



universität
wien

MASTERARBEIT

Titel der Masterarbeit

Proposed metabolic function of immune-responsive
gene 1 (IRG1) in terms of dendritic cell mediated
immune suppression

verfasst von

Natalie Knapp, BSc

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2014

Studienkennzahl lt. Studienblatt:

A 066 834

Studienrichtung lt. Studienblatt:

Masterstudium Molekulare Biologie

Betreut von:

Ao. Univ.-Prof. Dr. Erhard Hofer

Content

ABSTRACT	6
ZUSAMMENFASSUNG	7
1 INTRODUCTION	9
1.1 Dendritic cells in the immune system	9
1.1.1 Toll-like receptor signaling	11
1.1.2 The JAK-STAT Signaling Pathway	12
1.1.3 Kinetics of dendritic cell maturation	14
1.2 Role of Dendritic cells in T-cell Activation and differentiation	14
1.2.1 CD4+ T cell polarization	16
1.2.1.1 Th1 cells	17
1.2.1.2 Th17 cells	18
1.2.1.3 Treg cells	18
1.2.2 CD8+ T lymphocytes	19
1.3 Role of metabolism in the immune system	19
1.3.1 Regulation of Immune Responses by metabolism	20
1.3.2 Immune-responsive gene 1 (IRG-1)	23
1.3.2.1 Preliminary IRG1 study	24
2 WORKING HYPOTHESIS and AIMS	26
3 MATERIALS and METHODS	27
3.1 Mice	27
3.2 Cell culture	27
3.2.1 Isolation and preparation of murine bone marrow-derived dendritic cells (BM-DCs)	27
3.2.2 DC transfection	28
3.2.3 DC maturation	29
3.2.4 CD4 ⁺ and CD8 ⁺ T cell isolation and activation	30
3.2.5 CFSE labeling of T cells	30

3.2.6	T cell proliferation assays	31
3.2.7	In vitro apoptosis of effector T cells	32
3.2.8	CD4 ⁺ T cell phenotyping by quantitative real time PCR	32
3.3	Flow Cytometry	33
3.3.1	DC phenotype	33
3.3.2	T cell phenotype	33
3.4	Real-time Polymerase Chain Reaction (RT-PCR) Analysis	34
3.4.1	RNA Isolation	34
3.4.2	cDNA Reverse Transcription (cDNA-RT)	34
3.4.3	Real time PCR (RT-PCR)	35
3.5	Protein Detection by Western Blot	36
3.5.1	Sample preparation	36
3.5.2	SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	37
3.5.3	Western Blot	38
3.6	Cytokine Detection	39
3.6.1	Fluorescent Bead Immunoassay (FlowCytomix™ system)	39
3.7	High Performance Liquid Chromatography and Mass spectrometry (HPLC and MS)	39
4	RESULTS	41
4.1	Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells	41
4.1.1	Intracellular localization of IRG1	41
4.1.2	IRG1 expression in DCs stimulated via TLR ligands and cytokines	42
4.1.3	JAK/STAT mediated expression of IRG1 in DCs	46
4.1.4	Effect of IRG1 silencing on DCs	48
4.2	Effects of IRG1 silenced DCs on T cell functions	50
4.2.1	Effect of IRG1 silenced DCs on proliferation of CD8 ⁺ effector T cells	50
4.2.2	Effect of supernatants from IRG1 silenced DCs on the proliferation of effector CD8 ⁺ T cells	52

4.2.3	Effect of IRG1 silenced DCs on CD4 ⁺ T cell differentiation	55
4.3	Detection of enriched molecules in the supernatant of IRG1 ⁺ DCs by HPLC	57
5	DISCUSSION	61
5.1	Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells	61
5.1.1	Regulation of IRG1	61
5.1.2	Effect of IRG1 silencing on DCs	63
5.2	Effects of IRG1 silenced DCs on T cell functions	65
5.2.1	Effect of IRG1 silenced DCs on proliferation and viability of CD8 ⁺ effector T cells	65
5.2.2	Effect of IRG1 silenced DCs on of CD4 ⁺ T cell polarization	68
5.3	Detection of enriched molecules in the supernatant of IRG1 ⁺ DCs by HPLC	68
6	CONCLUSION	73
7	REFERENCES	75
8	LIST OF FIGURES	84
9	LIST OF TABLES	86
10	ABBREVIATIONS	87
11	ACKNOWLEDGEMENTS	89
12	CURRICULUM VITAE	90

ABSTRACT

Dendritic cells (DCs) are significant for a proper immune defense by orchestrating the innate and adaptive immunity. Upon receiving a danger signal, such as LPS, DCs start to undergo a differentiation process by switching from an immune-stimulatory to an immune-suppressive mode of action. The immune suppressive mechanisms initiated by DCs are needed to keep a balance in the immune system in order not to exaggerate immune responses. However, some of these immune suppressive mechanisms provided by DCs are not fully understood and characterized yet.

We consider the immune responsive gene (IRG) 1, which has high homology to the bacterial metabolite enzyme methylcitrate-dehydratase (PrpD), as an important regulator of the immune suppressive DC phenotype. The aim of this study was to investigate the regulation as well as the proposed immune suppressive function of IRG1 mediated via metabolites.

We found that IRG1 is induced by several Toll-like receptor (TLR) ligands as well as by cytokines and shows its highest expression at a time, when DCs become anti-inflammatory. Considering the function of IRG1, we observed in our in vitro analyses that the supernatants of IRG1 deficient, interferon (IFN)- γ activated DCs led to enhanced proliferation and viability of effector T cells as well as elevated numbers of the T helper (Th) 1, Th17 and regulatory T cell (Tregs) subsets. Furthermore, high performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses, revealed the presence of some enriched metabolites in the supernatant of IRG1 expressing DCs. Our results indicate that IRG1 links metabolism to immunity by providing an additional layer of immune regulation through the production of soluble immune suppressive metabolites.

Collectively, our study identified IRG1 as a novel target to improve the immune-stimulatory capacity of DCs by inhibiting its putative immune-suppressive function. Future studies are needed to elucidate the immune regulatory mechanisms provided by IRG1 and its metabolites. Its inhibition might be useful to improve immune therapeutic strategies.

ZUSAMMENFASSUNG

Dendritische Zellen (DCs) besitzen sowohl im angeborenen als auch im adaptiven Immunsystem wichtige Aufgaben und sind somit für eine normal funktionierende Immunabwehr unabdingbar. Die Erkennung eines Gefahrensignals, beispielsweise des Lipopolysaccharids (LPS), löst einen Differenzierungsprozess aus bei welchem die dendritische Zelle von einem stimulatorischen zu einem anti-inflammatorischen Zustand wechselt. Anti-inflammatorische Mechanismen werden zur Aufrechterhaltung der Homöostase initiiert. Viele dieser induzierten Mechanismen in den dendritischen Zellen sind jedoch noch nicht vollständig geklärt.

Wir betrachten das "immune responsive gene 1" (IRG1), welches eine hohe Homologie zum bakteriellen metabolischen Enzym der Methylcitrat-dehydratase (PrpD) besitzt, als einen wichtigen Regulator des anti-inflammatorischen Phänotyps einer DC. Das Ziel dieser Studie war es sowohl die Regulation als auch die vermutlich durch Metabolite herbei geführte anti-inflammatorische Funktion des IRG1 zu untersuchen.

Es konnte gezeigt werden, dass IRG1 sowohl durch verschiedene Toll-like Rezeptor (TLR) Liganden als auch durch Zytokine selbst induziert werden kann. Die höchste Expression des Gens wurde zu einem Zeitpunkt beobachtet in welchem die DC einen anti-inflammatorischen Phänotyp aufzeigte. Bezüglich der Funktion von IRG1 konnte in in-vitro Analysen gezeigt werden, dass die Überstände von IRG1 defizienten, Interferon (IFN)- γ aktivierten DCs zu einer gesteigerten Proliferation und Viabilität von Effektor T Zellen als auch zu einer erhöhten Anzahl der T Helfer (Th) 1, Th17 und regulatorischen T Zellen (Tregs) Subtypen, geführt haben.

Hochleistungs-flüssigkeitschromatographische (HPLC) und Massenspektrometrische (MS) Analysen deuteten auf angereicherte Metabolite im Überstand von IRG1 exprimierenden DCs hin. Unsere Resultate weisen auf eine durch IRG1 erzielte Kopplung des Metabolismus und des Immunsystems hin.

Diese Kopplung kommt durch eine zusätzlich induzierte Immunregulation, bei welcher immunsuppressive Metabolite ausgeschüttet werden, zustande.

In unserer Studie konnte IRG1 als ein neues Zielgen identifiziert werden, um die immun-stimulatorische Kapazität von DCs durch das Ausschalten seiner vermutlich immun-suppressiven Funktion zu verbessern. Zukünftige Studien zur Aufklärung der durch IRG1 induzierter immun-regulatorischer Mechanismen sind von großer Notwendigkeit. Die Inhibierung dieses Gens kann zur Verbesserung von immuntherapeutischen Strategien führen.

1 INTRODUCTION

In recent years, immunotherapy has become a new promising approach in treatment of various diseases; such as autoimmunity, infectious diseases and cancer.

Tumor immunology has seen many exciting developments in the last decade. Thus, in 2011, the original hallmarks of cancer have been revisited and one of the additionally added hallmarks was – evading immune destruction [5]. This new hallmark emphasizes the importance of establishing potential cancer immunotherapies. Two types of therapeutic strategies for the prevention and treatment of cancers can be distinguished: the passive and active immunotherapy. While passive immunotherapies are comprised of monoclonal antibodies or other immune system components that are administered to the patient, active immunotherapies aim to specifically activate the patients' own immune cells [6].

Dendritic cell immunotherapies have shown to be an efficient strategy to induce an antigen-specific T-cell response against the tumor. Several studies, in which dendritic cells (DC) were used to trigger a tumor-specific immune response, were published until now [7-9]. Nevertheless, DC based immunotherapies still need improvement and barriers, such as the suppressive tumor microenvironment, need to be overcome. In this perspective, our research is focused on improving the capacity of dendritic cells activating the adaptive immunity within a tumor microenvironment.

1.1 Dendritic cells in the immune system

Two systems provide host defense in vertebrates; the innate immune system and the adaptive immune system. Innate immunity is the phylogenetically oldest system of host defense. Important components of the innate immunity are physical and chemical barriers (skin, mucosal epithelia); phagocytic cells; blood proteins – complement system and cytokines that coordinate and regulate many activities of the cells of innate immunity. Adaptive immunity, also known as the

1 INTRODUCTION

acquired immune system, is a more specialized defense system and provides great variability, memory and adaption to infections. There are two types of adaptive immune responses; the humoral and the cell-mediated response. The key players in humoral immunity are B lymphocytes who produce and secrete antibodies in large amounts, whereas T lymphocytes contribute to cell-mediated immunity [10, 11].

Dendritic cells (DCs), regarded as the most potent antigen presenting cells, act at the interface of innate and adaptive immunity. At an immature state of development, DCs capture and phagocytose pathogens at site of infections and migrate to the lymphoid organs in order to present particular antigens via peptide-major histocompatibility complexes (MHC) to T cells. (Fig. 1.1) This way DCs initiate an adaptive immune response and thus both types of immune responses (adaptive and innate response) are interconnected, instructed and regulated by DCs.

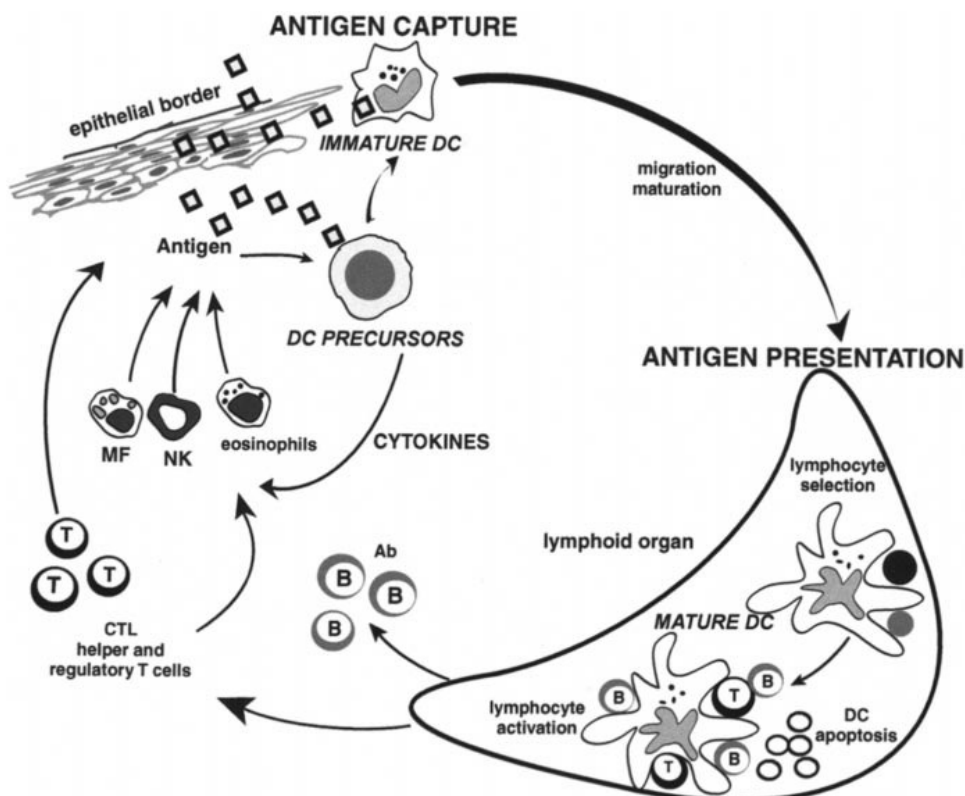


Figure 1.1: Life cycle of a dendritic cell. After encountering pathogens in tissues, immature DCs mature and migrate to the lymphoid organs. There they present the pathogen fragments to other immune cells, which get activated and migrate to the site of infection. This way adaptive immunity is initiated by dendritic cells [12].

DCs are heterogeneous and are divided into several subsets. Generally, DCs can arise from lymphoid-as well as myeloid-committed precursors. All subsets share the capacity of priming T cells but differ in the signaling that determines the outcome of a T cell response.

Importantly, mouse and human DCs share the basic functions but differ in some surface marker and cytokine production profiles. Some differences between mice and human DCs can be explained by the distinct microbial products and danger signals the species encounter. However, divergence is rather observed regarding culture-generated and freshly isolated DCs or between blood and lymphoid-tissue DCs and not between species [13].

1.1.1 Toll-like receptor signaling

As mentioned above, DCs link innate as well as adaptive immune responses. For eliciting a proper adaptive immune response, DCs first detect pathogens e.g. via Toll-like receptors (TLRs) by recognizing pathogen-associated molecular patterns (PAMPs) which are absent in mammals [14-16]. Each TLR binds its appropriate ligand and activates a signaling pathway resulting in a specific response. Most TLRs as TLR4, TLR5, TLR2 act in combination with TLR1 and TLR6 which are located on the cell surface, whereas TLR7/8, TLR9 and TLR3 are situated in the endosome and need acidification for ligand binding. (see Fig. 1.2) All TLRs are structurally composed of an extracellular domain consisting of leucine-rich repeats, a membrane-spanning region and an intracellular Toll-IL-1 R (TIR) domain.

The activation of TLR signaling pathways originates from the TIR domain and results in the recruitment of a specific adaptor molecule – myeloid differentiation factor 88 (MyD88). This adaptor molecule is common to all TLRs, except TLR3 and in part TLR4 signaling, and is necessary for inducing cytokine production.

Signaling via TLR3 and TLR4 is mediated by the adaptor molecule TIR domain-containing adaptor inducing IFN-beta (TRIF) and is involved in the expression of IFN-inducible genes and co-stimulatory molecules.

1 INTRODUCTION

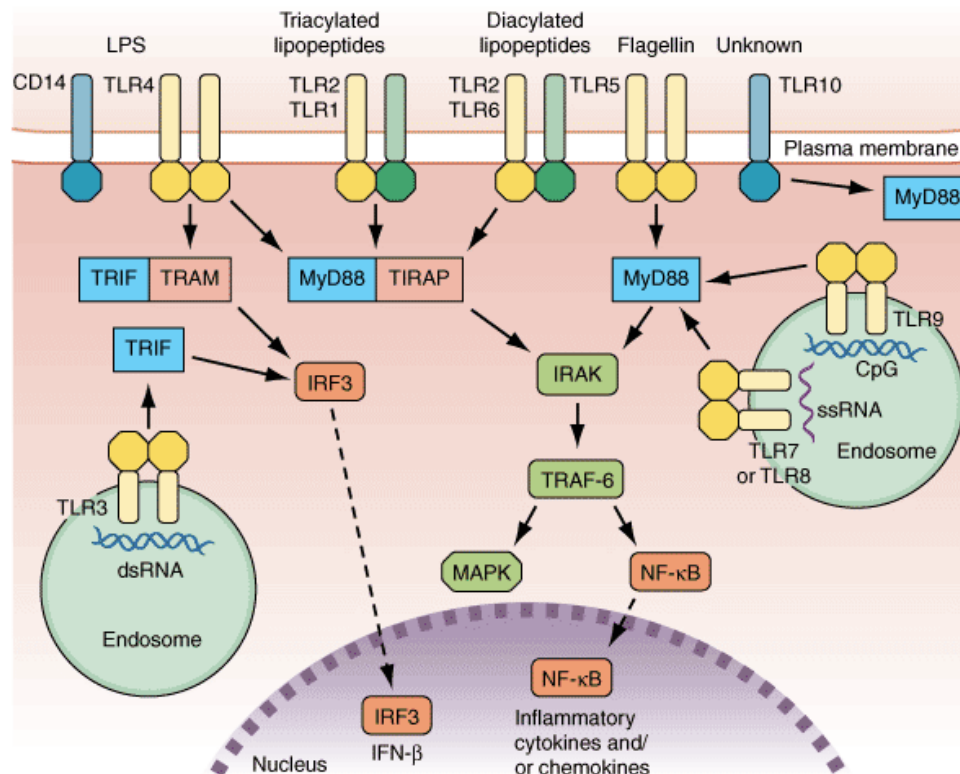


Figure 1.2: Toll-like receptor mediated immune responses. All TLRs, except for TLR3, lead to the activation of NFκB via MyD88 signaling, whereas TLR3 signaling results in the nuclear translocation of IRF3 via TRIF dependent signaling. The transcription factors NFκB and IRF3 are important for inducing a proper immune response – cytokine production, expression of co-stimulatory molecules and IFN-inducible genes [1].

Thus, we can distinguish between MyD88-dependent and MyD88-independent signaling cascades. TLR4 ligation in response to LPS, results in activation of both MyD88-dependent and independent signaling cascades. In the case of the MyD88 dependent signals, the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP-1) are activated, whereas TRIF dependent signals result in the activation of the transcription factor interferon-regulatory factor 3 (IRF3). The outcome of the specific TLR response is determined by the distinct adaptor proteins which account for differential gene expression [14-16].

1.1.2 The JAK-STAT Signaling Pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is essential for many cellular events as cell proliferation, differentiation,

cell migration and apoptosis that determine processes as hematopoiesis and immune development [17].

As discussed before, the first response of dendritic cells upon recognition of pathogens via TLRs is the production of various cytokines. There are about 36 cytokine receptors that respond to approximately 38 cytokines. In DCs most of these cytokines are important for eliciting a secondary signaling mechanism mostly via the JAK/STAT pathway. The principle components of this signaling cascade are the transmembrane cytokine receptor, the JAK kinase and the STAT molecules. Upon ligand binding, the JAKs get activated due to transphosphorylation and subsequently activate both, the receptors and STAT molecules. The STAT molecules dimerize and translocate to the nucleus to activate or repress target genes. (see Fig. 1.3) The number of JAKs and STATs is small compared to the number of cytokines that selectively induce gene expression [17, 18].

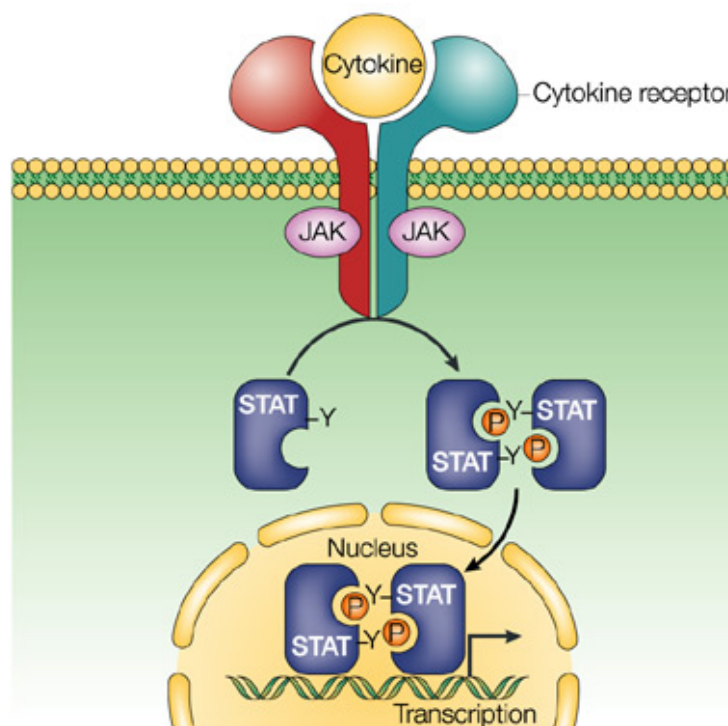


Figure 1.3: The JAK/STAT pathway. Cytokines bind to their appropriate receptors and activate the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. JAK kinases get activated, which is followed by the phosphorylation of the STAT molecules, which dimerize and translocate to the nucleus to induce gene expression [3].

