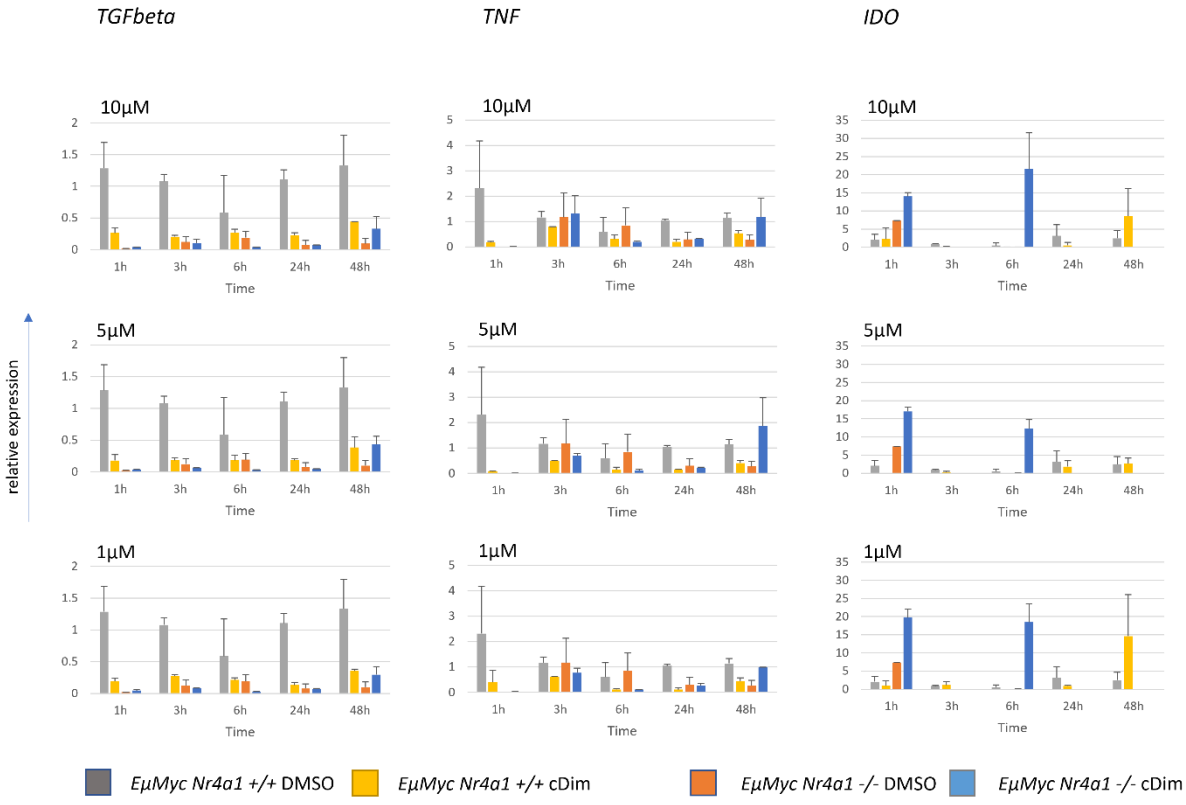

Figure 21: *Relative gene expression of immune regulatory genes after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* TNF *(after 24h) and* TGFβ *(after 3h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.3.2 Immune checkpoints (IC) and the co-inhibitory receptors

cDim treatment exhibited no effects on the gene expression of the immune checkpoint *CTLA4*, but it caused downregulation of its ligands *Cd80 (0.01-fold after 1h 10µM p=0.012; 5µMp=0.002; 0.1-Fold after 3h 5µM P=0.032) and Cd86* (0.007-fold after 6h 1µM p=0.37) in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused downregulation of *CTLA4 (*0.9-fold after 6h 10µM 0.017), upregulation of *Cd80* (8500-fold after 1h 5µM p=0.046), *and* no regulation can be seen in *Cd86* (Figure 22). This data indicates that regulation of *CTLA4* by cDim treatment might not be Nr4a1-dependent. Reduction of *Cd80* and *Cd86* by cDim treatment might be Nr4a1-dependent.

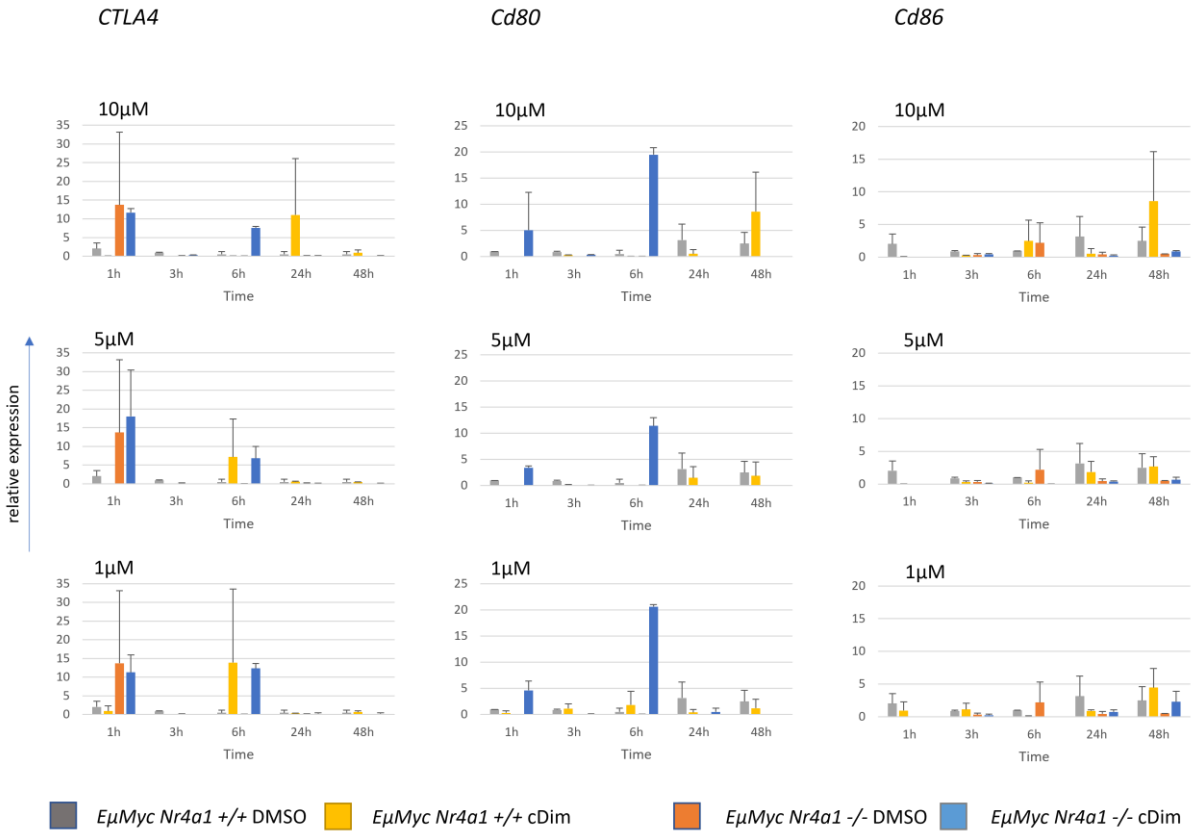

Figure 22: *Relative gene expression of immune checkpoint* CTLA4 *and its ligands* Cd80/Cd86 *after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Cd80 *(after 1h) and* Cd86 *(after 6h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

cDim treatment had no effects on the gene expression of the immune checkpoints *TigitR* and downregulation of its ligand *Cd112* (0.007-fold after 6h 1µM p=0.37). Interestingly, the treatment caused downregulation of the immune checkpoint *Cd160* (0.007-fold after 6h 1µM p=0.37) and no regulation of its ligand *Hvem*. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TigitR* (4-fold after 1h 10µM p=0.012) and did not affect *the expression of its ligand Cd112.* The treatment caused no deregulation of *Cd160* and upregulation of its ligand *Hvem* (3.4-fold after 1h 1µM p=0.03; 3-fold after 48h 5µM p=0.013) (Figure 23). This data indicates that the reduction of *Cd112* and *Cd160* by cDim treatment might be Nr4a1-dependent. On the other hand, regulation of *TigitR* and *Hvem* by cDim treatment might not be Nr4a1-dependent.

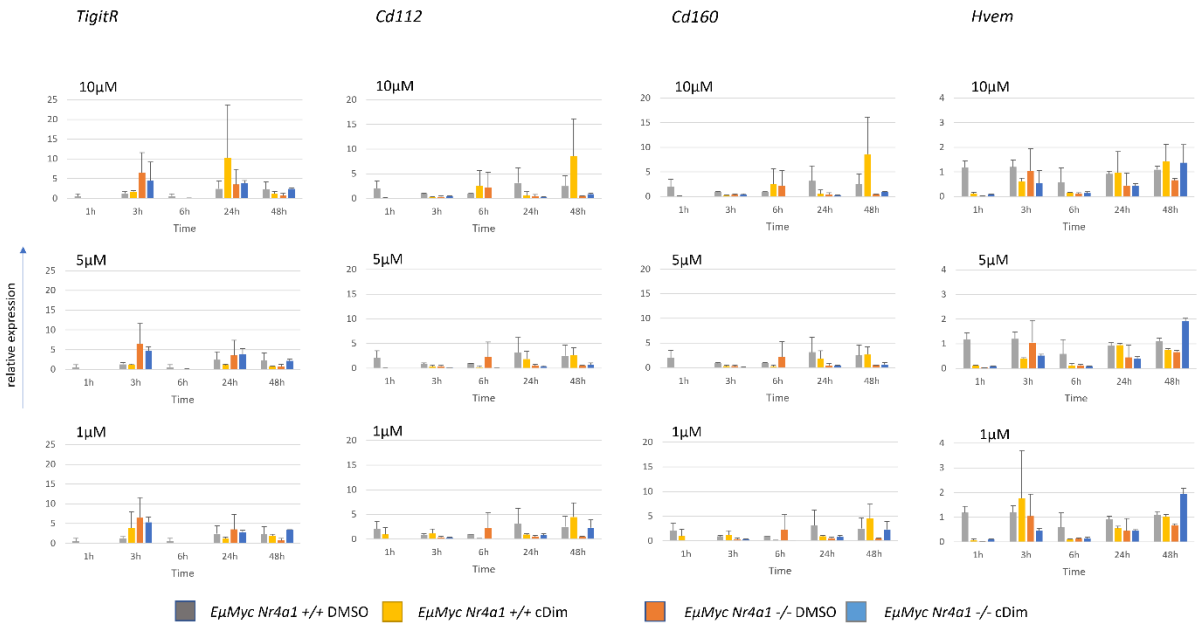

Figure 23: *Relative gene expression of immune checkpoints* TigitR/Cd112 *and* Cd160/Hvem *after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Cd112 *(after 6h) and* Cd160 *(after 6h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

cDim treatment caused downregulation of the gene expression of the immune checkpoints *Pdl1 (*0.4-fold after 3h 10µM p=0.022; 5µM p=0.029; 1µM p=0.048), *Pdl2 (*0.007-fold after 6h p=0.037), *Lag3* (0.007-fold after 6h p=0.037) and upregulation of the ligand *Gal9* (18-fold after 48h 10µM p=0.033) in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment exhibited no effects in *Pdl1, Pdl2,* and *Gal9* and caused downregulation of *Lag3* (0-fold after 48h p=0.012) (Figure 24). This data indicates that reduction of *Pdl1* and *Pdl2* and induction of *Gal9* by cDim treatment might be Nr4a1-dependent. Regulation of *Lag3* by cDim might not be Nr4a1-dependent.