1 INTRODUCTION

As in the case of IL-10/IL-10 receptor signaling, cytokines can act through autocrine signaling in order to dampen an immune response [19]. Cytokines secreted by other immune cells act on the cytokine receptors of DCs in order to strengthen the immune response [20].

1.1.3 Kinetics of dendritic cell maturation

Dendritic cells are located in so called peripheral microenvironments and are therefore exposed to cytokines, growth factors and other soluble mediators secreted by other cells. Additionally to these environmental stimuli, there are maturation stimuli that act directly on the TLRs and induce phenotypic maturation and cytokine secretion.

Studies have suggested that due to these stimuli, DCs differentiate into distinct functional subsets; for example into an inflammatory Interleukin-12 (IL-12) secreting DC subset or into a suppressive Interleukin-10 (IL-10) DC subset. These DC subsets might then selectively drive the development of T cell subsets [21, 22].

Other studies, including our own, have shown that DCs exposed to LPS, initiate a time-dependent differentiation program: First, rapid induction of pro-inflammatory cytokines, such as IL-12, as well as the upregulation of co-stimulatory molecules and major histocompatibility complex takes place. After about 24 hours of stimulation the same DC switches from a pro- into an anti-inflammatory phenotype characterized by the upregulation of immune-suppressive molecules as IL-10, soluble IL-2 receptor alpha molecules (sIL-2RA) and indoleamine-2, 3-dioxygenase (IDO) [23, 24] (see Figure 1.4). This observation does not rule out the presence of individual DC subsets but offers an additional layer of immune regulation.

1.2 Role of Dendritic cells in T-cell Activation and differentiation

Immature dendritic cells are specialized in capturing and processing pathogens. In this state DCs exhibit lower expression of surface molecules for T cell

1.2 Role of Dendritic cells in T-cell Activation and differentiation

activation. After ligation of TLRs, DCs become activated and mature during their migration to lymphoid organs where they activate other immune cells, as T cells. DCs are seen as the most effective antigen-presenting cells (APC) for being capable of initiating T cell responses. Additionally the formed peptide-MHC complexes after antigen processing are more concentrated on DCs than on other

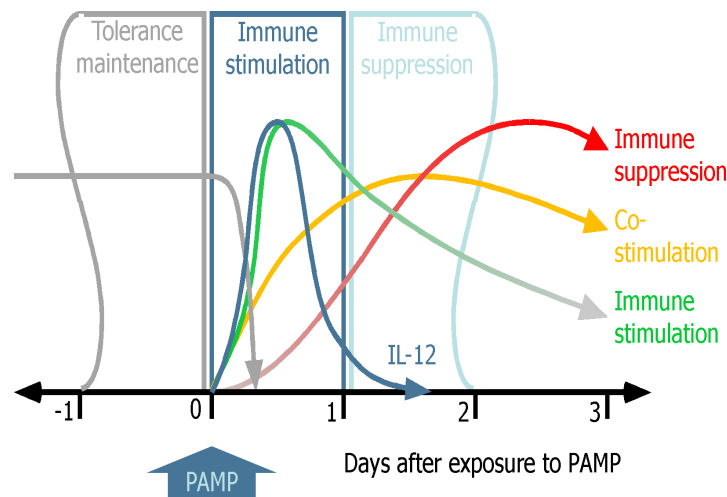


Figure 1.4: Differentiation of a pro- into an anti-inflammatory DC phenotype. Upon DC activation, the immune stimulation is characterized by the up regulation of antigen presenting molecules and co-stimulatory molecules, as well as the production and secretion of polarizing cytokines, such as Interleukin-12 (IL12), whereas in the immune suppression status high levels of Interleukin-10 are released. *Figure by Felzmann T et al., Laboratory for Tumor Immunology, CCRI, Vienna, Austria.*

APCs like B cells and monocytes [10, 25]. T cells are the mediators of immune responses but their effector functions are under instruction of dendritic cells. Proper T cell activation requires three dendritic cell derived signals: The first signal is provided by the peptide loaded MHC/ T cell receptor (TCR) complex. To sustain T cell activation, a second signal is given due to the interaction between co-stimulatory molecules CD80, CD86 on DCs and CD28 on T cells. In addition, multiple cytokines and membrane-bound molecules, such as IL-12 and CC-chemokine ligand 2 (CCL2), are secreted, referred to as third signal (see Fig. 1.5) [21]. The strength of stimulation that T cells receive is dependent upon three factors: First, the amount of peptide-loaded MHC/TCR complexes for the initiation of the signal; second, the amount of co-stimulatory molecules that bind to CD28 for signal amplification and third, the duration of the T cell-DC interaction [26].

1 INTRODUCTION

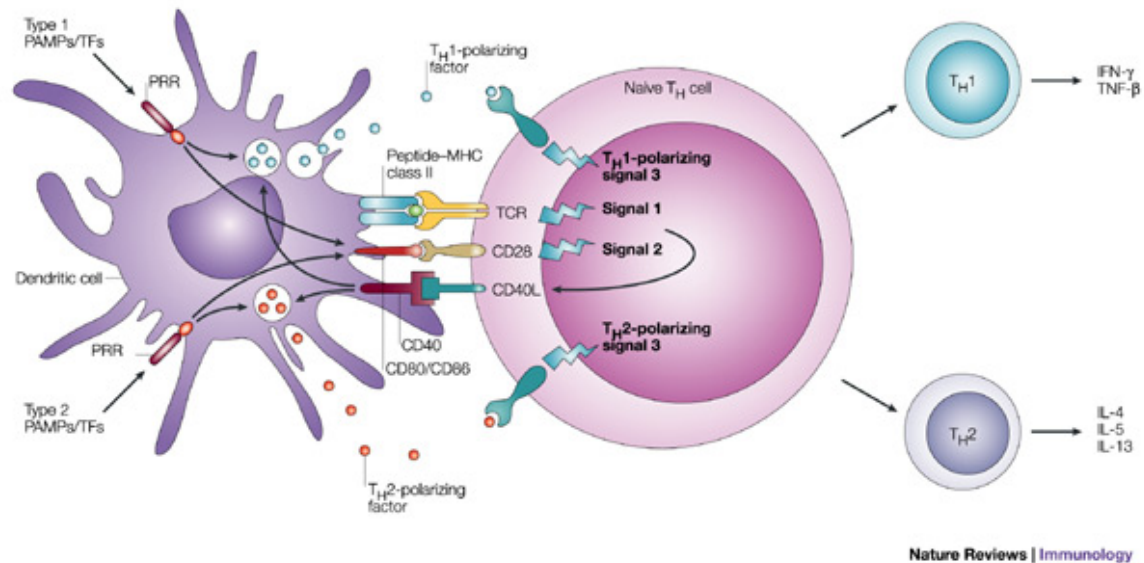


Figure 1.5: Formation of an immunological synapse leads to T cell activation. DCs activate T cells by forming an immunological synapse. Three dendritic cell-derived signals are necessary for a proper T cell activation. Signal 1 is provided by the TCR-MHC interaction, signal 2 is given by the co-stimulatory interaction and signal 3 is provided by the secretion of soluble factors. [21]

Studies using two-photon microscopy *in vivo* were performed to investigate the T cell priming by DCs in lymph nodes (LN). They observed that T cell priming occurs in three distinct phases: During the first 8 hours after entering the LN, T cells made transient serial encounters with DCs, followed by a second phase that lasted 12 hours with stable DC-T cell contacts and enhanced cytokine production. The third phase was characterized by high motility and increased proliferation of T cells [27].

Antigens are displayed either via the MHC II molecules that activate CD4⁺ T cells or via the MHC I molecule recognized by CD8⁺ T cells. Intracellular antigens within the cytosol are processed by proteasomes and displayed via MHC I, whereas extracellular antigens generally are presented via the MHC II [25].

1.2.1 CD4⁺ T cell polarization

Dendritic cells have the capacity to polarize naïve CD4⁺ T cells towards distinct T helper subsets. (see Fig. 1.6) Depending on the maturation stimuli DCs receive, a specific cytokine milieu is created which favors the differentiation into a specific T cell subtype. Additionally the kinetics of DC activation also plays a role in

determining T cell fates. Directly after TLR ligation, the pro-inflammatory cytokines secreted by DCs preferentially trigger a Th1 response, whereas after about 24 hours of stimulation, anti-inflammatory factors promote the development of regulatory T cells (Treg) [23, 24]. Besides the classical T helper subsets Th1 and Th2, many other subsets have been identified in the last years, including Th17, Treg, follicular helper T cell (Tfh), induced T-regulatory cells (iTreg), regulatory type 1 cells (Tr1) and the T helper subset 9 (Th9) [28].

1.2.1.1 Th1 cells

Th1 cells mediate immune responses in particular against intracellular pathogens and play an important role in cellular cytolytic activity. Their secreted cytokines are important for the cross-talk between immune cells. IFN- γ produced by Th1 cells activates macrophages. Interleukin-2 (IL-2) is important for stimulation of CD8⁺ T cells and formation of CD8⁺ and CD4⁺ cell memory [29, 30].

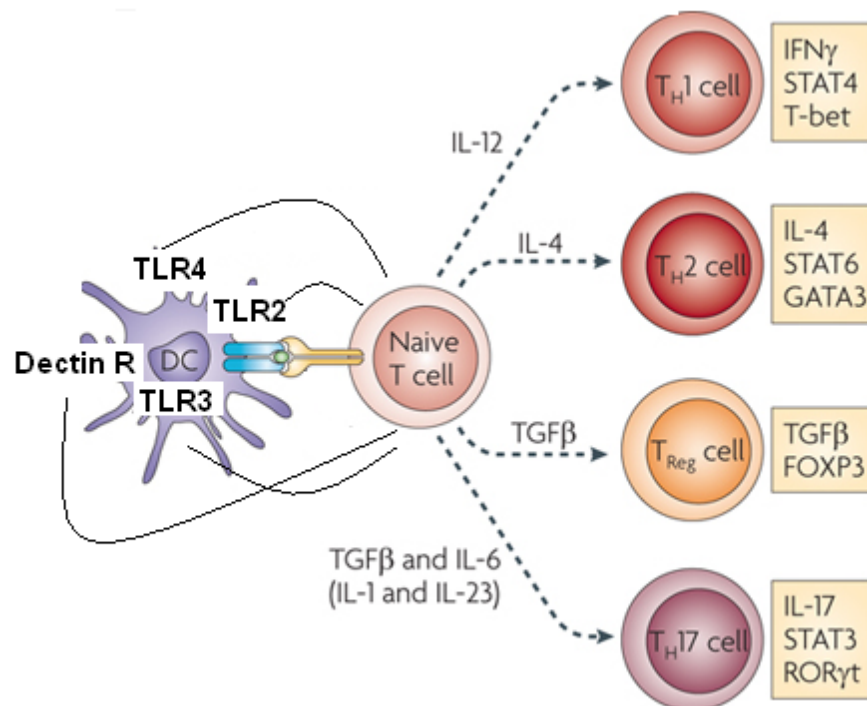


Figure 1.6: Polarization of naïve CD4⁺ T cells. Depending on the cytokine microenvironment provided by DCs, naïve CD4⁺ T cells are primed towards distinct T cell subsets; T_H1, T_H2, T_H17 and regulatory T cells.

1 INTRODUCTION

The key cytokines which are relevant for Th1 polarization are IL-12 and Interferon (IFN)- γ . Upon TLR4 as well as TLR3 ligation, DCs secrete IL-12 in high levels and additional presence of IFN- γ from other cells as natural killer cells (NK) supports the Th1 development. The transcription factor that is pivotal for the induction of the Th1 differentiation program, while suppressing the development of other T cell subsets, is T-box transcription factor (T-bet) [21, 28].

1.2.1.2 Th17 cells

Th17 cells are rather newly characterized T helper subsets. They are crucial for the elimination of extracellular bacteria and fungi but are also responsible for many autoimmune diseases [30]. Some studies have suggested that Th17 cells might mediate anti-tumor immunity by facilitating the recruitment of other effector immune cells. However, the promotion of tumor growth by Th17 cells has also been reported [31].

Th17-polarizing factors are mostly transforming growth factor β (TGF- β) and the cytokines IL-6, IL-23 and IL-1 β . Th17 cells produce IL-17a, IL-17f, IL-21, and IL-22. Retinoic acid-related orphan receptors (ROR) α and γ determine the Th17 cell fate [30].

1.2.1.3 Treg cells

Treg cells are important for the maintenance of tolerance to self and foreign antigen. They suppress immune responses in order to avoid immunopathology and re-establish immune homeostasis following pathogen elimination [29]. Cancer patients often have elevated numbers of Treg cells in the peripheral blood, which downregulate the anti-tumor immunity [32].

The master regulator of the Treg cell development is forkhead box P3 (FoxP3), which is specifically and highly expressed in Treg cells. Suppression of the immune system is especially mediated by following factors: IL-10, IL-35, granzyme B, IL-9, and TGF- β . Treg cells not only negatively affect the proliferation of effector T cells but they may also prevent maturation and co-stimulatory capacity of DCs with their ligand lymphocyte activation gene (LAG)-

that binds with high affinity to MHC II [33]. Interestingly, there seems to be a dynamic interaction between Th17 and Treg cells. It depends whether TGF- β acts alone or together with IL-6 and IL-21. Studies have shown that retinoic acid, a vitamin A metabolite, inhibits the generation of Th17 cells while enhancing the generation of Treg cells. It shows that a common metabolite like retinoic acid can regulate the balance between pro-inflammatory Th17 cells and anti-inflammatory Treg cells [34].

1.2.2 CD8⁺ T lymphocytes

CD8⁺ T cells contribute to immunological protection by eliminating intracellular pathogens. Under certain conditions, for example during strong innate immune responses, the help of CD4⁺ T helper cells is not necessarily needed for initiating an efficient cytotoxic CD8⁺ T cell response. The help of CD4⁺ T cells is of bigger importance during chronic viral infections and rejection of tumors, all of which elicit a weaker innate immune response. Multiple factors, as interactions between T cells and DCs, the inflammatory milieu, co stimulatory requirements and duration and strength of antigenic stimulation, influence the quality and extent of a CD8⁺ T cell response [10, 35]. CD8⁺ T cells proliferate rapidly and expand up to 10⁵-fold within a week and fulfill their effector functions through the production of cytokines such as IFN- γ and tumor necrosis factor (TNF) and/ or by cytolytic mechanisms [36].

1.3 Role of metabolism in the immune system

The interplay between the immune system and the metabolism, also termed “immunometabolism”, is becoming increasingly important in understanding diverse health disorders but also immune regulatory phenomena. The two primordially distinct systems have a lot in common; the modular architecture (sensing and transducing signals), challenges (confrontation with the external environment) and biological goals (maintaining the internal milieu) [37]. The study of immunometabolism incorporates the effects of immune cells on the whole-

1 INTRODUCTION

body metabolism but also the role of metabolic pathways determining immune cell functions [2].

The interconnectivity between immunity and metabolism is especially well studied in the white adipose tissue, the site of mammalian energy storage. In the adipose tissue, uptake and storage of glucose and fatty acids is regulated by the insulin signaling axis. Macrophages actively promote adipocyte insulin sensitivity by secreting IL-10 and other molecules. However, overnutrition causes cellular stress and dysfunction leading to the activation of various signaling cascades, as JNK and IKK- β pathways. These signaling pathways inhibit further nutrient uptake and initiate inflammation and insulin resistance by the recruitment of alternatively activated macrophages with an inflammatory phenotype. The regulatory macrophage population is overwhelmed by the huge number of recruited inflammatory macrophages [37]. Cancer cells also make use of metabolic enzymes, as IDO (indole-amine 2, 3-dioxygenase), to escape the tumor-specific immunity by depriving T cells of tryptophan which induces T cell tolerance [38].

1.3.1 Regulation of Immune Responses by metabolism

Cellular processes, as proliferation and production of cytokines, require energy but also the production of nutrients to produce a new cell. Most differentiated healthy cells perform glycolysis with the integrated tricarboxylic acid (TCA) cycle to fuel oxidative phosphorylation under aerobic conditions. This process leads to the highest yield of adenosine triphosphate (ATP), with minimal production of lactate.

Under anaerobic conditions, cells can produce ATP with pyruvate being converted directly to lactate after glycolysis without proceeding to TCA cycle and oxidative phosphorylation, which is referred as anaerobic glycolysis. Anaerobic glycolysis generates a low yield of ATP compared with oxidative phosphorylation. Otto Warburg revealed that cancer cells preferentially use glycolysis to produce large quantities of lactate even in the presence of oxygen, therefore referred as aerobic glycolysis or Warburg effect (see Fig. 1.7) [2, 4]. Before Warburg's discovery, glycolysis was described as an anaerobic process.

1.3 Role of metabolism in the immune system

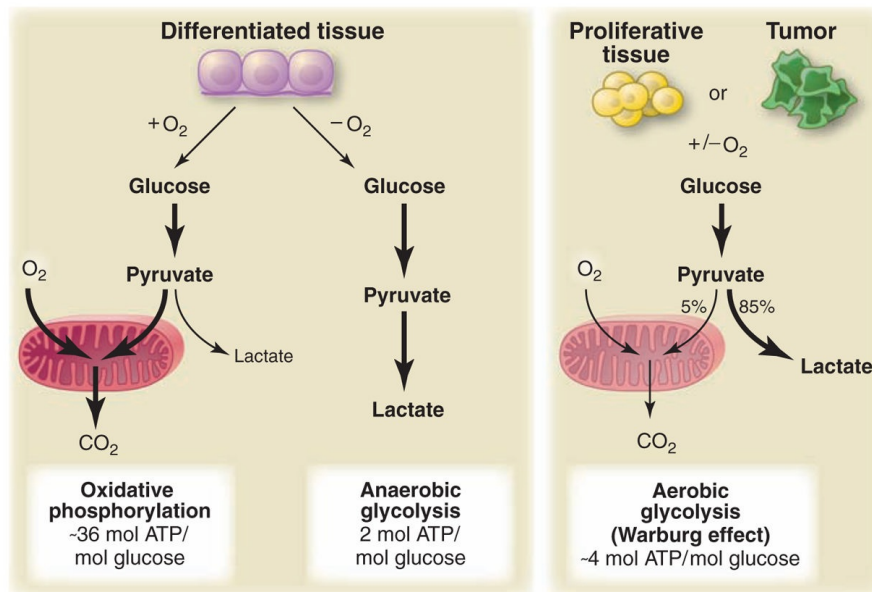


Figure 1.7: Schematic representation of oxidative phosphorylation, anaerobic and aerobic (Warburg effect) glycolysis. Differentiated cells primarily rely on oxidative phosphorylation to produce energy; whereas cancer cells but also proliferating cells instead rely on aerobic glycolysis for metabolite production [4].