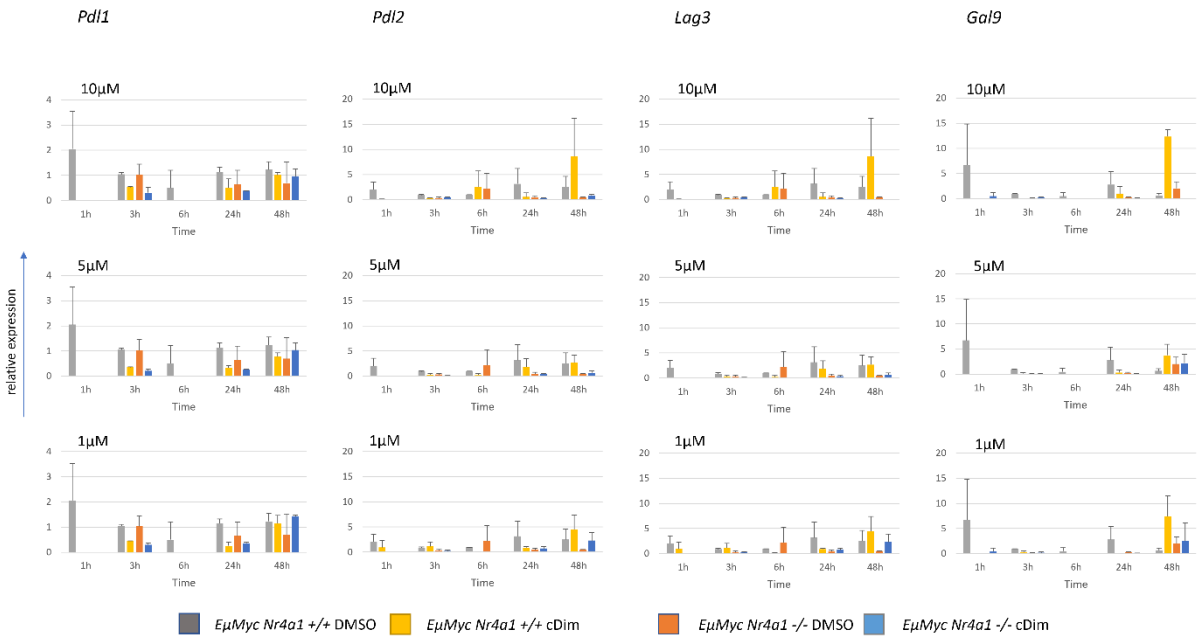

Figure 24: *Relative gene expression of immune checkpoints* Pdl2, Pdl2 *and* Lag3 *and the ligand Gal9 after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Pdl1 *(after 3h) and* Pdl2 *(after 6h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.3.3 Interleukins

cDim treatment caused *downregulation* of the gene expression of interleukine *Il10* (0.022-fold after 1h 10µM p=0.012; 5µM 0.002) and *Il13 (*0.2-fold after 24h 1µM p=0.017*) in EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Il10* (8.8-fold after 1h 5µM p=0.012; 1µM p=0.012) but did not affect *Il13* expression (Figure 25). This data indicates that the reduction of the anti-inflammatory interleukines *Il10* and *Il13* by cDim might be Nr4a1-dependent.

For cDim treatment, no effects were observed on gene expression of the anti-inflammatory interleukine *Il27*. Therefore, this data is whether shown nor interpreted in more detail.

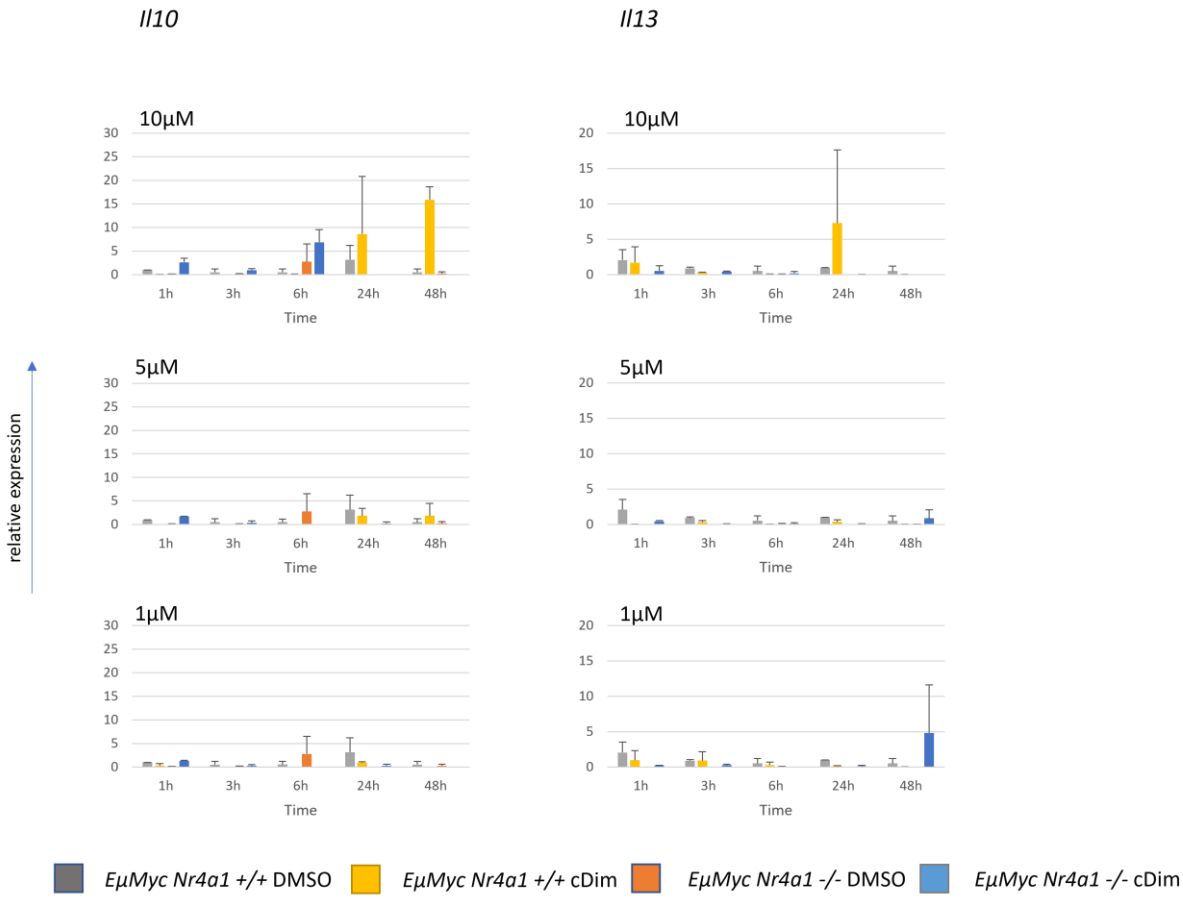

Figure 25: *Relative gene expression of anti-inflammatory interleukines after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Il10 *(after 1h) and* Il13 *(after 24) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

cDim treatment caused downregulation of *Il18Bp* (0.13-fold after 24h 5µM p=0,0.034;1µM p=0.045) and *Il23* (0.08-fold after 3h 5µM p<0.001) and no regulation of *Il1* and *Il6* in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused no regulation of *Il18Bp* and *Il23*, upregulation of *Il1* (4.6-fold after 48h 5µM p=0.003), and downregulation of *Il6* (0-fold after 48h 10µM p=0.012; 5µM 0.012) (Figure 26). This data indicates that reducing the pro-inflammatory interleukine *Il18Bp* and *Il23* by cDim might be Nr4a1-dependent contrary to the regulation of *Il1* and *Il6* .