Studies have shown that leukocytes also rely on aerobic glycolysis, similarly to the Warburg effect in cancer cells. Depending on whether cells are naïve, activated, antigen-experienced or anergic, cell's metabolism must adapt to follow these changes [2, 39, 40]. While activated effector T cells increase glucose metabolism by switching to aerobic glycolysis, regulatory [39] and memory T cells rather maintain a catabolic metabolism [2]. The observation that cancer cells and activated T cells rely on aerobic glycolysis, indicated that this process is necessary for cellular proliferation. However, it was proven that many innate immune cells, as dendritic cells and granulocytes, use the Warburg metabolism upon activation without proliferating [2]. Cells must constantly deal with environmental changes. Therefore it is important to consider that nutrients, substrates as well as growth factors or other mediators influence the immune cell metabolism favorable or unfavorable. (see Fig. 1.8)

1 INTRODUCTION

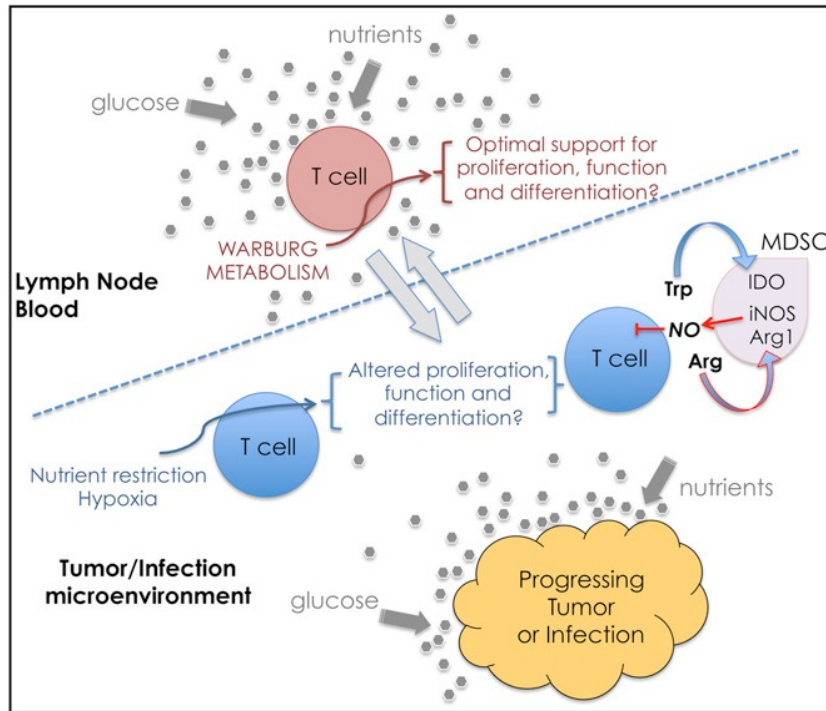


Figure 1.8: Suppressive tumor microenvironments affect proper T cell functionality. T cells require nutrients and other supportive signals for a proper activation. Tumors and sites of infections can inhibit T cell activation due to metabolic restrictions, inhibitory metabolic signals or recruitment of suppressive myeloid cells. [2]

As already mentioned above, a good example is the tryptophan catabolism used by DCs or cancer cells that negatively affects T cell functions [2]. Interestingly, the L-tryptophan metabolites secreted within the kynurenine pathway, are toxic to thymocytes and Th1 but not to Th2. This phenomenon shows that some cells are more susceptible to the regulation by some metabolites than others. This regulation might possibly occur to establish a specific immune cell response [41]. Myeloid suppressor cells (MSCs) might affect the activation of T cells by expressing the enzymes nitric-oxide synthase (NOS) and arginase (ARG), which catabolize the amino acid L-arginin [42]. Metabolic intermediates exiting the TCA cycle, as succinate, promote chemotaxis and support activation triggered by TLR agonists in DCs [2]. How metabolic pathways or metabolites act on immune cell functions still needs to be explored, since nothing is currently known about the metabolic state of some immune cells. Nevertheless there is great evidence that

the interplay of metabolism and immunity plays an important role in defeating some diseases and therefore opens new therapeutic avenues.

1.3.2 Immune-responsive gene 1 (IRG-1)

The IRG1 gene was first described in 1995 by Lee et al. in a study to identify genes which were expressed in macrophages upon activation with bacterial LPS [43]. Sequence analysis revealed that the IRG1 protein sequence is well conserved among many species; as mouse, rat, human and *Xenopus*. Mouse and human IRG1 amino acid sequences share more than 83% identity. Moreover, IRG1 was found to be orthologous to the bacterial metabolite enzyme, methylcitrate dehydrogenase (PrpD), which is responsible for converting 2-methylcitrate to 2-methylisocitrate within the methylcitrate cycle (see Fig. 1.9) [44].

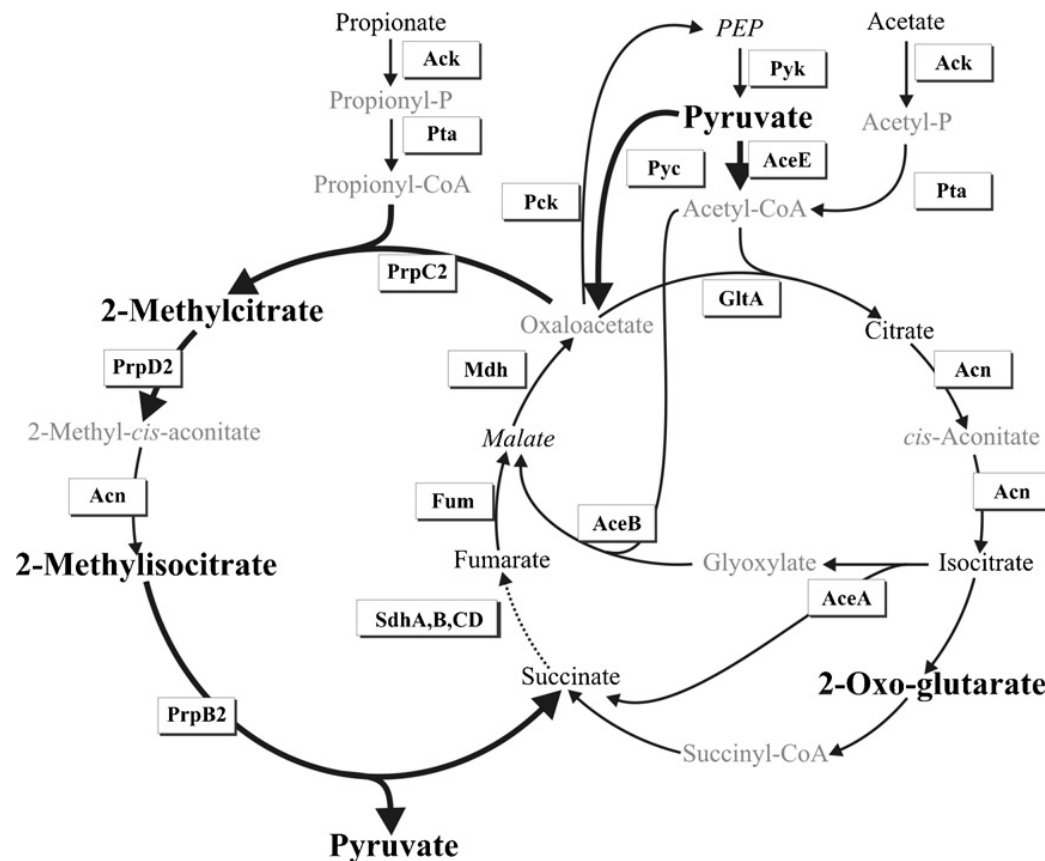


Figure 1.9: Metabolic map of the methylcitrate cycle and tricarboxylic acid cycle. Depending on the carbon source (acetate, propionate) bacteria utilize, different pathways become active. [45]

IRG1 was not only found to be highly expressed in peripheral blood mononuclear cells (PBMCs) isolated from human fetal tissue samples [46] but also in the mouse uterus at the time of implantation [44, 47]. Several studies addressed the question of how IRG1 protein might be regulated at the molecular level and which functional role it might have in an organism. In the context of immune responses not many studies are published until now. But it was shown by Degrandi et al. that IRG1 protein is associated with mitochondria and its expression is induced by several pro-inflammatory cytokines, in particular by IFN- γ and TNF, as well as by TLR agonists in activated murine macrophages [48]. The induction of a specific signaling pathway leading to IRG1 expression was not reported until now.

Recently it was revealed that IRG1, as an enzyme, catalyzes the formation of itaconic acid by decarboxylating cis-aconitate. Furthermore, itaconic acid produced by immune cells was shown to exhibit an antimicrobial activity. Thus, IRG1, by being linked to the TCA cycle and catalyzing the production of an antimicrobial metabolite, seems to have a crucial role during immune responses [49].

1.3.2.1 Preliminary IRG1 study

Previous expression profiling [50] and RNA interference experiments in human DCs (see Fig. 1.10) have identified various proteins, including IRG1, which might be crucial for the switch of DCs from the immune stimulatory to the immune-suppressive mode as described in 1.1.3. IRG1 was found to be highly expressed in human DCs after activation (data not shown). Moreover, co-cultivation of IRG1 siRNA-silenced DCs resulted in higher proliferation of CD4⁺ and CD8⁺ T cells compared to control silenced DCs (see Fig. 1.10).

1.3 Role of metabolism in the immune system

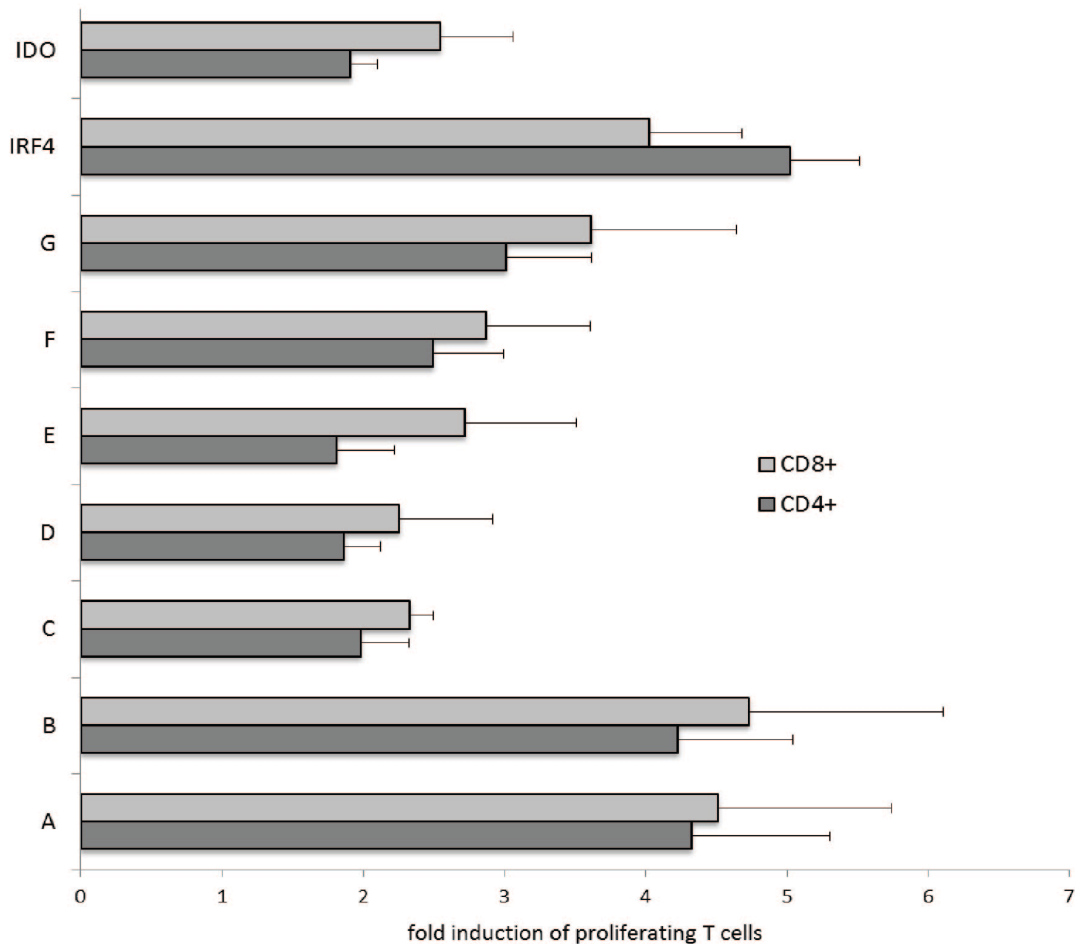


Figure1.10: siRNA-mediated silencing of selected candidate genes in human DCs. Knockdown of several potential immune suppressive genes in DCs resulted in a higher proliferation of CD4⁺ and CD8⁺ T cells in a co-cultivation setup of human T cells and DCs over 6 days. One of this siRNA silenced genes was IRG1, illustrated here as gene A. The ratio of absolute numbers of proliferating T cells activated with target silenced and control silenced DCs (non targeting siRNA) is shown. IDO and IRF4 – silenced DCs were used as a positive control. Bars indicate $\pm$ standard deviation of three independently analyzed experiments. *Figure by Alexander M. Dohnal et al., Laboratory for Tumor Immunology, CCRI, Vienna, Austria.*

2 WORKING HYPOTHESIS and AIMS

Based on the preliminary results, we hypothesize that IRG1 might be involved in immune suppression mediated by DCs. Furthermore, since IRG1 has strong homology to the bacterial enzyme PrpD, the immune suppressive effect might be triggered by soluble metabolites released by DCs.

The aim of this master thesis was to investigate the proposed immune suppressive function of IRG1 in murine DCs regarding their stimulatory potential on CD4⁺ and CD8⁺ T cells. Additionally, we explored the regulation of IRG1 expression in DCs.

For this purpose, the study aims to fulfill following objectives:

- Examination of IRG1 expression regulated by several cytokines and TLR agonists on mRNA and protein level in DCs
- Examination of how supernatants of/or IRG1-silenced DCs itself affect CD8⁺ T cell proliferation and apoptosis
- Examination of the way supernatants of IRG1-silenced DCs influence differentiation of naïve CD4⁺ T cells
- Analyses of supernatants of IRG1-silenced and control-silenced DCs for metabolites using high performance liquid chromatography (HPLC) in combination with mass spectroscopy (MS)

3 MATERIALS and METHODS

3.1 Mice

For the generation of bone marrow-derived dendritic cells (BM-DCs), C57BL/6N mice were purchased from Charles River and housed in the specific pathogen free (SPF) animal care unit at the Center of Biomedical Research, Medical University of Vienna, Austria. For the isolation of T cells, OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl) and OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) transgenic mice were purchased from the Department of Pharmacology, Medical University of Vienna.

3.2 Cell culture

3.2.1 Isolation and preparation of murine bone marrow-derived dendritic cells (BM-DCs)

Murine dendritic cells (DCs) were generated from wild-type C57BL/6N mice. Bone marrow cells were harvested from femurs and tibiae by rinsing the bone marrow and resuspending the cells in Iscove's Modified Dulbecco's Medium - IMDM (GIBCO by Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 10 ml/L penicillin/ streptomycin 100x (PAA, Pasching, Austria), 10 ml/L MEM non-essential amino acids 100x (GIBCO by Life Technologies, Paisley, UK) and 0.0002% β -mercaptoethanol (Sigma-Aldrich, Vienna, Austria) in following referred to as complete IMDM medium. After centrifugation (460 g, 7 min, 4°C), red blood cells were lysed in a hypotonic buffer, also referred as erylisis buffer (see table 3.1), on ice for 1-2 minutes.

Table 3.1 Erylisis buffer composition

Concentration	Substance	Company
0.15 M	NH ₄ Cl	ROTH, Germany
10 mM	KHCO ₃	MERCK, Germany
0.1 mM	Na ₂ EDTA	ROTH, Germany

3 MATERIALS and METHODS

Bone marrow cells were plated at a density of $0.3 - 0.5 \times 10^6$ cells/ cm^2 ml and cultivated for 6 days in complete IMDM medium supplemented with 1500 U/ ml recombinant murine GM-CSF (BD Pharmingen, San Diego, USA) and 50 U/ ml recombinant murine IL-4 (eBioscience, San Diego, USA). On days 3 and 5, 30 ml medium was exchanged by fresh complete IMDM medium supplemented with the same cytokines as on day 1.

3.2.2 DC transfection

Selective gene silencing using RNA interference (RNAi) was performed using the lipid-based transfection reagent DharmaFECT 1 (Thermo Scientific Dharmacon, USA). In brief, $4, 8 \times 10^6$ immatureDC/ 3, 6 ml complete medium without antibiotics were plated in a 21, 29 cm^2 petri dish. Four solutions were prepared, one containing the ON-TARGETplusSMARTpool, mouse IRG1 siRNA (solution B), second containing the non targeting siRNA control (solution C), and the last two containing the transfection reagent (solutions A), each with a total volume of 600 μl reduced serum medium (OptiMEM, Gibco, Invitrogen, Austria). After an incubation time of 5 min at room temperature (RT), the siRNA containing solutions B and C were each mixed with solution A, which contained the transfection reagent. Both solutions B and C were then incubated for another 30 min at RT. The mixed transfection solutions B and C were then added to the petri dishes containing the DC; petri dish B (on-target IRG1 siRNA) and C (non targeting control siRNA) to obtain an end concentration of 100nM siRNA. One petri dish with DC was left untransfected and only treated with the reduced serum medium as a control. DCs were then incubated over night (o/n) for around 14-16 hours at 37°C and 5% CO_2 .

Table 3.2: siRNAs used for the transfection procedure of DCs

siRNA	Target Sequence
ON-TARGET plus SMART pool, mouse IRG1	
J-048308-05	GCACAGAAGUGUCCAUAA
J-048308-06	GAAAUAAAGCAUCACUCUAA
J-048308-07	GAGCUUUGCUCCUAUCAUU
J-048308-08	GAAAGUGAACCACCUGACA
ON-TARGETplus Control siRNA	Not available

3.2.3 DC maturation

For co-culture assays, transfected DCs were loaded with 1 µg/ ml of MHC class I (H2-K^b restricted) OVA-(257-264)-peptide (SIINFEKL) or MHC class II OVA-(323-399)-peptide (from Bachem, Bubendorf, Switzerland) for 30 minutes before maturation. OVA/SIINFEKL-pulsed DC were further activated with 100 ng/ml LPS, 20 ng/ ml IFN-γ (BD Pharmingen, San Diego, USA) or a combination of IFN- γ and 100ng/ ml TNF(Pepro Tech, Rocky Hill, USA) at 37°C and 5% CO₂ for 4 hours. After four hours of stimulation the medium was removed and fresh complete medium without antibiotics was added and the cells were cultured for another 20 hours. IRG1 siRNA silencing efficiency was assessed using Western Blot analysis.

For time kinetics experiments, immature DCs were harvested on day 6 and plated at a density of 1 x 10⁶ cells/ cm² ml. In some experiments JAK 1 inhibition was performed by adding the JAK1 inhibitor (JAKAFI- Ruxolitinib – Selleck Chemicals, USA) at a concentration of 10 µM in complete medium prior to maturation. As a vehicle control, DCs were treated with the same amount of dimethylsulfoxide (DMSO – WAK-Chemie Medical GmbH, Steinbach, Germany).

DC maturation was induced by treating the cells with one or a combination of 2 following TLR ligands/cytokines: 10 µg/ ml polyinosinic:polycytidylic acid (Poly (I:C) – Sigma-Aldrich, St. Louis, USA), 100 ng/ ml lipopolysaccharide (LPS – E. coli strain O111:B4, Calbiochem – EMD Chemicals, San Diego, USA), 10 µg/ ml flagellin (FLA – InvivoGen, San Diego, USA), 2,0-2,5 µg/ ml resiquimod (R848 – Santa Cruz Biotechnology, Santa Cruz, USA), 20 ng/ ml IFN-γ, 100 ng/ ml TNF, 50 – 500 ng/ ml IL-6 and 500 ng/ ml IL-10.

Table 3.3: TLR ligands and cytokines used as stimuli for DC activation

stimuli	endconcentration
TLR ligands	
Poly (I:C)	10 µg/ ml
LPS	100 ng/ ml
FLA	10 µg/ ml
R848	2,0-2,5 µg/ ml
cytokines	
IFN-γ	20 ng/ ml
TNF	100 ng/ ml
IL-6	50 – 500 ng/ ml
IL-10	500 ng/ ml

3.2.4 CD4⁺ and CD8⁺ T cell isolation and activation

Murine spleenocytes were gained by smashing spleens of wild type C57BL/ 6N, as well as of OT-I and OT-II transgenic mice on a 70 µm cell strainer with a syringe plunger. The cells were resuspended in complete medium and disturbing connective-/fatty tissue was removed. Following erylisis (see table 3.1), CD4⁺ or CD8⁺ T cells were isolated using MACS® T cell isolation kits (MiltenyiBiotec, BergischGladbach, Germany) by depletion of non-CD8⁺ or non-CD4⁺ (negative selection) T cells. The magnetically labeled non-CD8⁺/ non-CD4⁺ T cells are depleted by retaining them on a MACS® Column (LS column) in the magnetic field of a MACS® Separator (MidiMACS magnets), while the unlabeled CD8⁺ and CD4⁺ T cells pass the column. For details see the MACS® T cell Isolation Kit data sheet. T cells, cultured without any antigen or antigen presenting cells, were activated by the addition of 4 x 10⁴ beads coupled to anti-CD3 and anti-CD28 antibodies (Dynabeads® Mouse T-Activator CD3/CD28 – GIBCO by Life Technologies, Oslo, Norway) per 100 000 T cells.

3.2.5 CFSE labeling of T cells

Carboxyfluoresceindiacetatesuccinimidyl ester (CFSE – Invitrogen, Lofer, Austria) staining was performed for determining the proliferation rate of T cells after several days of activation. Spleenocytes or T cells were resuspended in Dulbecco's PBS (PAA, Pasching, Austria) supplemented with 0, 1% bovine

serum albumin (BSA – Sigma-Aldrich, St. Louis, USA) in a concentration of 1×10^7 cells/ ml. The cells were then stained with 0, 7 μ M CFSE for 10 min at 37°C and 5% CO₂ by repeated shaking. Immediately thereafter, FCS was added in the same quantity as PBS before and incubated for 5 min at RT. At last, complete medium was added and the cells were centrifuged (460 g, 7 min, 4°C) and resuspended again in complete medium. The cell number of CFSE stained cells was determined by flow cytometry.

3.2.6 T cell proliferation assays

For DC/ T cell co-cultures, DCs that were siRNA transfected or untransfected, (see 1.1.2) were stimulated for 4 hours with LPS as described above. These pre-matured DCs were harvested and mixed with a fixed number of 5×10^4 CFSE-labeled CD8⁺ T cells at defined DC/T cell ratios (see table 3.4) in triplicates. In addition as a negative control, CFSE-labeled CD8⁺ T cells were cultured in the absence of DCs to assess background proliferation. The cells were incubated for 3 days in round-bottom 96-well culture plates at 37°C and 5% CO₂. The absolute number of proliferating T cells was assessed using Trucount™ system (BDBiosciences, San Jose, USA) by analyzing CFSE dilution mediated by cell division.

Table 3.4: Co-culture conditions of DCs and T cells

Ratio	DC number	T cell number	Total Volume
1:1	50 000	50 000	200 μ l
1:2	25 000	50 000	200 μ l
1:8	6 250	50 000	200 μ l

Additionally we assessed the effects of cell-free supernatants of IFN- γ stimulated IRG1 silenced or control silenced DCs on the proliferation of CD8⁺ T cells. For this, CFSE-labeled CD8⁺ T cells were treated with different dosages of cell-free supernatant of DCs which were either IRG1 silenced, control silenced or untransfected. (see table 3.5) T cells were stimulated with CD3/CD28 T-Activator Dynabeads as previously described in 3.2.4 and incubated for 3 days in round-bottom 96-well culture plates at 37°C and 5% CO₂. T cell proliferation was analyzed by flow cytometry the same way as in the DC/ T cell cultures.

3 MATERIALS and METHODS

Table 3.5: Supernatant dosages used for T cell incubation

Supernatant dosage	CD3/CD28 Dynabeads	T cell number	Total Volume
50 μ l	0.5 μ l/ 50 000 cells	50 000	200 μ l
100 μ l	0.5 μ l/ 50 000 cells	50 000	200 μ l
150 μ l	0.5 μ l/ 50 000 cells	50 000	200 μ l

3.2.7 In vitro apoptosis of effector T cells

CD8⁺ T cells were treated with 150 μ l of cell-free supernatants of IFN- γ stimulated IRG1 silenced or control silenced DCs and exogenously stimulated with CD3/CD28 T-Activator Dynabeads (see 3.2.4). The cells were incubated for 3 days in round-bottom 96-well culture plates at 37°C and 5% CO₂. For determination of apoptosis, cells were first stained with T cell mix A (see table 3.8) for 10 minutes at 4° and subsequently were stained with 2 μ l of Annexin V and DAPI (4'-6-diamidino-2-phenylindole) in an Annexin V 2x Buffer (BD Pharmingen) for another 10 minutes at 4°C. Following antibody for Annexin V staining was used: anti-Annexin V – APC (BD Pharmingen, San Diego, USA).

3.2.8 CD4⁺ T cell phenotyping by quantitative real time PCR

CD4⁺ T cells were co-cultured with IFN- γ stimulated IRG1 silenced DCs in 100 μ l of cell-free supernatant of either IFN- γ stimulated IRG1 silenced DCs or control silenced DCs (see table 3.6). On day 4, CD4⁺ T cells were harvested and frozen in trizol reagent (Ambion®) on -80°C. These samples were then used for RNA isolation and transcription factor gene expression analysis. (see 3.4.1)

Table 3.6: Co-culture setup for CD4⁺ T cell phenotyping

Ratio	DC number	T cell number	Total Volume
1:2	25 000	50 000	200 μ l

3.3 Flow Cytometry

3.3.1 DC phenotype

Flow cytometry was used to determine the immune phenotypic maturation status as well as cell number of viable DCs. DCs were incubated with 5 μ l of an antibody mix (see table 3.7) in a volume of 100 μ l cell suspension for 15-20 minutes at 4°C in complete darkness. Cells were analyzed using BD™ LSR II flow cytometer (Becton Dickinson, San Diego, USA). Data analysis was performed using FlowJo Flow Cytometry Analysis Software version 10.0.5 (Tree Star, Ashland, USA).

Table 3.7: Antibodies used for DC phenotyping

Antibody	Fluorochrome	Company
anti-CD80	PE-Cy5	eBioscience
anti-CD86	PE	eBioscience
anti-CD83	eFluor®660	eBioscience
anti-MHC I	FITC	eBioscience
anti-MHC II	PerCP-eFluor®710	eBioscience
anti-CD11b	APC-e Fluor®780	eBioscience
anti-CD11c	PE-Cy7	eBioscience

3.3.2 T cell phenotype

T cell phenotype of activated or inactivated T cells was analyzed using T cell mix A (see table 3.8). The staining procedure was the same as for DCs (see 3.3.1) using the TruCount™ system.

Table 3.8: Antibodies used for T cell phenotyping

	Antibody	Fluorochrome	Company
T cell mix A	anti-CD3 ϵ	APC	eBioscience
	anti-CD4	PerCP-Cy5.5	eBioscience
	anti-CD8 α	APC-e Fluor®780	eBioscience
	anti-CD25	PE-Cy7	eBioscience

3.4 Real-time Polymerase Chain Reaction (RT-PCR) Analysis

3.4.1 RNA Isolation

The stored samples in TRIzol® Reagent were thawed on ice and 200 µl of chloroform per 1 ml of TRIzol® Reagent were added for homogenization and incubated at RT. The samples were centrifuged (12 000 g, 15 min, 4°C) using an Eppendorf centrifuge. The mixture separates into three phases as follows; the upper aqueous phase (contains the RNA), the interphase (contains the DNA) and the organic phase (contains proteins and lipids). The upper aqueous phase is carefully removed and mixed with 500 µl of 100% Isopropanol and incubated for 10 min at RT. Subsequently the samples are centrifuged (12 000 g, 15 min, 4°C), the supernatant was discarded and the RNA Pellet was washed twice with 75% ethanol. The last washing step included a short centrifugation step (7 500g, 5 min, 4°C). After the ethanol evaporation, the RNA pellets were resuspended in 15 µl of RNase free water (Applied Biosystems) and incubated at 55–60°C for 10–15 minutes. RNA quantity and purity was measured with the Nanodrop (ND-1000 v3.5.2 software).

3.4.2 cDNA Reverse Transcription (cDNA-RT)

The materials required for the cDNA-RT procedure, were used from the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 1 µg of RNA per sample was mixed with a prepared 2x RT Master Mix (see table 3.9) in a 1:1 ratio. The reverse transcription was performed using a thermal cycler using the conditions shown in table. The cDNA samples were then stored at -20°C.

3.4 Real-time Polymerase Chain Reaction (RT-PCR) Analysis

Table 3.9: Master mix for cDNA reverse transcription

Components	Volume (µl)/reaction
Nuclease free water	3, 2
10XRT buffer	2
10XRT Random primers	2
25x dNTP Mix (100mM)	0, 8
RNase Inhibitor	1
MultiScribe RT	1

Table 3.10: Program for Thermocycler

	Temperature	Time
Step 1	25 °C	10 min
Step 2	37 °C	120 min
Step 3	85 °C	5 min
Step 4	4 °C	∞

3.4.3 Real time PCR (RT-PCR)

Gene expression analyses were performed using reagents from the TaqMan® Gene Expression kit (Applied Biosystems). Depending on the analyses we used different TaqMan® Gene Expression Assays provided by Applied Biosystems. 4 µl each of the cDNA samples were mixed with 16 µl of the prepared PCR reaction mix (see table 3.11) per gene expression assay. Gene expression was conducted on a 7500 Fast Real-Time PCR system cyclers (Applied Biosystems) according to manufacturer's instructions. All reactions were performed in duplicates. The amount of target, normalized to an endogenous reference was calculated by the formula: $2^{-\Delta\Delta CT}$.

Table 3.11: Master Mix for gene expression analysis

Components	Volume 20 µl /reaction
20X TaqMan gene Expression Assay	1 µl
2XTM buffer	10 µl
RNase free water	5 µl

Table 3.12: Gene expression assays used for RT-PCR

Gene expression assay	Product catalog Nr.	Company
Irg1 (Mm01224532_m1)	4331182	Applied Biosystems
Tnf (Mm00443260_g1)	4331182	Applied Biosystems
Ifng (Mm01168134_m1)	4331182	Applied
Tbx21 (Mm00450960_m1)	4331182	BiosystemsApplied
Rorc (Mm01261022_m1)	4331182	Biosystems
Foxp3 (Mm00475162_m1)	4331182	Applied
B2m (Mm00437762_m1)	4331182	BiosystemsApplied
reference gene		Biosystems

3.5 Protein Detection by Western Blot

3.5.1 Sample preparation

Cytoplasmic and nuclear fractions were prepared according to the methodology described in the Nuclei EZ Prep Nuclei Isolation Kit (SIGMA®). Cells were washed twice with Dulbecco's PBS (PAA, Pasching, Austria) and resuspended in 300 µl of Nuclei EZ Lysis Buffer (SIGMA®) and centrifuged in an Eppendorf centrifuge (2000 rpm, 2 min, 4°C). Supernatants that contain the cytoplasmic components are stored at -20°C. The nuclei pellet was washed twice with Dulbecco's PBS (PAA, Pasching, Austria) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) and resuspended in 10 µl Rippa Buffer (see table 3.13) containing the protease inhibitor cocktail as well. The nuclei were frozen at -80°C. Before loading the nuclei samples on the gel, they were thawed and mixed 1:1 with a 2x Novex® Tris-Glycine SDS Sample Buffer (Life Technologies™) supplemented with β-mercaptoethanol. The samples were then boiled at 95°C for 5 minutes and loaded on the gel.

For the preparation of whole cell fractions, DCs were harvested, centrifuged (460g, 7 min, 4°C) and the Pellet was dissolved 1:1 in Dulbecco's PBS (PAA, Pasching, Austria) and 2 x SDS loading buffer (see table 3.13) to a concentration of 30 000 cells per µl. These samples were then boiled at 95°C for 10 minutes, centrifuged (20 000 g, 10 min, 4°C) and frozen at -80°C. Before loading on the gel, the samples were thawed and centrifuged (20 000 g, 10 min, 4°C).

Table 3.13: Reagents used for sample preparation

	Concentration	Substance
Rippa Buffer	10mM	NaPO ₄
	0.3 M	NaCl
	0.1%	SDS
	1%	NP40
	1%	DOC (deoxycholate)
	2mM	EDTA
2 x SDS loading buffer	0.25M	TRIS (pH 6.8)
	3%	SDS
	6%	β-mercaptoethanol
	20%	glycerol
	0.005%	bromophenol blue

3.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples were either loaded onto Mini-PROTEAN® precast 4-15% gradient gels (Bio-Rad Laboratories, Hercules, USA) or on a gel composed of a 5% stacking gel and a 12.5% separating gel (see table 3.14). Additionally to the samples, 5 µl of a PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, Rockford, USA) was loaded.

Settings for electrophoresis: 30 mA (stacking gel)/ 60mA (separating gel) in 1x running buffer (see table 3.15)

Table 3.14: Composition of stacking and separating gel for SDS-PAGE

	5% Stacking gel	12.5% separating gel
40% Acrylamide	623 µl	3.2 ml
H ₂ O	3.6 ml	4.15 ml
1.5M Tris pH 8.8	-	2.5 ml
1M Tris pH 6.8	630 µl	-
20% SDS	25 µl	50 µl
10% APS	50 µl	100 µl
TEMED	5 µl	12 µl

Table 3.15: 10 x Running Buffer composition

Concentration	Substance
0.25M	TRIS
1.92M	glycine
1 %	SDS

3.5.3 Western Blot

Proteins that were separated by SDS-PAGE were further transferred onto Whatman™ Protran nitrocellulose membranes (GE Healthcare, Dassel, Germany) for 1.5 – 2 hours at 300 mA in 1 x transfer buffer on ice (see table 3.16).

Table 3.16: 1 x Transfer Buffer composition

Concentration	Substance
25 mM 187 mM 20%	TRIS glycine methanol

Table 3.17: 1 x TBS composition

Concentration	Substance
50 mM 150 mM	TRIS NaCl

The transfer of proteins on membranes was visualized by Ponceau S (Sigma-Aldrich, Vienna, Austria) staining. The with dH₂O destained membranes were further blocked with 1x Blocking Reagent (Roche Diagnostics, Mannheim, Germany) in 1x TBS (see table 3.17) for one hour at RT or overnight at 4°C. Further on, the membranes were incubated with the primary antibody diluted in 0.5x Blocking Reagent in 0.1% TBS supplemented with Tween® (Sigma-Aldrich, Saint-Quentin Fallavier, France) - TBST- for one hour at RT or overnight at 4°C. At the end of the incubation time, the membranes were washed with 0.3% TBST and subsequently incubated either with the horseradish peroxidase (HRP) -or fluorophore conjugated secondary antibody in 0.5x Blocking Reagent in 0.1% TBST for one hour at RT. The membranes were washed with 0.3% TBST and treated either with FEMTO or PICO chemiluminescent substrate (Thermo Scientific, Rockford, USA) for 5 min. The proteins that were detected via HRP conjugated secondary antibodies were exposed to chemiluminescent detection films (Roche Diagnostics, Mannheim, Germany). Proteins that were detected via fluorophore-conjugated secondary antibodies were analyzed with a LI-COR Odyssey imaging system. Preparation of chemiluminescent substrates: the two available solutions were mixed in a 1:1 ratio.

Table 3.18:Antibodies used for Western Blot

	Antibody	Dilution	Company
1 st Antibody	rabbit - IRG1	1: 1000	Atlas Antibodies, Stockholm, Sweden
	mouse - GAPDH	1 : 20000	Ambion, CA, USA
	mouse - pSTAT3	1 : 1000	Cell Signaling Technology, Denvers, USA
	rabbit - Lamin A/C	1 : 1000	Cell Signaling Technology, Denvers, USA
2 nd Antibody	goat anti-mouse	1 : 50000	Thermo Scientific. Rockford, USA
	goat anti-rabbit (HRP-conjugated)	1 : 50000	Thermo Scientific. Rockford, USA
	goat anti-rabbit (DyLight™ 800)	1: 10000	Pierce Biotechnology, Rockford, USA

3.6 Cytokine Detection

3.6.1 Fluorescent Bead Immunoassay (FlowCytomix™ system)

Cytokines released into the supernatant by DCs 6 and 24 hours upon activation with various TLR ligands (see table 3.3) were measured by flow cytometry using multiplex bead-based assays. The method was carried out using the kit FlowCytomix™ (eBioscience, San Diego, USA) according to manufacturer instructions, except that half of the given dosages of reagents and samples were used. The kit uses multiple bead sets with an internal fluorescent dye. Each of the beads is pre-coated with a unique antibody specific for a single analyte. Each cytokine is detected via and antibody-coated bead, which is subsequently captured by a biotin-conjugated antibody. Addition of Streptavidin-phycoerythrin (PE) is used to measure the different intensities that represent different cytokine concentrations using FACS LSR II flow cytometer. Since the kit also provides a set of standards for each analyte, the intensities can be translated into concentration units using the FlowCytomix™ Pro Software (Bender MedSystems, Vienna, Austria).

3.7 High Performance Liquid Chromatography and Mass spectrometry (HPLC and MS)

Our collaborators at the Institute of Organic Chemistry at the Johannes Kepler University (JKU) profiled our supernatants of IRG1 deficient and IRG1 expressing

3 MATERIALS and METHODS

DCs by HPLC and MS. Following equipment was used for the HPLC; Finnigan Surveyor Autosampler, Finnigan Surveyor MS Pump, Finnigan Surveyor PDA and MS; LCQ Deca XPPlus. Solvent: A: 0,1% (NH₄)₂CO₃ pH 2,75, B: Acetonitril (AcCN)

4 RESULTS

4.1 Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells

The main purpose of this thesis was to investigate the role of the IRG1 protein in DC-based immune regulation. So far, regulation and function of IRG1 during infection, autoimmune diseases or anti-tumor immunity is not known. Previous expression profiling [50] and RNA interference experiments in human DCs (Fig. 1.10) have identified various proteins, including IRG1, which might be crucial for the switch of DCs from an immune stimulatory to an immune-suppressive mode in terms of effector T cell functions. Based on initial data, we predicted IRG1 as an important regulator of an immune suppressive DC phenotype. The results are divided into three parts as follows:

- the regulation of IRG1 in DCs
- the function of IRG1 regarding T cell functions
- the identification of enriched molecules secreted by activated IRG1-expressing compared to IRG1-deficient DCs

In order to investigate these tasks, we used siRNA-mediated gene silencing to target IRG1 in murine wild-type DCs since there was no IRG1 knockout mouse available.

4.1.1 Intracellular localization of IRG1

Determining the localization of proteins within the cell is important for understanding protein functions as well as the development of inhibitors that specifically target the protein. Here, we investigated the intracellular localization of IRG1 protein in murine DCs. We separated the nuclear and cytoplasmic cell fractions of immature and LPS stimulated DCs and compared these to whole cell fractions. The fractions were then applied for Western Blot analysis. As can be seen in Fig. 4.1, IRG1 protein was found only in LPS stimulated cells within the

4 RESULTS

cytoplasm. The purity of subcellular fractions was verified by the detection of pSTAT3 and Lamin A/C as nuclear proteins.

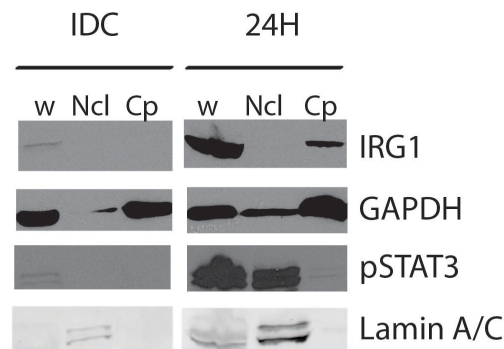


Figure 4.1: Localization of IRG1 in LPS stimulated DCs. DCs were stimulated with LPS for 24 hours. Unstimulated DCs (IDC) served as a control. Cells were harvested at the given time point and nuclear (Ncl), cytoplasm (Cp) and whole cell (W) fractions were produced and applied for Western Blot analysis. GAPDH served as loading control, whereas pSTAT3 and Lamin A/C were used as a control for protein expression within the nucleus.

4.1.2 IRG1 expression in DCs stimulated via TLR ligands and cytokines

It is known that IRG1 is highly expressed after LPS treatment, a TLR4 ligand, in various cell types [43, 46, 48, 51, 52]. TLR4 engagement, leads to the activation of the MyD88-dependent and -independent pathways [14], therefore we especially were interested whether IRG1 expression is also induced by TLR3 ligation with Poly(I:C), in a MyD88 independent manner. In addition we tested whether other TLR ligands as resiquimod (R848), a TLR7/8 ligand or Flagellin, a TLR5 ligand trigger IRG1 expression.

IRG1 expression was compared by real-time PCR and Western Blot analysis. We observed that LPS and R848 are the most potent TLR stimuli leading to the highest IRG1 expression on mRNA level (20-to 25-fold) after 6 hours of stimulation. Induction of IRG1 transcripts was also observed after stimulation with Poly(I:C), though at much lower levels (Fig.4.2A – upper panel). This expression pattern was consistent with the detection of IRG1 protein; LPS and R848 were the strongest IRG1 inducers, whereas Flagellin and Poly(I:C) showed weak IRG1 expression (Fig. 4.2B- upper panel).