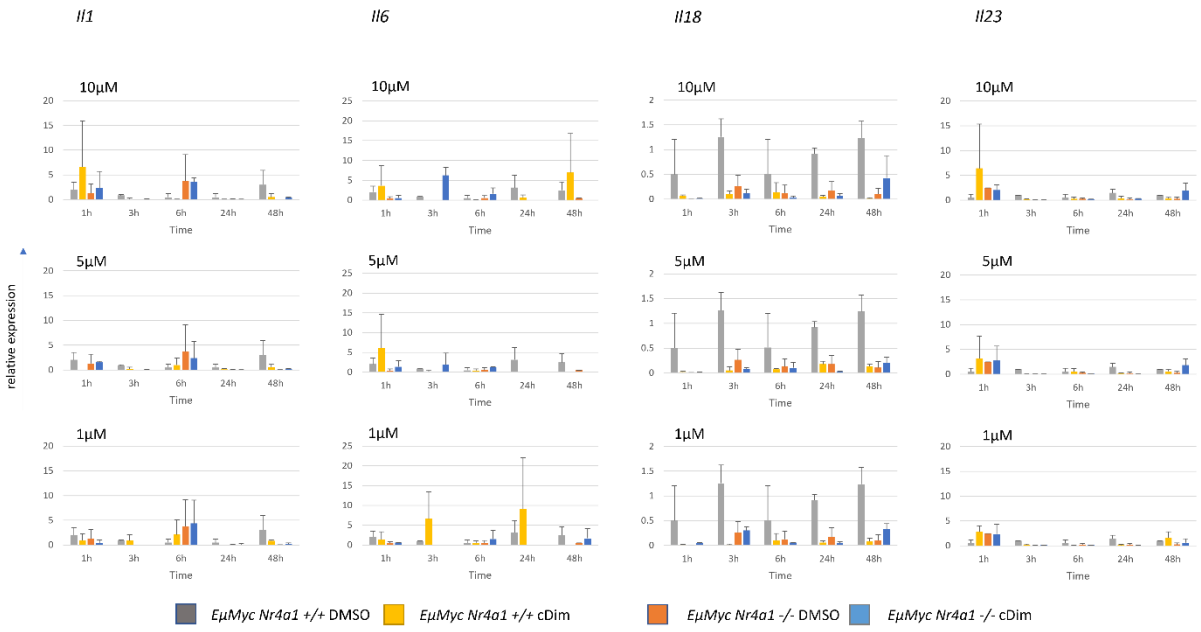

Figure 26: *Relative gene expression of pro-inflammatory interleukines after treatment with different concentrations of CDim and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Il18Bp *(after 1h) and* Il23 *(after 3h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.4 THPN treatment

5µM THPN treated samples did not survive because of its high cell toxicity.

THPN treatment induced *Nr4a1* expression later compared to the other agonists in *EµMyc Nr4a1+/+* lymphoma cells. 26-fold upregulation was observed 6h (1µM p=0,004) and 5-fold upregulation 48h (0.5µM p=0.005) after treatment (Figure 27). As expected, the treatment did not induce *Nr4a1* expression in the *EµMyc Nr4a1-/-* mice lymphomata.

THPN treatment showed no effects on *Nr4a2* and *Nr4a3* expression in *EµMyc Nr4a1+/+* lymphoma cells. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Nr4a2* (2.5-fold after 24h 1µM p=0.013; 0.5µM p=0.037; 3.4-fold after 48h 1µM p=0.04) and downregulation of *Nr4a3* (0-fold 48h 1µM p=0.012) (Figure 27). This data indicates that regulation of gene expression of *Nr4a2* and *Nr4a3* by THPN treatment might not be Nr4a1-dependent.

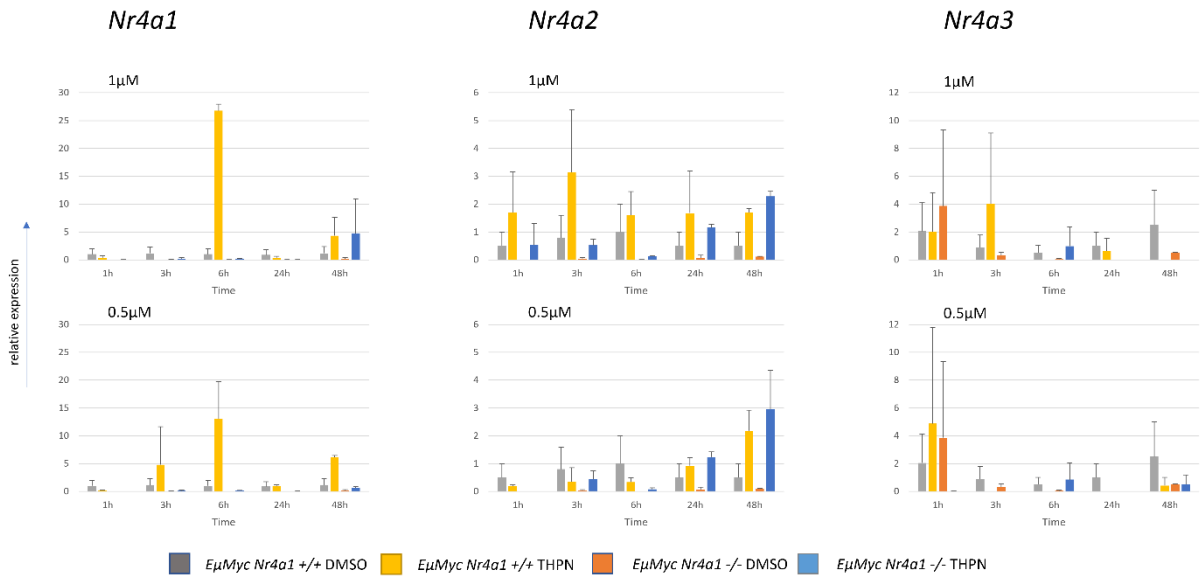

Figure 27: *Relative gene expression of* Nr4a *receptors after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a significant upregulation of* Nr4a1 *6h and 48h after treatment can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.4.1 Immunoregulatory genes

THPN treatment caused downregulation of *TNF* (0.31-fold after 24h 0.5µM p=0,002) and *TGFβ (0.4-fold after 24h 0.5µM p=0.048),* and upregulation of *IDO* (5-fold after 48h 0.5µM p=0,049) in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TGFβ* (5-fold after 24h 0.5µM p=0.041; 10-fold 48h 1µM p=0.041) and exhibited no effects on *TNF and IDO* expression levels (Figure 28). This data indicates that reducing pro-inflammatory cytokine *TNF* and anti-inflammatory cytokine *TGFβ* and inducing pro-inflammatory enzyme *IDO* by THPN treatment might be Nr4a1-dependent.