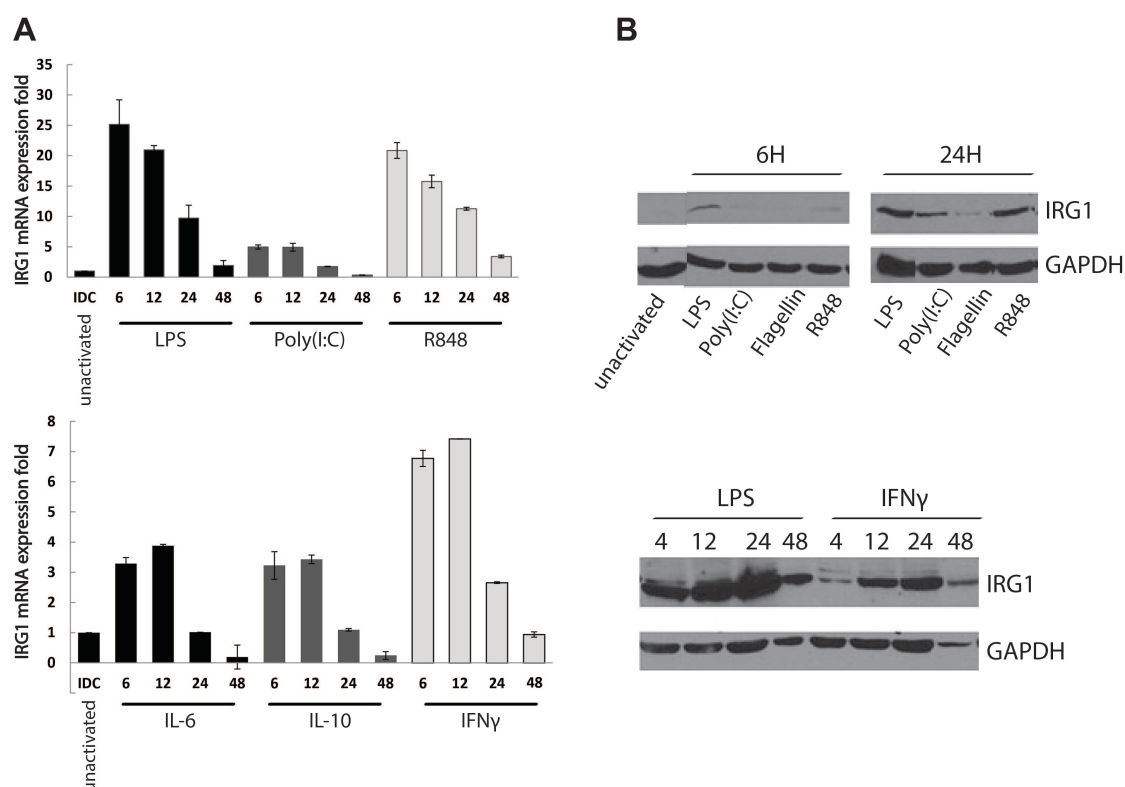


Figure 4.2: IRG1 expression kinetics in DCs stimulated with different TLR ligands and cytokines. (A) IRG1 mRNA was measured by real-time PCR at the indicated time points (6, 12 and 24 hours) in DCs activated with TLR ligands as LPS, Poly(I:C), R848 or the cytokines IL-6, IL-10 and IFN- γ . Beta2microglobulin mRNA was used as a reference control. Error bars indicate variances within technical duplicates. (B) Analysis of IRG1 protein levels in DCs upon TLR stimulation with LPS, Poly(I:C), Flagellin, R848 or IFN- γ at given time points. GAPDH served as a loading control. Shown is one representative experiment using bone marrow derived DCs from 2 mice.

TLR activation represents a primary (immediate) immune response followed by the release of cytokines as a secondary (cytokine-mediated) response. Therefore we tested whether cytokines, as IL-6, IL-10 and IFN- γ themselves, without the action of any TLR ligands, lead to the induction of IRG1.

4 RESULTS

The predominantly pro-inflammatory cytokines IL-6 and IFN- γ , as well as the anti-inflammatory cytokine IL-10 induced IRG1 expression at early time points. IL-6 and IL-10 induced IRG1 mRNA to a similar extent up to approximately 4-fold, while IRG1 mRNA upon IFN- γ stimulation was higher increased up to 8-fold after 6 and 12 hours of stimulation (see Fig. 4.2A– lower panel).

Concerning the kinetics of IRG1 expression, highest IRG1 mRNA levels were detected 6 and 12 hours after stimulation and decreased at later time points. Likewise, IRG1 protein was also detected in a time-dependent manner with its highest expression achieved after 24 hours of stimulation with R848 and LPS. Poly(I:C) and Flagellin were weak IRG1 inducers at all time points (see Fig.4.2B-upper panel). Thus, these data show a correlation between IRG1 mRNA and protein expression levels. Moreover the expression of IRG1 protein at late time points might reflect that IRG1 is present and active during the anti-inflammatory DC mode.

Taken together, IRG1 expression is activated by MyD88-dependent and independent TLR pathways and cytokines (IL-6, IL-10 and IFN- γ), in a time-dependent manner.

Our observation that IRG1 is induced by different TLR ligands as well as cytokines at distinct expression levels raises the question whether secondary autocrine cytokine signaling after TLR ligation triggers TLR-mediated expression of IRG1.

Therefore we treated DCs with the same TLR ligands as in the previous experiments; LPS, R848, Poly(I:C) and FLA, and collected the supernatants after 6 and 24 hours of stimulation and measured the level of cytokines secreted from DCs. Various TLR ligands show a distinct cytokine profile upon TLR ligation (Fig. 4.3).

4.1 Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells

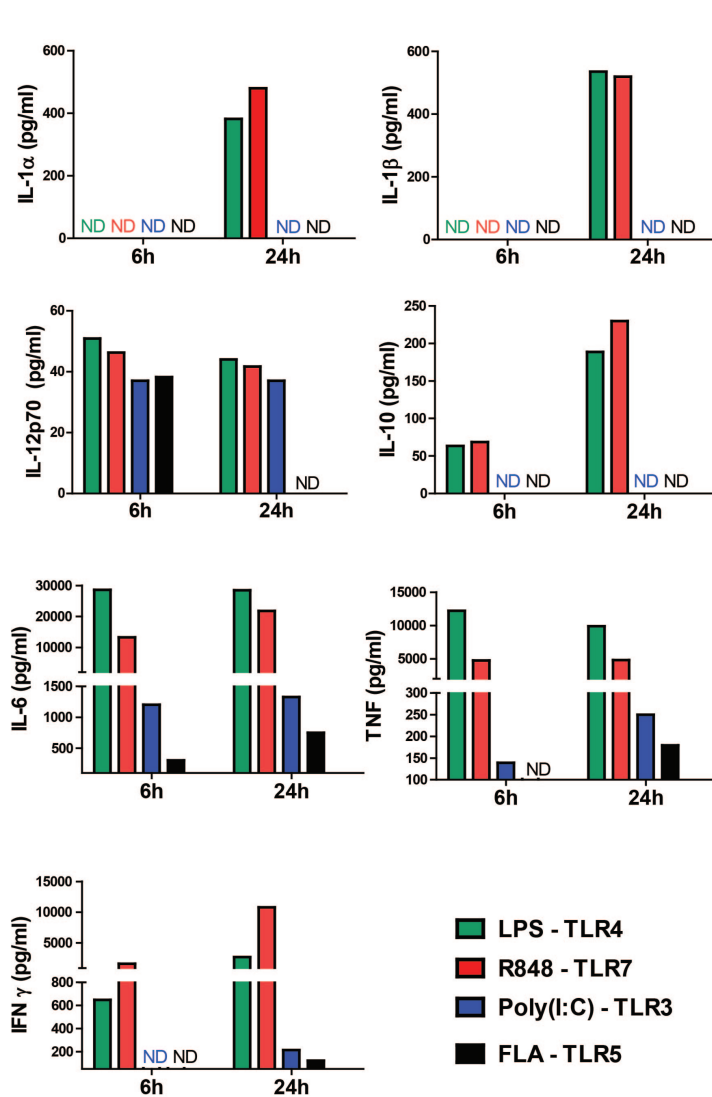


Figure 4.3: Production of cytokines upon TLR ligation.

The supernatant of DCs stimulated with various TLR ligands (LPS, R848, Poly(I:C), FLA) was collected after 6 and 24 hours of stimulation and the concentration of cytokines (IL-1α, IL-1β, IL-12p70, IL10, IL-6, TNF, IFN-γ) was measured using FlowCytomix™ Bead-based assay. ND: not detectable

When we stimulated DCs with LPS and R848, comparable amounts of cytokines were detected after 6 and 24 hours. The highest levels of IL-6, TNF and IFN-γ, were secreted upon LPS and R848 stimulation, whereas DCs exposed to Poly(I:C) and FLA have weaker abilities to induce these cytokines.

These results can be linked to our previous findings, which showed highest IRG1 expression upon TLR4 and TLR7/8 ligation on mRNA as well as on protein level compared to the other TLR ligands. Thus we propose a dominant cytokine mediated IRG1 induction after LPS or R848 stimulation.

4.1.3 JAK/STAT mediated expression of IRG1 in DCs

Upregulation of IRG1 likely due to TLR-induced cytokine secretion in DCs guided us to further investigate cytokine receptor signaling in more detail. Janus kinases (JAK) are crucial for cytokine receptor signaling during immune responses which triggers the JAK-STAT pathway [53].

In order to inhibit the Janus kinases JAK1 and JAK2, which mediate the signaling of a number of cytokines, we made use of a clinically approved kinase inhibitor JAKAFI, also known as Ruxolitinib.

By Blocking JAK1 and JAK2 we prevented the stimulatory action of cytokines mediated by its appropriate receptors. DCs were stimulated with the cytokines IL-6, IL-10 and IFN- γ , known to induce IRG1 (see Fig.4.4 A-upper panel) in the presence of JAKAFI or DMSO, used as the vehicle control and harvested after 24 hours. Additionally DCs were treated with different TLR ligands in the presence of JAKAFI or DMSO in order to show TLR-induced cytokine signaling by acting on the JAK-STAT pathway (Fig.4.4 A-lower panel).

Cytokine mediated induction of IRG1 protein was abrogated in the presence of JAKAFI, whereas TLR-induced IRG1 expression was only slightly affected. These findings underline the importance of cytokines in the induction of IRG1 expression.

Since IRG1 expression was not completely abrogated upon TLR ligation in the presence of JAKAFI, it was not clear whether other cytokines, as TNF, that do not act through the JAK-STAT pathway, are responsible for triggering IRG1 expression or rather primary TLR signaling without the action of cytokines contributes to IRG1 induction.

4.1 Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells

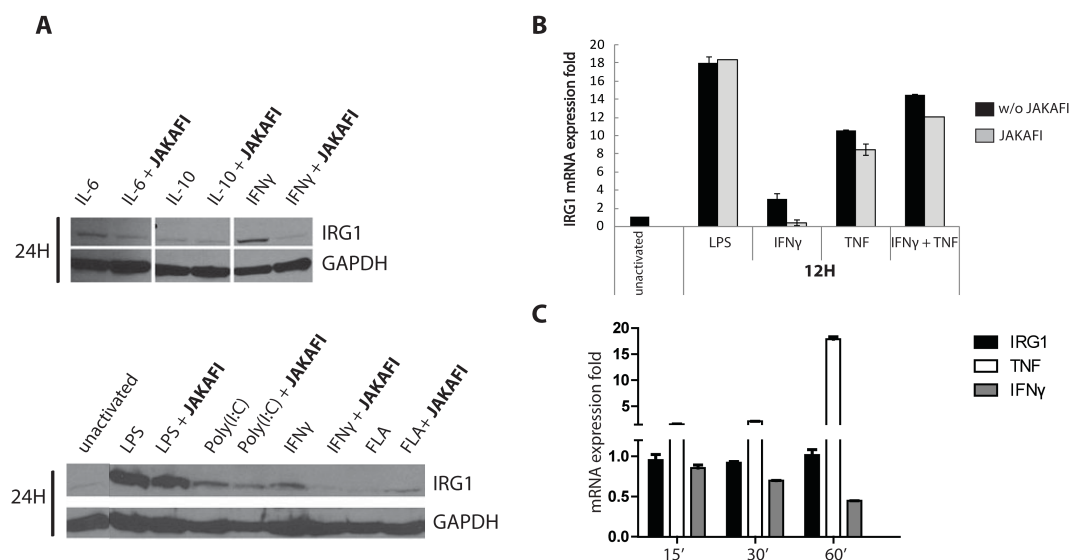


Figure 4.4: Effect of JAK1/JAK2 inhibition on IRG1 expression. (A) Protein expression of IRG1 in DCs that were stimulated with either cytokines (IL-6, IL-10, IFN- γ) or TLR ligands (LPS, Poly(I:C), FLA) for 24 hours in the presence of a JAK1/2 inhibitor (JAKAFI). As a vehicle control, DCs were treated with the same amount of DMSO solvent. **(B)** Real-time PCR was performed to assess IRG1 mRNA transcription after 12 hours of stimulation with LPS, IFN- γ , TNF and a combination of IFN- γ and TNF incubated with either JAKAFI or vehicle control. **(C)** Expression of IRG1, TNF and IFN- γ mRNA was measured by real-time PCR at early time points (15', 30' and 60') after stimulation of DCs with LPS.

The results prompted us to investigate whether IRG1 expression could be triggered after stimulation with TNF. Hence, DCs were stimulated with TNF, IFN- γ or a combination of both cytokines additionally treated or untreated with JAKAFI for 12 hours and subsequently IRG1 transcripts were measured by real-time PCR. LPS stimulated DCs were used as a control (Fig. 4.4 B).

TNF strongly induced IRG1 mRNA and a synergistic effect of IFN- γ and TNF on IRG1 expression was observed. Notably, the presence of JAKAFI hardly affected IRG1 expression induced by TNF (see Fig. 4.4 B), suggesting that TLR induced IRG1 expression might be the result of TNF mediated signaling. Based on this observation we next investigated whether TNF mRNA is upregulated earlier than IRG1 mRNA, shortly after LPS activation, in order to exclude the possibility of direct TLR mediated IRG1 expression.

4 RESULTS

As indicated in Fig. 4.4 C, TNF mRNA can be detected 15 minutes after LPS stimulation and increases up to 20 fold after one hour of stimulation, whereas IRG1 expression is barely detectable at this time point.

Taken together, these results indicate that IRG1 expression might be the result of the activation of secondary cytokine-mediated signaling pathways (as TNF mediated signaling) upon TLR ligation.

4.1.4 Effect of IRG1 silencing on DCs

Gene silencing through the use of siRNA has become a primary tool for characterizing genes involved in different pathways and diseases. Since all protein-protein interactions within cells are part of integrated networks, the loss of one gene can have an impact on cell viability, communication and phenotype. As mentioned earlier, in our assays we used siRNA-mediated gene silencing to achieve a knockdown of our gene of interest, IRG1, in order to investigate its role in immune responses.

We evaluated the expression of co stimulatory molecules (CD80 and CD86), activation marker (CD83) and molecules associated with antigen presentation as MHC class I and II under IRG1 deficient conditions in LPS or IFN- γ activated DCs.

The phenotype analysis (Fig. 4.5A) revealed that the control silenced and target silenced DCs independent of the stimulus showed a very similar upregulation of maturation markers. We observed slight differences in the surface expression of MHC class I/II and CD80 between untransfected (UT) and siRNA transfected DCs (IRG1⁻, IRG1⁺) that were exposed to IFN- γ . On the contrary, LPS stimulated DCs that were not treated with any transfection compounds (UT) showed a decreased upregulation of all surface markers, except MHC I, compared to the transfected DCs. Moreover, the stimulation of DCs with LPS was associated with enhanced DC activation and maturation compared to DCs stimulated with IFN- γ . Non-stimulated DCs showed a typical immature DC phenotype with a low level of

4.1 Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells

co-stimulatory molecules as well as antigen presenting molecules MHCII/MHCI and no activation marker upregulated.

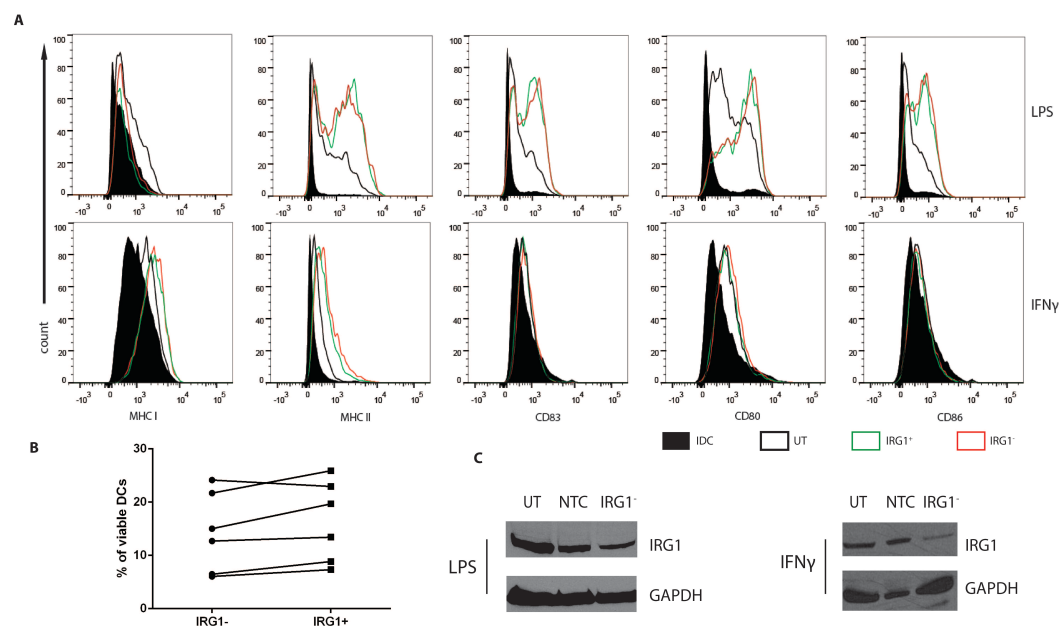


Figure 4.5: Phenotype of activated DCs targeted with IRG1 specific siRNA. (A) DCs were exposed to lipid-based transfection reagents and siRNA specific for IRG1 and stimulated afterwards for 4 hours with either LPS or IFN γ . After medium exchange DCs were cultured for another 20 hours. Surface marker expression was analyzed by flow cytometry and compared between untransfected DCs (UT), control transfected DCs (NTC) and IRG1 silenced DCs (IRG1-). Black filled histograms represent the expression of maturation-associated molecules on immature DCs used as a control. (B) The viability of transfected DCs was calculated as the ratio of the counted cell number after 24 hours of IFN γ stimulation to the initially plated cell number. The viability was compared between IRG1 silenced DCs (IRG1-) and control silenced DCs (IRG1+). Results from 6 independent transfection experiments are depicted. (C) The efficiency of IRG1 silencing in DCs was analyzed by Western Blot. IRG1 protein expression was compared between UT, NTC and IRG1- after 24 hours of LPS or IFN γ stimulation. GAPDH was used as a loading control. One representative silencing experiment is shown.

These results indicate that transfection compounds influence the maturation status of DCs but also that specific stimuli, as LPS, may serve as stronger activation inducers than others.

We further investigated whether the transfection compounds had an impact on DC cell mortality (Fig.4.5B). Survival of DCs was similar between target (IRG1-)

4 RESULTS

and non-target (IRG1⁺) siRNA transfected DCs suggesting that silencing of IRG1 had no influence on the viability.

With regard to the silencing efficiency IFN- γ stimulated DCs which were exposed to target siRNA (IRG1⁻) showed a specific reduction in IRG1 protein expression compared to cells that were treated with the control siRNA (NTC) (Fig. 4.5C). Interestingly, a weak silencing was observed when DCs were stimulated with LPS after the transfection procedure. A difference in IRG1 protein expression can also be seen between control transfected and non transfected DCs. This might be linked to siRNA mediated signaling in DCs.

4.2 Effects of IRG1 silenced DCs on T cell functions

Having investigated the regulation of IRG1 silenced DCs, the second part of this study focused on the impact of IRG1 silenced DCs on CD4⁺ T cell differentiation and stimulation of cytotoxic CD8⁺ T cells. Since DCs play a pivotal role in eliciting a proper immune response, changes in the immunoregulatory activities of DCs may differently affect the outcome of a T cell response. We propose IRG1 belonging to the immune-suppressive molecules secreted by DCs in order to maintain peripheral tolerance. As our goal is to improve the immune stimulatory capacity of DCs, we want to enhance effector T cell activation by silencing IRG1 in DCs.

4.2.1 Effect of IRG1 silenced DCs on proliferation of CD8⁺ effector T cells

In order to measure the extent of T cell responses, we determined the proliferation and survival of CD8⁺ T cells. First, we investigated the proliferative potential of CD8⁺ T cells co cultured with IRG1 silenced DCs using CFSE staining.

For this purpose, siRNA transfected DCs were loaded with the MHC class I OVA-(257-264)-peptide (SIINFEKL) and stimulated with LPS for 4 hours. In parallel, splenic CD8⁺ T cells which possess the transgenic TCR specific for the SIINFEKL

peptide, were CFSE labeled and then co-cultured with DCs at defined DC/T cell ratios (1:1 = 50 000:50 000, 1:2 = 50 000:25 000, 1:8 = 50 000:6 250) for three days.

On day three, the cells were harvested and the proliferation and activation of CD8⁺ T cells was assessed by FACS analysis. The gating strategy is depicted in Fig.4.6.

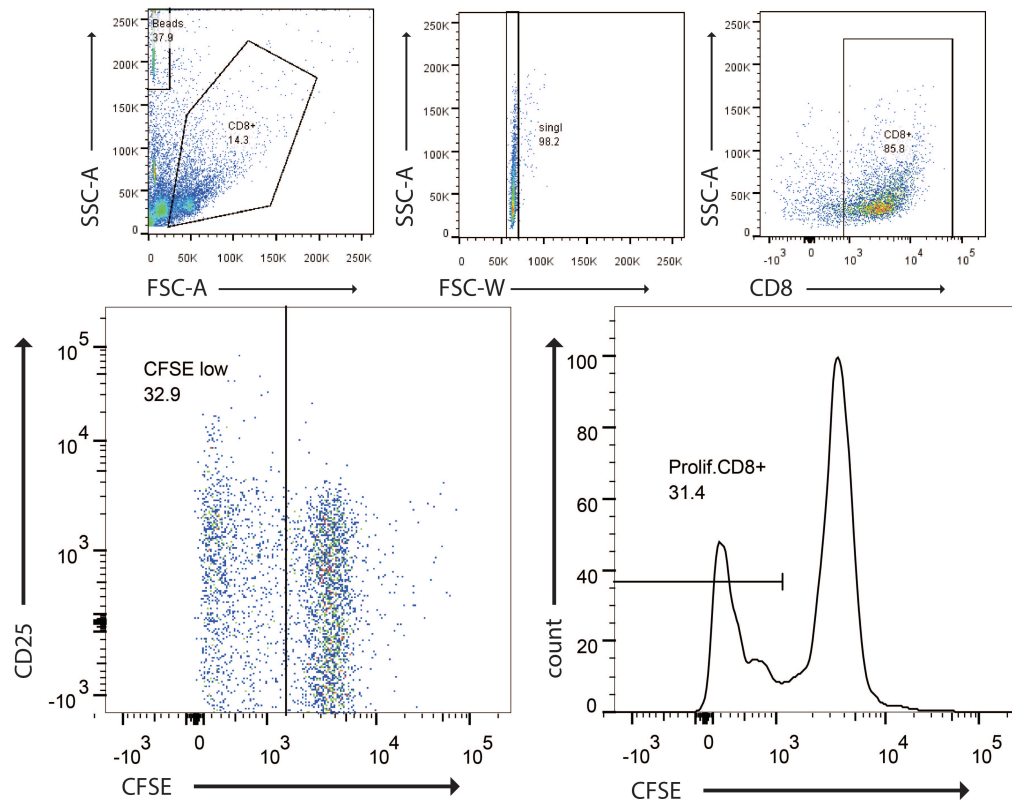


Figure 4.6: Assessment of CD8⁺T cell proliferation. CD8⁺T cells were harvested on day 3 of co-cultures with transfected DCs or different supernatant conditions of transfected DCs. T cells were gated based on morphology and selected upon expression of the surface marker CD8⁺. These cells were further analyzed regarding the activation marker CD25⁺ and the percentage of proliferating T cells was determined by gating on the CFSE low population. One representative experiment of T cells activated with anti-CD3⁺/CD28⁺ Dynabeads is shown.

Fig. 4.7 shows the absolute cell count of proliferated CD8⁺ T cells at different DC/T cell ratios. The absolute cell number of proliferating CD8⁺ T cells cultured with IRG1 silenced DCs was remarkably higher than those that were cultured with control silenced DCs in a 1:1 DC/ T cell ratio. However, at higher DC/ T cell ratios (1:2/ 1:8), reduced T cell expansion was observed upon encounter of transfected or untransfected DCs. Untransfected DCs appeared to have the lowest capacity in activating T cells in the 1:1 and 1:2 DC /T cell mixture. Thus, the 1:1 ratio

4 RESULTS

seems to represent the ideal condition for supporting T cell proliferation when they are co cultivated with IRG1 silenced DCs.

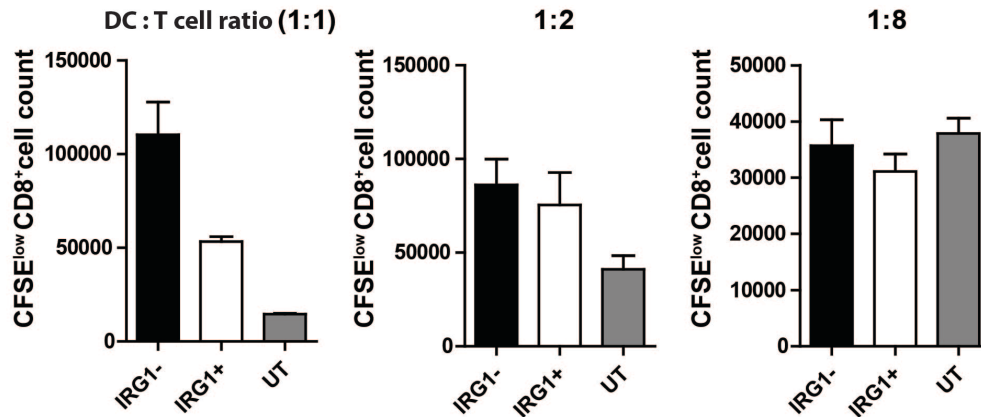


Figure 4.7: Proliferation of CD8⁺ T cells upon co-culture with LPS activated IRG1-silenced DCs at different ratios. The proliferation of CD8⁺ T cells was determined after 3 days of a T cell-dendritic cell co-culture system. DCs, either untransfected (UT), control transfected (IRG1⁺) or IRG1⁻ silenced, were used after LPS stimulation to investigate the relevance of IRG1 expression in DCs for T cell proliferation. The number of proliferating CD8⁺ T cells was analyzed according to the decrease of CFSE fluorescence signal as a function of cell division over time. Distinct ratios of dendritic cell and T cells (1:1, 1:2 and 1:8) were used during the co-culture system. Error bars indicate variances within technical triplicates. One representative experiment is shown.

4.2.2 Effect of supernatants from IRG1 silenced DCs on the proliferation of effector CD8⁺ T cells

IRG1 has been previously shown to have enzymatic function [49]. It further shares high homology to the bacterial metabolic enzyme 2-methylcitrate-dehydratase [44]. Therefore we tested whether supernatant of IRG1 silenced DCs might have an impact on the proliferation of CD8⁺ T cells.

We collected supernatants of IRG1 silenced, control silenced and non transfected DCs which were stimulated with IFN- γ . Wild-type CD8⁺ T cells were then co cultured with defined volumes of these supernatants for three days. Unlike in the prior proliferation experiments, we used anti-CD3⁺/CD28⁺Dynabeads for T cell activation. After three days of co cultivation, the proliferation rate of CD8⁺ T cells was assessed by flow cytometry (Figure 4.8A). We found that the proliferation of CD8⁺ T cells was enhanced (more than 30%) when treated with the supernatant

of IRG1 silenced DCs (IRG1⁻), compared to T cells cultured with supernatants from control silenced (IRG1⁺) (about 20%) or untransfected (UT) (about 11%) DCs.

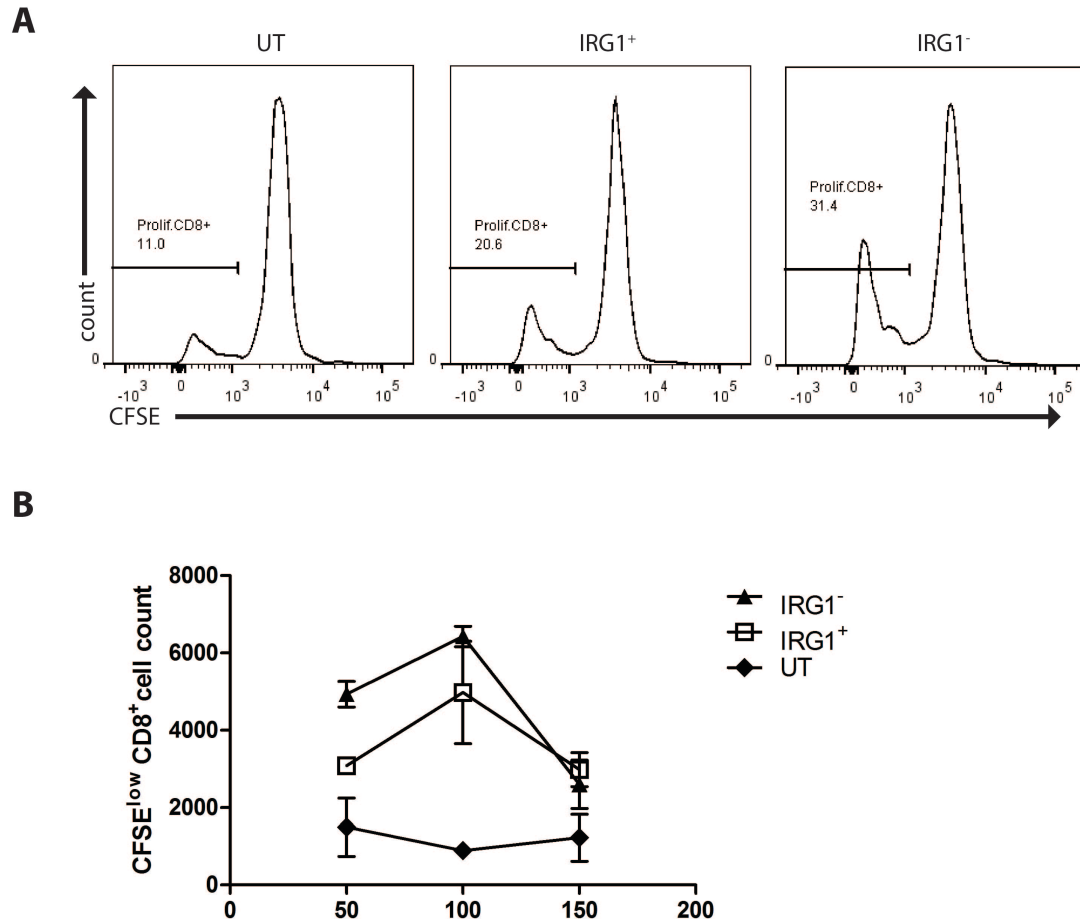


Figure 4.8: Proliferation of CD8⁺ T cells upon treatment with cell-free supernatants of IRG1 deficient DCs. (A) CD8⁺ T cells were harvested after 3 days upon treatment with supernatants of untransfected DCs (UT), control silenced DCs (IRG1⁺) and IRG1 silenced DCs (IRG1⁻). Since CFSE was used as a proliferation marker, the percentage of proliferated CD8⁺ T cells was assessed by gating on the CFSE low population. One representative example of T cells treated with 50 µl supernatant is shown. (B) Shown are the absolute numbers of proliferating CD8⁺ T cells (CFSE^{low}) cultured either with 50 µl, 100 µl or 150 µl cell-free supernatants of untransfected DCs (UT), control silenced DCs (IRG1⁺) and IRG1 deficient DCs (IRG1⁻). Error bars indicate variances within technical triplicates. Data shown are representative for one experiment.

Further the absolute numbers of proliferating T cells were higher when T cells were treated with 100 µl of supernatants derived from transfected DCs (Fig. 4.8B). At a lower dosage (50 µl) we observed a clear increase in T cell proliferation considering the when CD8⁺ T cells were incubated with supernatants of IRG1 silenced DCs (IRG1⁻). At higher dosages (150 µl) proliferation for all

4 RESULTS

conditions was strongly decreased showing no differences between IRG1⁺ and IRG1⁻ conditions.

Next we examined the apoptosis rate of CD8⁺ T cells upon incubation with supernatants collected from IRG1 silenced DCs for three days. In this setting, CD8⁺ T cells were treated with 150 μ l of IRG1 silenced DCs and control silenced DCs which were stimulated with IFN- γ . The percentage of pro-apoptotic CD8⁺ T cells was determined by Annexin V (Ann V) staining, a proapoptotic marker.

We observed a higher rate of viable, Ann V⁻ T cells (around 40%) when incubated with the supernatant of IRG1 silenced DCs (IRG1⁻ supernatant) (Fig. 4.9). T cells treated with the supernatant of control silenced DCs (IRG1⁺ supernatant) were less viable (around 24%). When we analyzed absolute cell counts, almost 2-fold more non-apoptotic CD8⁺ T cells were detected when incubated with IRG1⁻ compared to IRG1⁺ supernatants.

In summary we demonstrated that proliferation of CD8⁺ T cells was enhanced when IRG1 was not expressed in DCs. This finding indicates that DCs lacking IRG1 have a stronger capacity of activating CD8⁺ T cells, moreover support their survival on the basis of altered production of molecules secreted by DCs.

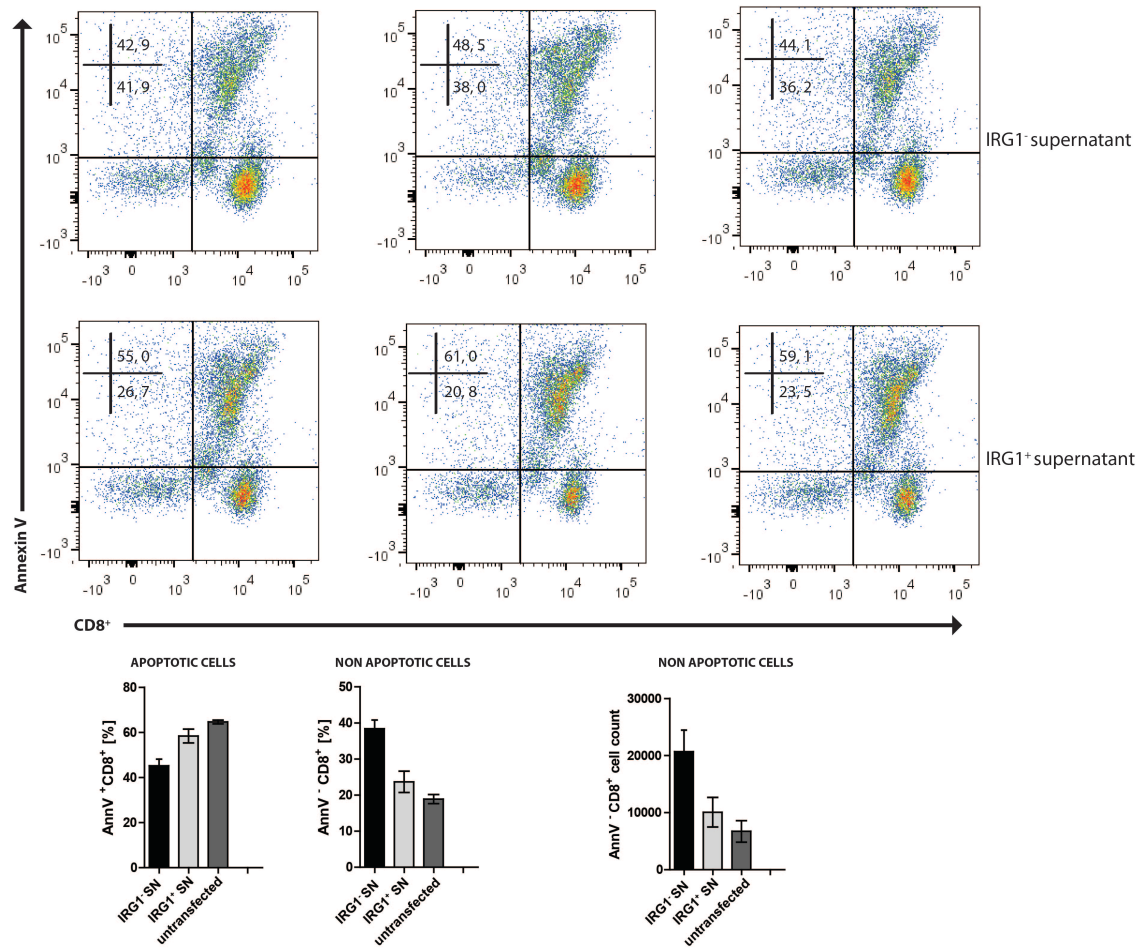


Figure 4.9: Annexin V staining of CD8⁺ T cells after treatment with supernatant of IRG1 deficient DCs. (A) Using flow cytometry to assess apoptosis, T cells were stained with antibodies for the pro-apoptotic marker Annexin V and the surface marker CD8⁺ after 3 days of treatment with 150 μ l of supernatant from IRG1 silenced DCs (IRG1⁻ SN) or control silenced DCs (IRG1⁺). One representative triplicate is shown. (B) The percentage of apoptotic and non apoptotic T cells as well as absolute number of non apoptotic T cells was calculated by selecting CD8⁺ and Annexin V positive/negative T cells. Error bars indicate variances within technical triplicates.

4.2.3 Effect of IRG1 silenced DCs on CD4⁺ T cell differentiation

Since DCs are important for directing the T helper cell (Th-cell) response, we were further interested in what direction IRG1 deficient DCs affect the Th-cell polarization. The development into a particular Th phenotype indicates what type of immune response is initiated.

4 RESULTS

We used IRG1 silenced DCs loaded with the MHC class II OVA-(323-399)-peptide and activated them with IFN- γ . CD4⁺ T cells, specific for the OVA peptide, were then incubated with IRG1 silenced DCs together with 100 μ l of IRG1⁻ supernatant or IRG1⁺ supernatant for four days. This experiment was performed in a DC/ T cell ratio 1:2. In Figure 4.10 mRNA expression of the transcription factors ROR γ t, Tbet and FoxP3 defining Th17, Th1 and Treg subsets is illustrated.

Noticeably, the mRNA expression of all transcription factors was at least 2 fold higher when CD4⁺ T cells were treated with IRG1⁻ supernatant than those treated with IRG1⁺ supernatant. This finding suggests that the absence of IRG1 leads to elevated numbers of all three T cell subsets.

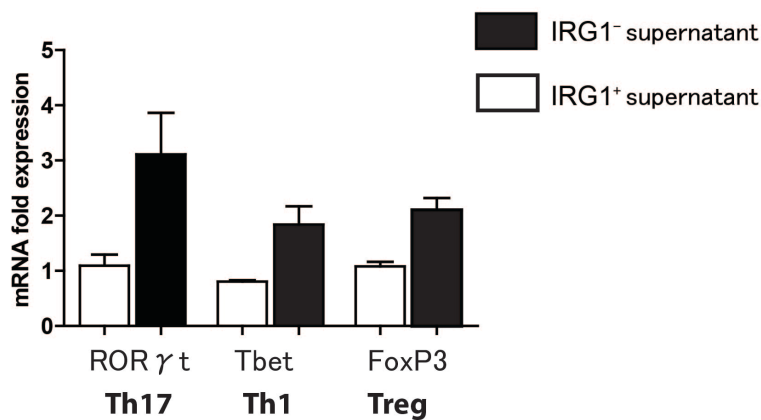


Figure 4.10: Differentiation of CD4⁺ T cells after treatment with supernatant of IRG1 deficient DCs. IRG1-silenced DCs were used as antigen presenting cells in co-cultures with CD4⁺ T cells. CD4⁺ T cell differentiation was measured after a 4-day treatment with supernatants from either IRG1-silenced (IRG1⁻ SN) or control silenced DCs (IRG1⁺ SN). mRNA expression of the transcription factors ROR γ t, Tbet and FoxP3 defining Th17, Th1 and Treg subsets, respectively, was analyzed using quantitative real time PCR. Error bars indicate variances between technical duplicates. One representative experiment is shown.

4.3 Detection of enriched molecules in the supernatant of IRG1⁺ DCs by HPLC

In the last part of this study we investigated the metabolome profile secreted by activated IRG1⁺, IRG1⁻ DCs using HPLC (High Performance Liquid Chromatography) in combination with mass spectroscopy (MS).

In previous experiments we observed that the cell-free supernatant of IRG1-deficient DCs has an overwhelming impact on the proliferation and viability of CD8⁺ T cells (Fig.4.8 and 4.9) as well as the stimulatory potential on different effector T cell subsets. (Fig.4.10) Thus, we propose a soluble immune suppressive molecule involved in IRG1 mediated metabolism.

For this purpose, supernatants of IRG1-deficient DCs were profiled by our collaborators at the Institute of Organic Chemistry at the Johannes Kepler University (JKU), Linz for low molecular metabolites.

The HPLC chromatograms contain peaks from metabolites that were separated within 15.95 minutes (Fig. 4.11). During this period, one peak between the retention time of 7.33 and 10.92 minutes is overrepresented in the chromatogram of IRG1⁺ supernatant (see red line in the 1st chromatogram – Fig.4.10). The corresponding peak is missing in the IRG1⁻ supernatant as well as in the negative control. This result indicates that some enriched molecules are present in the supernatant of IRG1⁺ DCs.

Further fractions with enriched metabolites in the IRG1⁺ supernatant were characterized by Quadrupole Ion Trap Mass Spectrometer (Fig.4.12). Two mass spectrometric analyzes of HPLC fractions isolated after a retention time of 8.74 and 10.43 min of the IRG1⁺ supernatant are represented.