THPN treatment did not influence the expression levels of the anti-inflammatory enzyme *COX2.* Therefore, this data is whether shown nor interpreted in more detail.

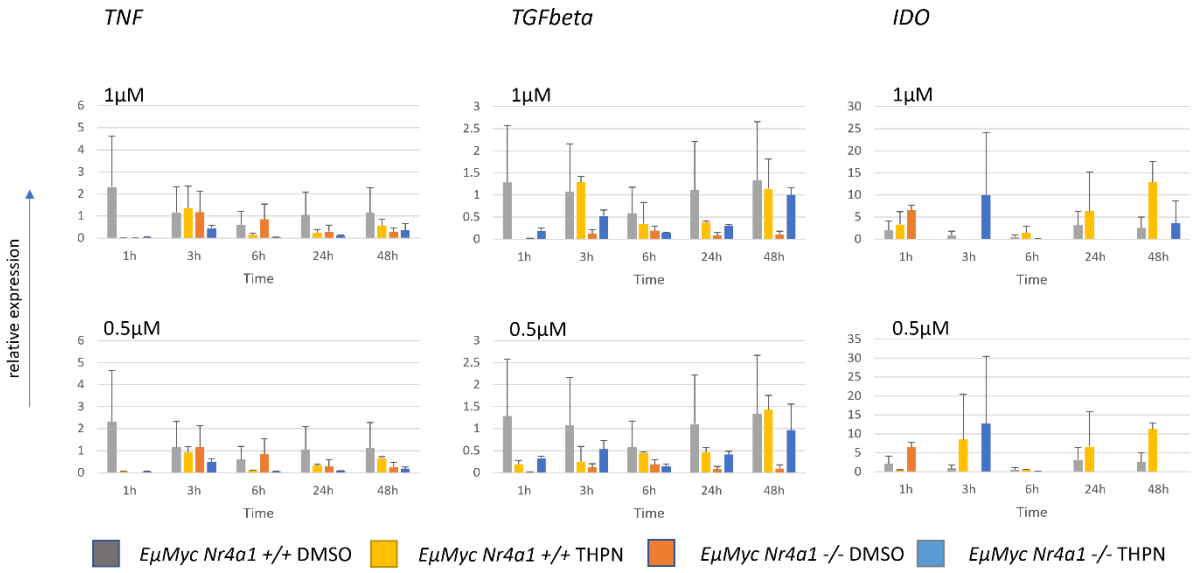

Figure 28: *Relative gene expression of immune regulatory genes after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* TNF *(after 24h) and* TGFβ *(after 24h) and induction of* IDO *(after 48h)can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

3.2.4.2 Immune checkpoints (IC) and the co-inhibitory receptors

THPN treatment caused no deregulation of the *CTLA4 expression,* and it caused downregulation of its ligands *Cd80* (0.03-fold after 1h 0.5µM p=0.004) and no expression of *Cd86* (after 6h 1µM p<0.001; 0.5µM p<0.001) in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused downregulation of *CTLA4* (0.07-fold after 48h p=0.012) and no expression *Cd86* (after 48h 1µM p=0.012; 0.5µM p=0.012), but no effects can be seen for *Cd80* (Figure 29). This data indicates that regulation of *CTLA4*/*Cd86* by Csn-B treatment might not be Nr4a1-dependent. However, the reduction of *Cd80* by Csn-B treatment might be Nr4a1-dependent.

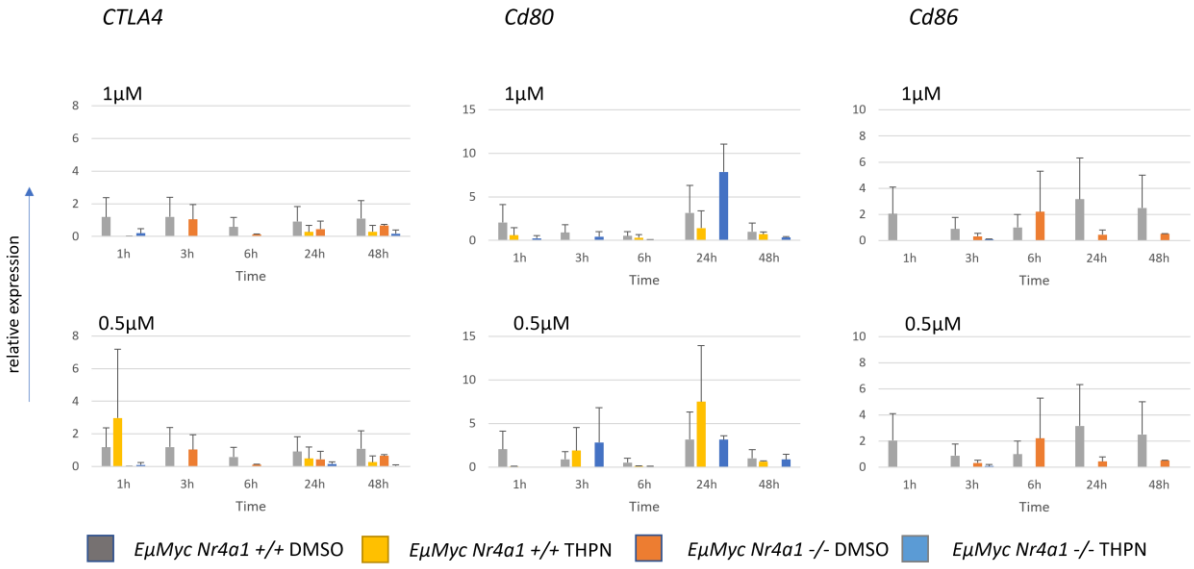

Figure 29: *Relative gene expression of immune checkpoint* CTLA4 *and its ligands* Cd80/Cd86 *after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Cd80 *(after 1h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

THPN treatment exhibited no effects on the *TigitR* expression and caused upregulation of its ligand *Cd112* (6.7-fold after 3h 1µM p=0.001) in *EµMyc Nr4a1-/-* lymphoma cell lines. The treatment caused downregulation of the immune checkpoint *Cd160* (0.1-fold after 3h 1µM p=0.032) and upregulation of its ligand *Hvem* (8.5-fold after 48h 0.5µM p=0.046). Interestingly, in the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *TigitR* (1.7-fold after 3h 1µM p=0.0004) and did not affect the expression level of its ligand *Cd112*. The treatment caused downregulation of *Cd160* (0.06-fold after 48h 0.5µM p=0.012) and upregulation of its ligand *Hvem* (70-fold after 24h 1µM p= 0.029) (Figure 30). This data indicates that regulation of *TigitR* and *Cd160/Hvem* by THPN treatment might not be Nr4a1-dependent. The upregulation of *Cd112* by THPN treatment might be Nr4a1-dependent.

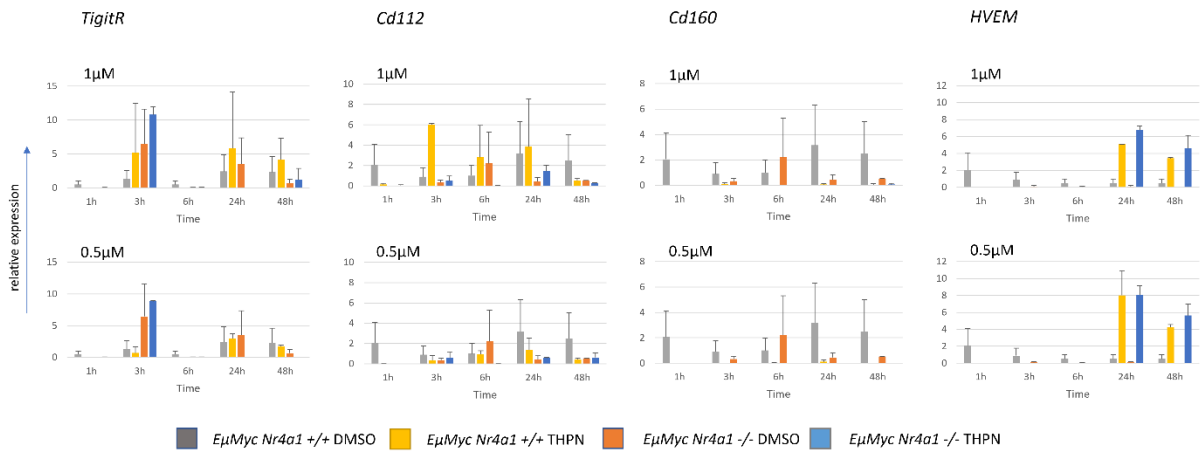

Figure 30: *Relative gene expression of immune checkpoints* TigitR/Cd112 *and* Cd160/Hvem *after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending induction of* Cd112 *(after 3h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

THPN treatment caused upregulation of the gene expression of the immune checkpoint Pdl1 (10-fold after 6h 0.5µM p=0.044), no regulation of *Pdl2,* and downregulation of *Lag3* (0.0003-fold after 6h 1µM p<0.001; 05µM p<0.001) in *EµMyc Nr4a1+/+* cell lines. However, in the *Eµ Myc Nr4a1-/-* setting, the treatment caused no regulation of *Pdl1*, downregulation of *Pdl2* (0.06-fold after 48h 1µM p=0.029) and *Lag3* (0.0001-fold after 48h 1µM p=0.012; 0.5µM p=0.012) (Figure 31). This data indicates that induction of *Pdl1* by THPN treatment might be Nr4a1-regulated. The regulation of *Pdl2* and *Lag3* by THPN treatment might not be Nr4a1-dependent.

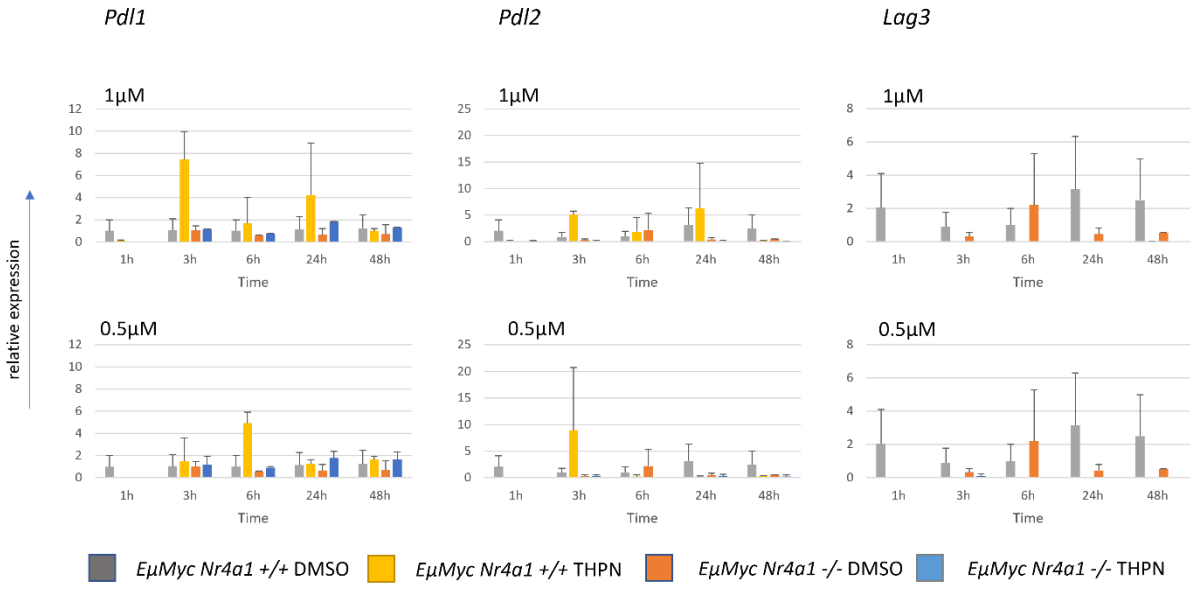

Figure 31: *Relative gene expression of immune checkpoints* Pdl1, Pdl2*, and* Lag3 *after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending induction of* Pdl1 *(after 6h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

For THPN treatment, no effects were observed on gene expression of *Galectin-9* (*Gal9*). Therefore, this data is whether shown nor interpreted in more detail.

3.2.4.3 Interleukines

THPN treatment caused downregulation of the gene expression of *Il10* (0.02-fold after 1h 0.5µM p=0.00) and upregulation of interleukine *Il27* (6-fold after 3h 1µM p=0.002) in *EµMyc Nr4a1+/+* lymphoma cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Il10* (8-fold after 6h 1µM p=0.032) but no regulation in *Il27* (Figure 32). This data indicates that induction of the anti-inflammatory interleukine *Il27* and reduction of *Il10* might be Nr4a1-dependent.