4 RESULTS

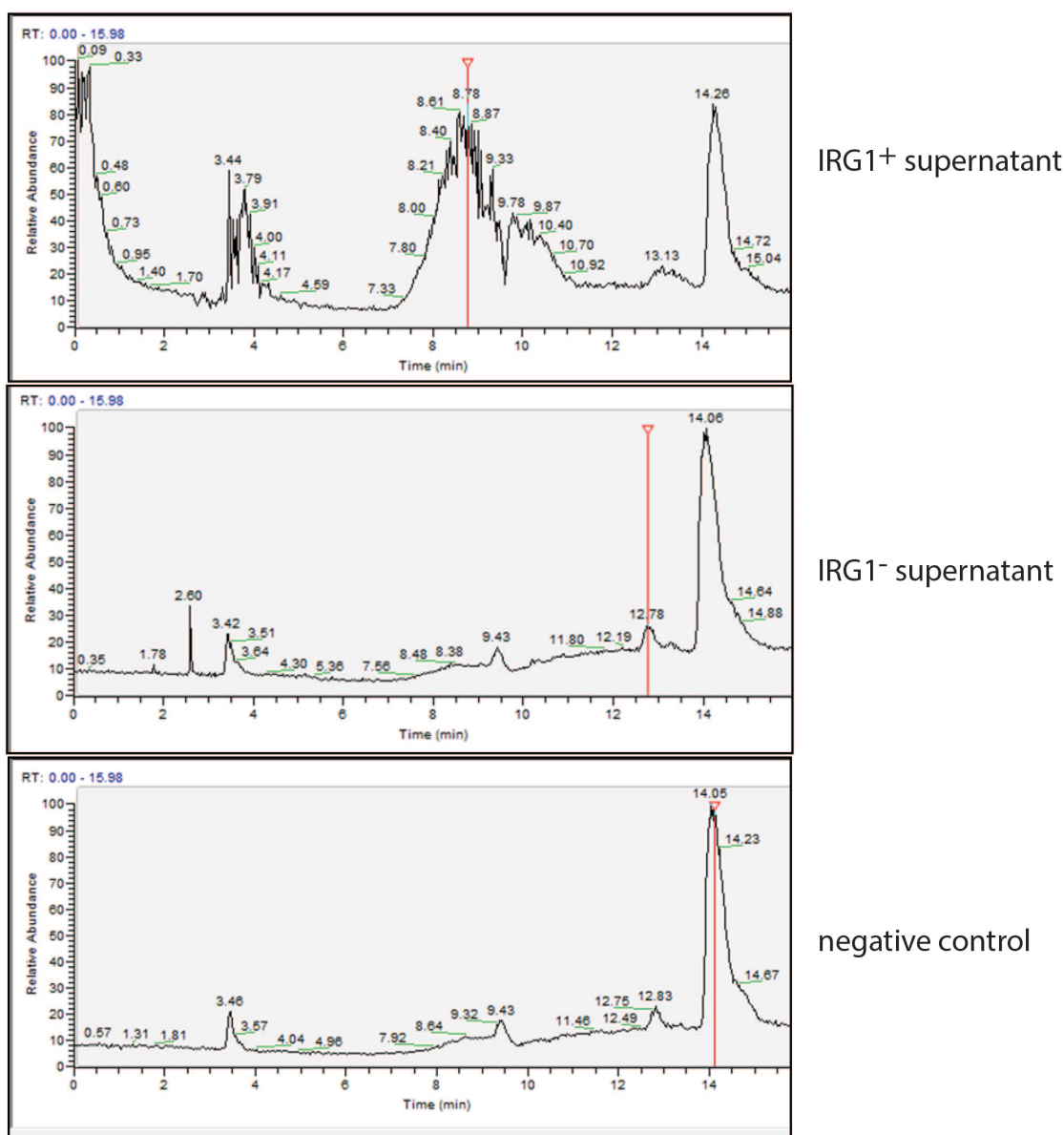


Figure 4.11: Analysis of differentially secreted molecules in the supernatants of IRG1 silenced DCs by HPLC. These chromatograms show the relative abundance of enriched molecules in the supernatant of IRG1 silenced DCs (IRG1⁻ SN), control silenced DCs (IRG1⁺ SN) after 24 hours of IFN- γ stimulation. As a negative control SN of iDCs was analyzed. High performance liquid chromatography conditions: solvent A - 0, 1% (NH₄)₂CO₃ pH 2, 75; solvent B- Acetonitril, flow rate= 1mL/min (ambient conditions)

The fraction at the retention time of 8.74 minutes showed a high peak for a metabolite with the mass to charge ratio (m/z) 546.66 (Fig. 4.12 A). The other fraction at the retention time, 10.43 minutes contained a mixture of metabolites with 2 dominant m/z values 339.66 and 355.57 which were over represented in this fraction (Fig. 4.12 B). Using different databases (E.coli Metabolome Database and Human Metabolome Database), we obtained several predictions

4.3 Detection of enriched molecules in the supernatant of IRG1+ DCs by HPLC

for the identity of the metabolites based on the m/z ratio: 546, 66 – Guanosine- 5 triphosphate, 339, 66 – 2-Maleylacetate and 355, 57 – NAD/Diadenosine hexaphosphate). However, full characterization of these metabolites will require repetition of these procedures and further analysis.

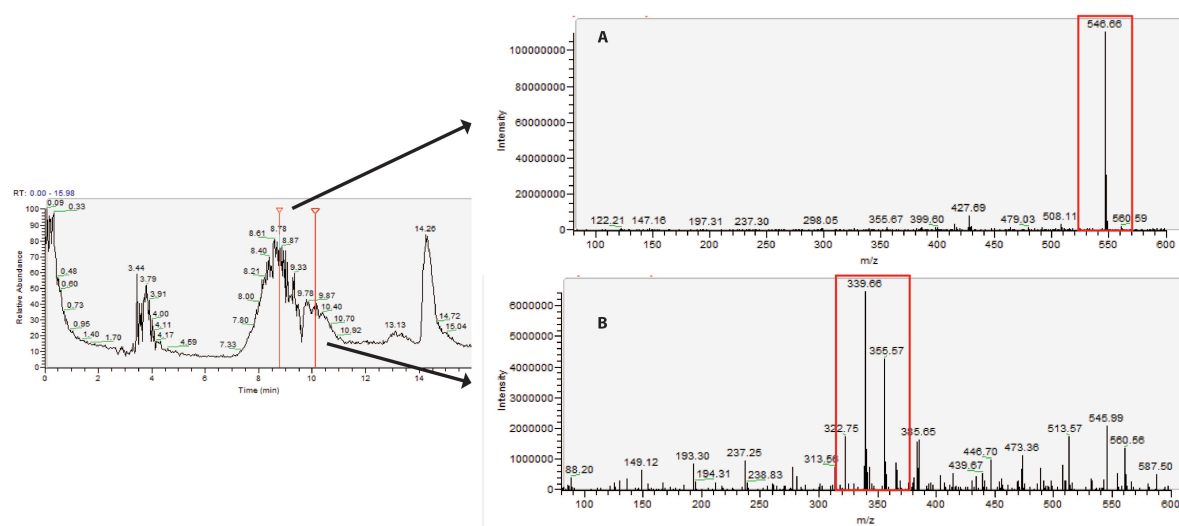


Figure 4.12: Mass spectrometric analyzes of HPLC fractions (IRG1⁺ supernatant). Two HPLC fractions present in the supernatant of control silenced DCs and absent in the other supernatants at a retention time of 8.74 min and 10.43 min were further analyzed by the Quadrupole Ion Trap Mass Spectrometer. The distinct and sharp metabolite profile of the mass spectrometric analysis of these 2 fractions is shown.

In this study, we show that IRG1 expression might be the result of the activation of cytokine-mediated signaling pathways. Given that multiple independent signals provided via TLRs as well as cytokines can activate IRG1 expression, makes the regulation of IRG1 even more complex and might reflect its importance in generating an immune response. Furthermore, inhibition of IRG1 in DCs led to increased numbers of CD8⁺ T cells in co culture assays. Supernatants of IRG1 deficient IFN- γ stimulated DCs enhanced the proliferation and viability of CD8⁺ T cells and led to elevated numbers of Th1, Th17 and regulatory T cells. Since our HPLC/MS analyzes revealed the presence of some enriched metabolites in the supernatant of IRG1 expressing DCs, which are missing when IRG1 is silenced, we conclude that IRG1 provides its immune suppressive function by the secretion of metabolites acting anti-inflammatory on T cells. Therefore, it can be assumed

4 RESULTS

that triggering IRG1 activation in DCs might provide an additional layer of immune regulation to maintain tolerance.

5 DISCUSSION

To get more information about immune-suppressive mechanisms mediated by DCs, we were interested in characterizing genes, which could be important regulators of the switch of DCs from an immune stimulatory to an immune-suppressive mode in terms of effector T cell functions. Among others, IRG1 was chosen for further investigation, because knockdown of this gene in human DCs resulted in higher T cell proliferation. (see Fig. 1.9)

Thus, the aim of this master thesis was to study the regulation of IRG1 expression, the effect of its absence in DCs in particular on T cells as well as the presence of immune suppressive metabolites produced by IRG1.

5.1 Characterization of Immune-responsive gene 1 (IRG1) in murine dendritic cells

In order to gain insight into regulation of IRG1 and to address the question of its possible function, we were first interested in the sub cellular localization of IRG1. Knowing the sub cellular location that a protein resides in is important to elucidate protein function [54].

We detected IRG1 protein in LPS stimulated cells within the cytoplasm and not in the nucleus. Although no mitochondria targeting signal sequence was detected in the IRG1 amino acid sequence, the cytoplasmic location of IRG1 correlates with the reported association of IRG1 to mitochondria by Degrandi et al [48]. Since mitochondrial proteins often play key roles in metabolic and survival pathways [55], IRG1 might be part of these protein networks.

5.1.1 Regulation of IRG1

In this context, we demonstrated that the expression kinetics of IRG1 in DCs correlates with that of these molecules secreted during the anti-inflammatory DC mode, showing highest expression after 24 hours of stimulation independent on

5 DISCUSSION

what type of stimuli. Dendritic cells have shown to go through a time-dependent maturation process by switching from a pro-inflammatory to an anti-inflammatory DC phenotype upon activation [23, 24]. Several molecules, as IDO [56], IL-10, soluble IL-2 receptor alpha molecules (sIL2RA) [24] as well as phosphorylated STAT3 [57] are linked to this anti-inflammatory DC maturation status.

We observed upregulation of IRG1 transcripts and protein after stimulation of DCs with several cytokines or TLR agonists. Interestingly, IRG1 expression was detected via MyD88 involved in TLR4, TLR7/8 and TLR5 signaling, but also in a MyD88 independent manner via TLR3 signaling. This observation is in contrast to the findings of Degrandi et al. [48], who did not detect any IRG1 transcripts upon TLR3 stimulation by Poly(I:C). It has to be taken into account that these findings were true for macrophages and not DCs which were stimulated for 16 hours by Poly(I:C). In our experiments we observed a huge decline of IRG1 transcription in DCs already after 16 hours of Poly(I:C) stimulation. Moreover, other studies performed in DCs have reported unaltered IRG1 gene induction in MyD88 knockout DCs, suggesting that IRG1 gene expression is indeed independent of MyD88 [52].

The finding that IRG1 is differently induced by a variety of TLR ligands but also cytokines, suggests the possibility that secondary autocrine cytokine signaling after TLR ligation triggers TLR-mediated expression of IRG1. Consistent with the literature, the cytokine response varied with the type of TLR being activated [22]. TLR4 and TLR7/8 ligation resulted in an elevated production of the cytokines IL-6, TNF and IFN- γ , which in turn seemed to be associated with the observed stronger IRG1 expression. Contrary, activation of TLR5 and TLR3 elicited a decreased cytokine response, which also resulted in a weaker IRG1 expression. To assess the effect of cytokine stimulation on IRG1 expression, we blocked the cytokine-mediated activation of the JAK-STAT pathway with the JAK1/JAK2 (JAKAFI) inhibitor. Indeed, IRG1 expression was abrogated upon stimulation with the cytokines IL-6, IL-10 and IFN- γ but not in the absence of the inhibitor. Since, TLR-induced IRG1 expression was not completely abrogated in the presence of JAKAFI, additional cytokines, as TNF might mediate IRG1 signaling. Notably,

TNF induced remarkably high IRG1 expression levels even in the presence of the JAK1/JAK2 inhibitor compared to other cytokines. Furthermore, TNF mRNA is upregulated early after LPS activation before IRG1 expression is even detectable. Therefore, altogether it can be assumed that IRG1 expression is the result of secondary cytokine-mediated signaling pathways (as TNF mediated signaling) upon TLR ligation.

It is noteworthy that IRG1 was highly expressed upon the synergistic action of TNF and IFN- γ . This is in line with the study reported by Degrandi et al. [48]. Although these two cytokines act through two distinct signaling pathways, this synergism is a well known phenomenon in antibacterial activities [58]. Increased transcriptional activity of IDO, which is a well described antimicrobial and immune-suppressive molecule [59] secreted by DCs, has also been observed in response to TNF and IFN- γ . Thus, IRG1, whose catalyzing products recently have been reported as acting antimicrobial [49] shows similarity to other immune-suppressive molecules associated with the change in DC phenotype over time.

5.1.2 Effect of IRG1 silencing on DCs

In our study siRNA-mediated gene silencing was used to explore the particular function of IRG1 in DCs. Cells were siRNA transfected prior to 24 hours of LPS or IFN- γ stimulation to assure that IRG1 mRNA is quickly degraded upon stimulation. Since IRG1 protein levels are the highest after 24 hours of stimulation, the efficiency of silencing was tested after 24 hours of stimulation. Although the transfection procedure generally led to high cell mortality, we detected no differences in the viability of IRG1 silenced and control silenced DCs, indicating that lack of IRG1 expression does not affect DC viability.

Interestingly, IRG1 gene silencing in LPS stimulated dendritic cells was not as efficient as in IFN- γ stimulated DCs. Additionally, although LPS stimulated DCs were treated with the control-siRNA, we observed weaker IRG1 expression compared to untransfected DCs. Many studies focused on the undesirable “off-target” effects mediated by siRNAs. [60-62] In these studies it was reported that

5 DISCUSSION

siRNAs beyond their capacity of selective gene silencing, can stimulate innate immune responses. In this context, siRNA can signal through TLR3 and induce type I interferons and cytokines as interferon (IFN)- α , TNF, IL-6 and IL-12. Moreover, the activation of serine–threonine protein kinase R (PKR) and RNaseL, resulting in non-specific degradation of cellular RNA and general repression of protein synthesis, was observed. This might explain why our control silenced DCs exhibited lower levels of IRG1 protein, compared to untransfected cells. The inefficient IRG1 silencing in LPS stimulated DCs might be due to inappropriate effects caused by the combination of TLR3 and TLR4 ligation. Interestingly, TLR4 engagement was shown to inhibit TLR3 signaling[63]. Thus, DCs receiving a TLR signal twice, once via siRNA and secondly via LPS, might affect gene silencing by an unknown mechanism. We found efficient silencing by avoiding a second TLR stimulus and stimulated DCs with IFN- γ , which is known to induce IRG1 expression via JAK-STAT signaling.

Phenotypic analysis of dendritic cells by flow cytometry revealed that siRNA transfected DCs independent of the stimulus showed enhanced upregulation of maturation marker compared to untransfected DCs. The reason for this enhanced upregulation of surface markers might be due to additional siRNA induced TLR3 signaling [60-62] in combination with the actual LPS or IFN- γ stimulus. Untransfected DCs did not receive any additional siRNA triggered TLR signaling. Since the upregulation of DC surface molecules was similar between IRG1 silenced and control silenced DCs, the absence of IRG1 does not seem to affect DC maturation and activation.

Moreover, the stimulation of DCs with LPS was associated with enhanced DC activation and maturation compared to DCs stimulated with the cytokine IFN- γ . Maturation of DCs can be triggered by multiple stimuli, however LPS, a traditional stimulus, proved to have a significant stimulatory effect by inducing a marked up-regulation of co-stimulatory molecules CD80 and CD86, [64, 65] but also CD83 [66]. These phenotypic changes occurred already at low concentrations of LPS [65] and greatly increased their stimulatory ability for T cells [64]. This explains why we observed dramatic upregulation of the co-stimulatory molecules CD80 and CD86 and the activation marker CD83, whereas IFN- γ stimulation was more

efficient in the upregulation of the class I MHC complex. Upregulation of MHC-I by IFN- γ is important for cytotoxic T cell recognition to establish an antiviral state [67].

Taken together, these data suggest that IRG1 knockdown has no impact on DC viability, maturation and activation. Moreover, efficient silencing seems to depend on the type of stimulus used during the transfection procedure, highlighting the difficulty of siRNA-mediated silencing.

5.2 Effects of IRG1 silenced DCs on T cell functions

The function of IRG1 in dendritic cells or its impact on T cells has not been reported yet. Although it's critical role during implantation of embryos [47], indicates that IRG1 is important to maintain maternal tolerance as has been shown for IDO [68]. Moreover, very recently it was published that IRG1 catalyzes the production of itaconic acid, an antimicrobial metabolite, in macrophages [49]. We were especially interested whether the absence of IRG1 in DCs affects the priming of naïve CD4⁺ T cells and the activation or viability of CD8⁺ T cells. As our overall goal is to enhance the effector responses of T cells, IRG1 might be used as a target gene in DCs to improve DC-based cancer immunotherapies.

5.2.1 Effect of IRG1 silenced DCs on proliferation and viability of CD8⁺ effector T cells

One way of investigating the stimulatory potential of IRG1 deficient dendritic cells on T cells, was by assessing T cell proliferation. The proliferation of CD8⁺T cells is important for mounting an effective adaptive immune response in order to elicit an efficient anti-tumor immunity [69].

In this study we have demonstrated that the absence of IRG1 in DCs co-cultured with CD8⁺ T cells led to enhanced proliferation of these cells at a 1:1 DC/ T cell ratio. At higher DC/ T cell ratios (1:2/ 1:8), resulting in lower DC numbers, reduced T cell expansion was observed upon encounter of transfected or untransfected DCs. The decreased T cell proliferation at higher DC/ T cell ratios

5 DISCUSSION

led to the assumption that a lower density of DCs is not sufficient enough in stimulating T cells since the amount of immune-stimulatory cytokines is diluted and thus fails to trigger T cell activation. Furthermore, the immune-suppressive effect of IRG1 on T cells was only detectable at the 1:1 DC to T cell ratio. At higher DC/ T cell ratios, T cells regardless whether co cultured with control silenced or IRG1-deficient DCs exhibited a quite similar proliferation profile. Hence, not only the immune-stimulatory but also immune-suppressive molecules secreted by DCs lose their ability to affect T cell responses due to a low quantity of DCs. Interestingly at the 1:1 and 1:2 DC to T cell ratios, untransfected DCs appeared to have the lowest capacity to induce T cell proliferation when compared with the transfected DCs. Notably; untransfected DCs displayed lower expression of maturation and co-stimulatory molecules than transfected DCs. Thus, the distinct DC phenotype of untransfected DCs might be responsible for the diversity in the proliferative capacity of T cells.

In vitro analyses that explain how, why and when T cells respond to DC activation, truly depends on the methodology. It is essential to consider that the ratio of stimulating DCs and responding T cells in our experiments, determines the outcome of T cell responses. Many variables, as the DC number, antigen dose, TCR avidity and duration of DC-T cell encounter play a role in regulating immune responses in experimental systems. Thus, it is important to be cautious with interpreting results from experimental model systems, since they may not be representative of what happens in vivo [70, 71]. Nevertheless, we can conclude from our results that there is a difference in activating T cells depending on whether T cells encounter IRG1 deficient or IRG1 expressing DCs. Moreover, the immune suppressive effect of IRG1 on T cell proliferation was highest at the 1:1 DC to T cell ratio, leading to a decreased number of proliferating T cells.

We further tested whether the anti-proliferative effect of IRG1 expressing DCs on T cells is mediated by the secretion of an immune suppressive molecule into the supernatant. Such a mechanism was observed for the IDO enzyme in DCs, whose immunoregulatory effects were mediated by the production of kynurenine [59].

Supplementing our T cell culture with 50 μ l of cell-free supernatant derived from IRG1 deficient DCs led to enhanced proliferation of CD8⁺ T cells compared to

supernatants collected from IRG1 expressing DCs. The augmented T cell proliferation suggests the depletion of an immune suppressive molecule, which is present in the supernatant of IRG1 expressing DCs. At higher dosages, 100 and 150 μ l of cell-free supernatant, this proliferative effect gets lost showing no differences between T cells incubated with supernatants from IRG1 expressing or deficient DCs. At the highest dosage, 150 μ l, proliferation for all conditions was strongly decreased, showing no differences between T cells kept in culture with supernatant from transfected or untransfected DCs. It is unclear why the middle dosage, 100 μ l of supernatant, led to the highest proliferation of CD8⁺ T cells. It will be necessary to test whether lower dosages, as 25 μ l of cell-free supernatants derived from IRG1 deficient DCs, improve even more the proliferation of T cells.

Concerning the apoptosis rate of CD8⁺ T cells upon incubation with supernatants of IRG1 deficient DCs or control silenced DCs we detected more viable CD8⁺ T cells when cultured with the supernatant collected from IRG1 deficient DCs. The percentage of Annexin V positive cells was therefore lower and indicated that the absence of IRG1 increases to some extent the viability of the CD8⁺ T cells. However, the underlying mechanisms for reduced viability of CD8⁺ T cells in the presence of IRG1 are unclear. We suggest that an immune suppressive molecule secreted by IRG1 expressing DCs, acts negatively on the T cell metabolism. Whether the concentration of the proposed immune suppressive factor plays a role on its effects on T cells must be elucidated.