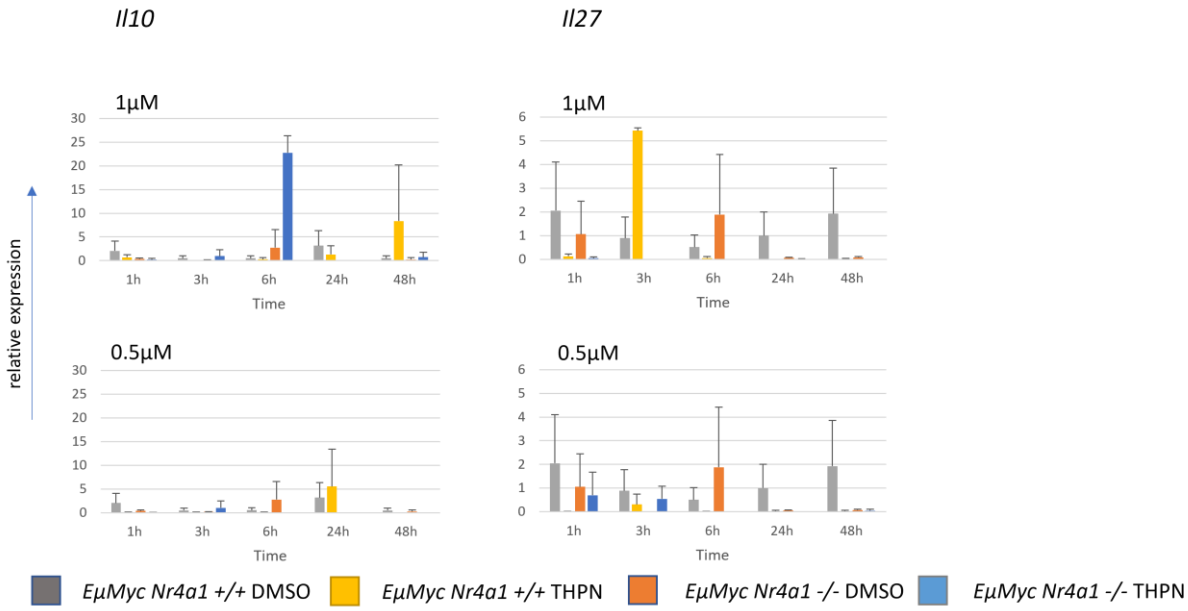

Figure 32: *Relative gene expression of anti-inflammatory interleukins after treatment with different concentrations of THPN and DMSO control at different spots in time in* EµMyc Nr4a1-/- *and EµMyc Nr4a1+/+ mice lymphoma cells; a Nr4a1-depending induction of* Il27 *(after 3h) and* Il10 *(after 1h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

THPN treatment did not affet the expression levels of *Il1, Il6, and Il23 and* downregulation of *Il18Bp* (0.3-fold after 24h 1µM p=0,032) in *EµMyc Nr4a1+/+* cell lines. In the *EµMyc Nr4a1-/-* setting, the treatment caused upregulation of *Il1* (8.7-fold after 48h 1µM p<0.001), downregulation of *Il6* (0.4-fold after 48h 1µM p=0,009; 0.5µM p=0.011) and *Il23 (*0.02-fold after 1h 0.5µM p=0,001) and no regulation of *Il18Bp* (Figure 33). This data indicates that the reduction of the pro-inflammatory interleukine *Il18Bp* might be Nr4a1-dependent. The regulation of the pro-inflammatory interleukine *Il1, Il6,* and *Il23* by THPN might not be Nr4a1-dependent.

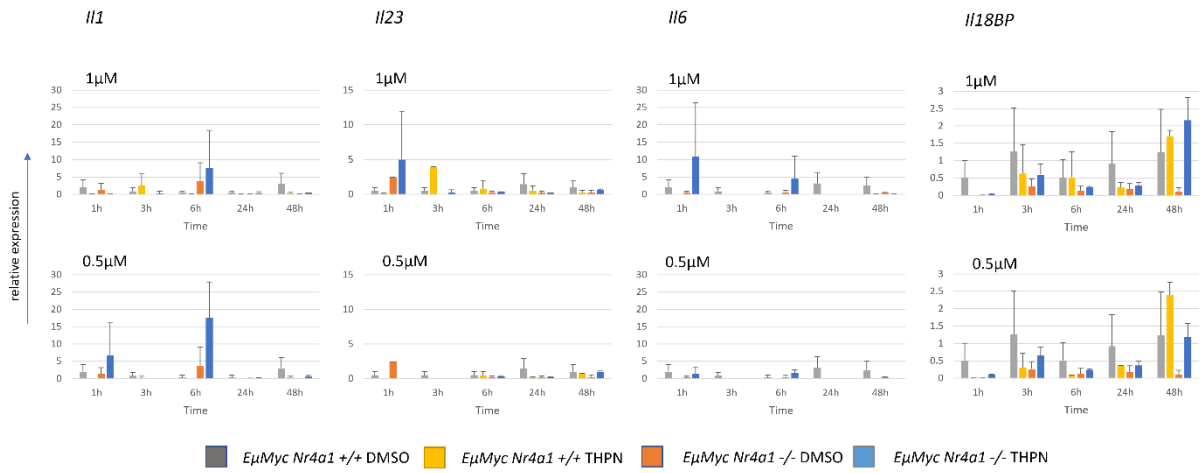

Figure 33: *Relative gene expression of pro-inflammatory interleukines after treatment with different concentrations of THPN and DMSO control at different spots in time in EµMyc Nr4a1-/- and* EµMyc Nr4a1+/+ *mice lymphoma cells; a Nr4a1-depending reduction of* Il18Bp *(after 24h) can be seen in* EµMyc Nr4a1+/+ *mice lymphoma cells*

For THPN treatment, no effects were observed on gene expression of anti-inflammatory interleukine *Il13*. Therefore, this data is whether shown nor interpreted in more detail.

3.2.5 Celastrol treatment

To determine the effects of Celastrol on lymphoma cell growth, we treated *EµMyc Nr4a1+/+* and *EµMyc Nr4a1-/-* lymphoma cells with this agent. After 72h of Celastrol treatment, concentration-dependent growth inhibition in all investigated cell lines was detected by the EZ4U assay (Figure 34). This data indicates Celastrol possesses cytotoxic effects, which are Nr4a1-independent.

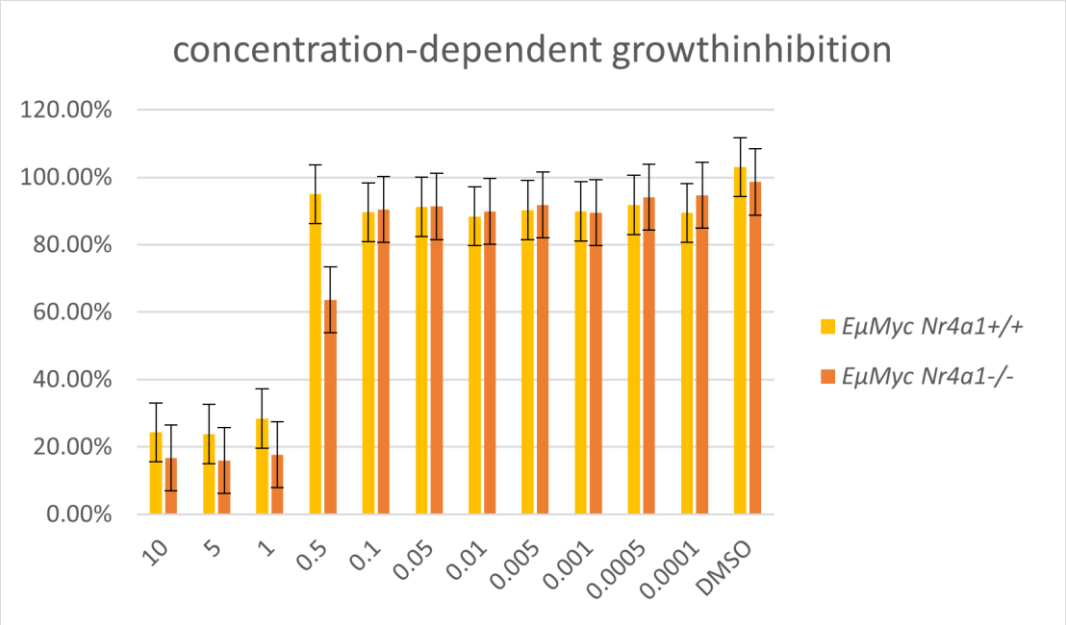

Figure 34: *EZ4U analysis of the toxicity of Celastrol showed a concentration-dependent growth inhibition up to 0.5μM, which is Nr4a1-independent*

4 Discussion

This study aimed to investigate the influence of *Nr4a1* on the expression of immune regulatory genes. Studies have shown that the presence of *Nr4a1* has direct or indirect effects on the immune system [108]. Probably, the results are skewed because of the outliers and the low number of probed samples. Therefore, it would be necessary to retry the study with more samples in each of the groups.

4.1 Primary tumor

None of the genes showed a significant difference in the expression rate between *EμMyc Nr4a1-/-* and *EμMyc Nr4a1+/+* mice lymphoma in this study. *In conclusion, Nr4a1* might not impact *Il-27, Il-10, Il-6, Il-18Bp, Il-23, Il-1rnTX, TNF*, and *TGFβ*.

4.2 Treatment

All treatment leads to an expression and thus to an increased level of *Nr4a1* in lymphoma cells. Additionally, they have a different impact on several pro- and anti-inflammatory processes, Nr4a1-dependent and -independent. According to several studies, the interaction between agonists and Nr4a1 leads to a rise of immune response induced by regulation of gene expression of immune-regulatory components [104–107].

4.2.1 Cytosporone B (Cns-B)

In this study, Cns-B treatment downregulated pro-inflammatory genes *TNF, Il18*, and *Il23* and upregulated *Il6* in Nr4a1-dependent manner. In addition, Csn-B treatment, reduced the expression of anti-inflammatory factors *Il10* and *TGFβ* and induced *Il27* gene expression in the Nr4a1+/+ condition.

In conclusion, after Cns-B treatment, Nr4a1-dependent pathways induce immune response via reduction and/or induction of pro- and anti-inflammatory molecules. This data indicates that Cns-B might be a potential treatment to induce anti-tumor responses in aggressive lymphoma. However, no rise in an apoptotic co-factor was seen like in the literature [104].

4.2.2 N-Amyl

n-Amyl treatment downregulated pro-inflammatory genes *IDO, Il18*, and *Il23* Nr4a1-dependent and *Il6* Nr4a1-independent. Interestingly, *TNF* was the only upregulated immune-activating cytokine in Nr4a1 dependent manner. The treatment decreased the expression of anti-inflammatory factors *Il13* and *TGFβ* Nr4a1-dependent manner. n-Amyl downregulated the immune checkpoints *Lag3, Pdl1, Pdl2, Cd160* and the co-inhibitory factors *Cd86* and *Cd112* and upregulated *TigitR* in Nr4a1 dependent manner.

In conclusion, after n-Amyl treatment, Nr4a1-dependent pathways induce an immune response by reducing immune inhibitory molecules and immune checkpoints and their ligands. Additionally, a Nr4a1-dependent pathway might regulate gene expression of *Nr4a3* by n-Amyl. Furthermore, N-Amyl seems to have a more significant impact on Nr4a1 than Cns-B and thus a higher anti-tumor activity seen in further studies [106].

4.2.3 cDim

cDIM treatment downregulated pro-inflammatory genes *TNF, Il18*, and *Il23* and anti-inflammatory factors *Il10*, *Il13,* and *TGFβ* Nr4a1-dependent manner and *Il6* Nr4a1-independent way. cDIM also downregulated the immune checkpoints *Lag3, Pdl1, Pdl2, Cd160* and the co-inhibitory factors *Cd80, Cd186*, and *Cd112* and upregulated *Gal9* Nr4a1-dependent manneer.

In conclusion, after n-Amyl treatment, Nr4a1-dependent pathways induce an immune response by reducing immune inhibitory molecules and immune checkpoints and their ligands. This data indicates that n-Amyl deregulates the invisibility of tumor cells against T-cells. Furthermore, cDIM binds to multiple other receptors [104].

4.2.4 THPN

THPN treatment downregulated gene expression of pro-inflammatory factors *TNF*, and *Il18*, and *Il23* Nr4a1-dependent manner. Interestingly, *Il1* was the only upregulated immune-activating cytokine via the Nr4a-independent pathway. the expression of anti-inflammatory factors *Il10* and *TGFβ* were reduced The *Il27*

expression level was induced gene expression of *Il27* by the treatment depending on the occurance of Nr4a1. In addition, THPN downregulated the immune checkpoints *Lag3* and the co-inhibitory factors *Cd80* and *Cd186* and upregulated *Cd112* and *Pdl1* Nr4a1-dependent manner.

In conclusion, after THPN treatment, Nr4a1-dependent pathways induced immune response as seen in the literature [106] is not detectable in this study.

4.2.5 Celastrol

To determine the effects of Celastrol on lymphoma cell growth and the correlation with Nr4a1, *EμMyc Nr4a1+/+*, and *EμMyc Nr4a1-/-* lymphoma cells were treated with different concentrations, and cell growth was detected. This data shows a concentration-dependent growth inhibition which was Nr4a1-independent.

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