One known soluble factor that mediates T cell suppression by regulating cell death is nitric oxide (NO) [72]. NO is generated out of L-arginine by the enzyme nitric oxide synthase (NOS) [73]. This mechanism was postulated to be reminiscent to the antimicrobial effects provided by IRG1 [49]. Thus, showing a causal relation between NO and IRG1, can provide insight into immune suppressive effects mediated by IRG1.

5.2.2 Effect of IRG1 silenced DCs on of CD4⁺ T cell polarization

Further we raised the question whether T cell polarization might be affected using IRG1 deficient DCs instead of IRG1 expressing DCs. As already mentioned, DCs provide various T cell polarizing signals depending on their activation or maturation state. For example some tryptophan metabolites have been reported to suppress Th1 and Th17 cells and on the other hand support regulatory T cell differentiation [74]. Thus, DCs can determine whether an inflammatory or rather an immune suppressive T cell response is induced.

Interestingly, we found that the mRNA expression level of all transcription factors specific for Th1, Th17 and regulatory T cells was elevated, when activated CD4⁺ T cells where incubated with the supernatant of IRG1 deficient DCs. This finding suggests that the absence of IRG1 in DCs leads to elevated numbers of all three T cell subsets. Thus, IRG1 expressing DCs are supposed to suppress both effector and regulatory T cell subsets in vitro. It is not clear whether the elevated numbers of these T cell subsets are caused by enhanced proliferation or reduced apoptosis in the absence of IRG1. Hence, the secretion of T cell specific cytokines and other factors must still be investigated to fully understand this mechanism. Moreover, it can be assumed that IRG1 has an impact on other T cell subsets, which were not tested in our experimental model. Additionally, the concentration of the putative immune suppressive molecule produced by IRG1 and secreted into the supernatant might be relevant and determine T cell fate. Therefore dosage dependent T cell differentiation needs to be investigated.

5.3 Detection of enriched molecules in the supernatant of IRG1⁺ DCs by HPLC

We discovered that the supernatant of IRG1 deficient DCs had an impact on the viability and proliferation of T cells due to an improved stimulatory potential of DCs. This led to the conclusion that IRG1 mediated metabolism produces some soluble immune suppressive molecules that are transported into the extracellular matrix. To determine extracellular metabolites differentially secreted into the

supernatant of IRG1 expressing versus deficient DCs, we analyzed supernatants of IFN- γ stimulated IRG1 silenced and control silenced DCs by HPLC at the Johannes Kepler University in Linz. UV light active metabolites were separated in HPLC columns that separated low molecular weight metabolites.

A peak was observed in the HPLC chromatogram obtained from the supernatant of IRG1 expressing DCs that was absent in supernatants of IRG1 deficient and immature DCs, which we used as the negative control. The fraction at the retention time of 8.74 minutes showed a dominant peak for a metabolite with the mass to charge ratio (m/z) 546.66. The other fraction at the retention time of 10.43 minutes contained a mixture of metabolites with 2 dominant m/z values 339.66 and 355.57.

We used the E.coli Metabolome Database (ECMDB) and the Human Metabolome Database (HMDB) to predict the formulations based on the detected mass peaks. We identified guanosine-5, triphosphate (GTP) for the 546.66 mass to charge ratio using the ECMDB and HMDB. Interestingly, 2-Maleylacetate was further predicted for the m/z value 339.66 by the ECMDB, whereas the HMDB could not identify any metabolite fitting to this m/z value. The ECMDB predicted the chemical structure of NAD accurate to the 355.57 m/z value, whereas the HMDB suggested the chemical structure of Diadenosine hexaphosphate.

It is striking that all these above mentioned metabolites identified in the supernatant of IRG1 expressing DCs are complete missing in the supernatant collected from IRG1 deficient DCs; especially since our silencing procedure did not lead to a 100% reduction of IRG1 expression. One possible explanation for this might be that the concentration of these metabolites is lower than the detection limit.

Another reason might be that silencing of IRG1 affects the energy coupling in chemical reactions within metabolic cycles. Some reactions proceed spontaneously, while others require a lot of energy to be induced. Therefore, non-spontaneous reactions are often coupled to spontaneous occurring reactions to make the overall reaction favorable. So if an enzyme that catalyzes a favorable reaction is for some reason absent, it could in turn prevent the induction of the

5 DISCUSSION

following coupled reaction [75]. IRG1, as an enzyme [49], might be an important regulator of such chemically coupled reactions. Thus, already partial removal of IRG1 from the energy exchange cycle might decrease the amount of products needed for the completion of other following reactions. Therefore It might be reasonable that products of coupled reactions are absent in the supernatant of IRG1 deficient DCs.

Recently, itaconic acid was found to be most affected by IRG1 silencing compared to all other measured intracellular metabolites in RAW264.7 cells [49]. Although high intracellular concentrations of itaconic acid were determined, it is unclear what quantities of this compound were found in the supernatant. Having identified other metabolites besides itaconic acid in the supernatant of IRG1 expressing DCs, we suggest that these metabolites are produced downstream of itaconic acid and subsequently secreted into the supernatant. However, Michelucci et al. detected itaconic acid as one of 5 metabolites affected by IRG1 silencing. The other metabolites were not mentioned in the literature [49]. We also have to consider that their metabolite analyses were performed in a macrophage cell line and not in dendritic cells. Thus, it will be necessary to verify the presence of itaconic acid in our analysis.

If indeed itaconic acid is found in the IRG1 mediated metabolism in DCs, it will be of great interest to investigate the missing link between its antimicrobial and immunoregulatory properties. Previously, it has been shown that such an antimicrobial and immunoregulatory effect can be incorporated by the concentration of a metabolite. In case of IDO, tryptophan degradation leads first to the inhibition of bacteria, and later on, at a lower concentration, T cell responses are shut down. The different amounts of tryptophan that are required for bacteria growth and T cell growth make it possible to incorporate two such contradictory roles. The antimicrobial activity of itaconic acid has been shown to be exhibited by inhibiting isocitrate lyases, which are only present in the glyoxylate shunt of bacteria [49]. The question arises how itaconic acid if secreted might inhibit T cell growth, since the glyoxylate shunt is not present in T cells. It is possible that itaconic acid interferes with the glucose metabolism in T cells, since it has been shown that it inhibits phosphofructokinase [76]. Blocking of phosphofructokinase leads to a decrease of the concentration of fructose-2, 6-

bisphosphate, which is crucial for the stimulation of glycolysis in T cells [77]. Therefore itaconic acid might affect T cell activation processes by blocking the glucose metabolism. That could be one way of itaconic acid linking antibacterial effects, by inhibiting lyases, and immunoregulatory effects, by blocking glycolysis in T cells.

In our study we have indications for increased amounts of GTP in the supernatant of IRG1 expressing DCs. GTP is an important energetic factor bound by GTPases, which switch from an inactive, GDP-bound to an active, GTP-bound state. Rho GTPases have been shown to play an important role in the coordination of immune responses, especially by affecting the regulation of TCR signaling, T cell cytoskeletal reorganization, T cell migration and T cell apoptosis [78]. In the context of immune responses nothing was reported about the action of extracellular GTP. Nevertheless, studies have implicated extracellular ATP (adenosine triphosphate) in the regulation of programmed cell death. Extracellular ATP was shown to rapidly increase cytoplasmic calcium levels which lead to apoptosis [79]. Other roles for extracellular ATP have been found regarding the differentiation of intestinal T helper 17 cells and co-stimulatory signals to T cells [80]. Interestingly, extracellular ATP was shown to induce the expression of the immune suppressive molecule IDO in DCs. Moreover depending on the microenvironment, extracellular ATP might contribute to an immunogenic or tolerogenic activity of DCs [81]. Hence, extracellular nucleotides appear to have important roles in the regulation of innate immunity. Since, ATP and GTP share many similarities [82] it is conceivable that extracellular GTP might fulfill similar tasks to these mediated by ATP. Specific binding sites for extracellular GTP that are not bound by other nucleotides were found on the surface of cells [83]. A better understanding in the effects mediated by extracellular GTP may thus provide critical insights into the regulation of immunity by nucleotides. Moreover IRG1 might be an important regulator of controlling the secretion and effects of extracellular GTP by DCs. According to data published by the group of Michelucci [49] and our HPLC/MS analysis, induction of IRG1 leads to the secretion of various metabolic products, which are absent when IRG1 is silenced. A novel metabolic pathway for the biosynthesis of itaconic acid catalyzed by IRG1 was discovered [49].

5 DISCUSSION

Furthermore, the identification of other pathways as well as downstream metabolite products that are part of IRG1-mediated metabolism, need to be discovered. Our study highlights the importance of IRG1 in regulating immune responses by its metabolic products. The precise molecular mechanisms that lie behind the immune regulatory effects of IRG1 still need to be explored.

6 CONCLUSION

Dendritic cell immunotherapy is acquiring increasing importance in clinical immunology and holds great promise in the treatment of many immune diseases. The application of DCs into medicine requires detailed knowledge on DC functionality, in particular on DC activation status, to make DCs highly efficient in generating effective immune responses.

Dendritic cells have been shown to undergo a maturation process in a time-dependent manner, by switching from a pro-inflammatory to an immune suppressive mode of action. Targeting genes that are essential factors in DC-mediated immune suppressive mechanisms, might improve their immune-stimulatory capacity.

In this study, we have investigated the proposed immune suppressive function of IRG1 in DCs. We showed a strong upregulation of IRG1 protein at late time points in response to cytokines or various TLR ligands, what might reflect that IRG1 is active during the anti-inflammatory DC mode. Furthermore, we found that supernatants collected from IRG1-deficient DCs led to enhanced proliferation and viability of CD8⁺ T cells as well as elevated numbers of the T cell subsets Th1, Th17 and Tregs, compared to supernatants from IRG1 expressing DCs. In addition, HPLC/MS analysis revealed the presence of various metabolites as GTP in the supernatant of IRG1 expressing DCs, pointing at immune suppressive molecules being released by DCs.

Very recently, IRG1 was identified as an enzyme linked to the TCA cycle by catalyzing the production of an antimicrobial metabolite, termed itaconic acid, in macrophages. Our data as well as the discovery of IRG1 catalyzing the formation of an antimicrobial metabolite support the idea that these IRG1 produced metabolites have an immune-modulatory role. In this context, it would be interesting to test the effects of these metabolites, by adding them directly into T cell cultures. However, it remains unclear how the antimicrobial and

6 CONCLUSION

immunoregulatory activities of IRG1 can be linked. Since IRG1 silenced DCs were observed to be potent inducers of an immune response, inhibition of IRG1 might offer a novel therapeutic strategy for the treatment of cancer. Thus, research for identifying a potent inhibitor specific for IRG1 needs to be done. Revealing the crystal structure of IRG1 might be helpful in designing a potent clinically applicable IRG1 inhibitor. All in all, we need to carefully consider the biological function before targeting IRG1 in therapy. In this context the effects of IRG1 metabolites on the immune system in tumor mouse models needs to be examined.

Having investigated the regulation and function of IRG1 in dendritic cells, we moved one step closer to the goal of improving DC-based cancer vaccines. Moreover, this study underlines the importance of the interaction of two primordially different but closely overlapping systems, the metabolome and the immune system.

7 REFERENCES

7 REFERENCES

1. FAUCI, A.S., *HARRISON'S PRINCIPLES OF INTERNAL MEDICINE*. 17. ED. 2008, NEW YORK, N.Y.: MCGRAW-HILL MEDICAL PUB. DIVISION. 2 BD.
2. PEARCE, E.L. AND E.J. PEARCE, *METABOLIC PATHWAYS IN IMMUNE CELL ACTIVATION AND QUIESCENCE*. IMMUNITY, 2013. **38**(4): P. 633-43.
3. SHUAI, K. AND B. LIU, *REGULATION OF JAK-STAT SIGNALLING IN THE IMMUNE SYSTEM*. NAT REV IMMUNOL, 2003. **3**(11): P. 900-11.
4. VANDER HEIDEN, M.G., L.C. CANTLEY, AND C.B. THOMPSON, *UNDERSTANDING THE WARBURG EFFECT: THE METABOLIC REQUIREMENTS OF CELL PROLIFERATION*. SCIENCE, 2009. **324**(5930): P. 1029-33.
5. HANAHAN, D. AND R.A. WEINBERG, *HALLMARKS OF CANCER: THE NEXT GENERATION*. CELL, 2011. **144**(5): P. 646-74.
6. WALDMANN, T.A., *IMMUNOTHERAPY: PAST, PRESENT AND FUTURE*. NAT MED, 2003. **9**(3): P. 269-77.
7. BANCHEREAU, J., ET AL., *DENDRITIC CELLS AS VECTORS FOR THERAPY*. CELL, 2001. **106**(3): P. 271-4.
8. HSU, F.J., ET AL., *VACCINATION OF PATIENTS WITH B-CELL LYMPHOMA USING AUTOLOGOUS ANTIGEN-PULSED DENDRITIC CELLS*. NAT MED, 1996. **2**(1): P. 52-8.
9. STEINMAN, R.M. AND M. POPE, *EXPLOITING DENDRITIC CELLS TO IMPROVE VACCINE EFFICACY*. J CLIN INVEST, 2002. **109**(12): P. 1519-26.
10. ABBAS, A.K., A.H. LICHTMAN, AND S. PILLAI, *CELLULAR AND MOLECULAR IMMUNOLOGY*. 7. ED. 2012, PHILADELPHIA: SAUNDERS/ELSEVIER. X, 545 S.
11. JANEWAY, C.A., JR. AND R. MEDZHITOV, *INNATE IMMUNE RECOGNITION*. ANNU REV IMMUNOL, 2002. **20**: P. 197-216.
12. BANCHEREAU, J., ET AL., *IMMUNOBIOLOGY OF DENDRITIC CELLS*. ANNU REV IMMUNOL, 2000. **18**: P. 767-811.

7 REFERENCES

13. SHORTMAN, K. AND Y.J. LIU, *MOUSE AND HUMAN DENDRITIC CELL SUBTYPES*. NAT REV IMMUNOL, 2002. **2**(3): P. 151-61.
14. AKIRA, S. AND K. TAKEDA, *TOLL-LIKE RECEPTOR SIGNALLING*. NAT REV IMMUNOL, 2004. **4**(7): P. 499-511.
15. AKIRA, S., K. TAKEDA, AND T. KAISHO, *TOLL-LIKE RECEPTORS: CRITICAL PROTEINS LINKING INNATE AND ACQUIRED IMMUNITY*. NAT IMMUNOL, 2001. **2**(8): P. 675-80.
16. TAKEDA, K. AND S. AKIRA, *TLR SIGNALING PATHWAYS*. SEMIN IMMUNOL, 2004. **16**(1): P. 3-9.
17. RAWLINGS, J.S., K.M. ROSLER, AND D.A. HARRISON, *THE JAK/STAT SIGNALING PATHWAY*. J CELL SCI, 2004. **117**(PT 8): P. 1281-3.
18. MURRAY, P.J., *THE JAK-STAT SIGNALING PATHWAY: INPUT AND OUTPUT INTEGRATION*. J IMMUNOL, 2007. **178**(5): P. 2623-9.
19. SARAIVA, M. AND A. O'GARRA, *THE REGULATION OF IL-10 PRODUCTION BY IMMUNE CELLS*. NAT REV IMMUNOL, 2010. **10**(3): P. 170-81.
20. TRINCHIERI, G., *INTERLEUKIN-12 AND THE REGULATION OF INNATE RESISTANCE AND ADAPTIVE IMMUNITY*. NAT REV IMMUNOL, 2003. **3**(2): P. 133-46.
21. KAPSENBERG, M.L., *DENDRITIC-CELL CONTROL OF PATHOGEN-DRIVEN T-CELL POLARIZATION*. NAT REV IMMUNOL, 2003. **3**(12): P. 984-93.
22. MAZZONI, A. AND D.M. SEGAL, *CONTROLLING THE TOLL ROAD TO DENDRITIC CELL POLARIZATION*. J LEUKOC BIOL, 2004. **75**(5): P. 721-30.
23. LANGENKAMP, A., ET AL., *KINETICS OF DENDRITIC CELL ACTIVATION: IMPACT ON PRIMING OF TH1, TH2 AND NONPOLARIZED T CELLS*. NAT IMMUNOL, 2000. **1**(4): P. 311-6.
24. LUGER, R., ET AL., *TOLL-LIKE RECEPTOR 4 ENGAGEMENT DRIVES DIFFERENTIATION OF HUMAN AND MURINE DENDRITIC CELLS FROM A PRO- INTO AN ANTI-INFLAMMATORY MODE*. PLOS ONE, 2013. **8**(2): P. E54879.

7 REFERENCES

25. BANCHEREAU, J. AND R.M. STEINMAN, *DENDRITIC CELLS AND THE CONTROL OF IMMUNITY*. NATURE, 1998. **392**(6673): P. 245-52.
26. LUTZ, M.B., N. ROMANI, AND A. STEINKASSERER, *HANDBOOK OF DENDRITIC CELLS : BIOLOGY, DISEASES, AND THERAPIES*. 2006, WEINHEIM: WILEY-VCH ; [CHICHESTER : JOHN WILEY, DISTRIBUTOR].
27. MEMPEL, T.R., S.E. HENRICKSON, AND U.H. VON ANDRIAN, *T-CELL PRIMING BY DENDRITIC CELLS IN LYMPH NODES OCCURS IN THREE DISTINCT PHASES*. NATURE, 2004. **427**(6970): P. 154-9.
28. LUCKHEERAM, R.V., ET AL., *CD4(+)T CELLS: DIFFERENTIATION AND FUNCTIONS*. CLIN DEV IMMUNOL, 2012. **2012**: P. 925135.
29. WAN, Y.Y., *MULTI-TASKING OF HELPER T CELLS*. IMMUNOLOGY, 2010. **130**(2): P. 166-71.
30. ZHU, J. AND W.E. PAUL, *CD4 T CELLS: FATES, FUNCTIONS, AND FAULTS*. BLOOD, 2008. **112**(5): P. 1557-69.
31. ZOU, W. AND N.P. RESTIFO, *T(H)17 CELLS IN TUMOUR IMMUNITY AND IMMUNOTHERAPY*. NAT REV IMMUNOL, 2010. **10**(4): P. 248-56.
32. WOLF, A.M., ET AL., *INCREASE OF REGULATORY T CELLS IN THE PERIPHERAL BLOOD OF CANCER PATIENTS*. CLIN CANCER RES, 2003. **9**(2): P. 606-12.
33. JOSEFOWICZ, S.Z., L.F. LU, AND A.Y. RUDENSKY, *REGULATORY T CELLS: MECHANISMS OF DIFFERENTIATION AND FUNCTION*. ANNU REV IMMUNOL, 2012. **30**: P. 531-64.
34. KORN, T., ET AL., *IL-17 AND Th17 CELLS*. ANNU REV IMMUNOL, 2009. **27**: P. 485-517.
35. COX, M.A. AND A.J. ZAJAC, *SHAPING SUCCESSFUL AND UNSUCCESSFUL CD8 T CELL RESPONSES FOLLOWING INFECTION*. J BIOMED BIOTECHNOL, 2010. **2010**: P. 159152.

7 REFERENCES

36. SEDER, R.A. AND R. AHMED, *SIMILARITIES AND DIFFERENCES IN CD4+ AND CD8+ EFFECTOR AND MEMORY T CELL GENERATION*. NAT IMMUNOL, 2003. **4**(9): P. 835-42.
37. ODEGAARD, J.I. AND A. CHAWLA, *THE IMMUNE SYSTEM AS A SENSOR OF THE METABOLIC STATE*. IMMUNITY, 2013. **38**(4): P. 644-54.
38. VON BERGWELT-BAILDON, M.S., ET AL., *CD25 AND INDOLEAMINE 2,3-DIOXYGENASE ARE UP-REGULATED BY PROSTAGLANDIN E2 AND EXPRESSED BY TUMOR-ASSOCIATED DENDRITIC CELLS IN VIVO: ADDITIONAL MECHANISMS OF T-CELL INHIBITION*. BLOOD, 2006. **108**(1): P. 228-37.
39. GERRIETS, V.A. AND J.C. RATHMELL, *METABOLIC PATHWAYS IN T CELL FATE AND FUNCTION*. TRENDS IMMUNOL, 2012. **33**(4): P. 168-73.
40. MCGETTRICK, A.F. AND L.A. O'NEILL, *HOW METABOLISM GENERATES SIGNALS DURING INNATE IMMUNITY AND INFLAMMATION*. J BIOL CHEM, 2013. **288**(32): P. 22893-8.
41. FALLARINO, F., ET AL., *T CELL APOPTOSIS BY TRYPTOPHAN CATABOLISM*. CELL DEATH DIFFER, 2002. **9**(10): P. 1069-77.
42. BRONTE, V. AND P. ZANOVELLO, *REGULATION OF IMMUNE RESPONSES BY L-ARGININE METABOLISM*. NAT REV IMMUNOL, 2005. **5**(8): P. 641-54.
43. LEE, C.G., ET AL., *CLONING AND ANALYSIS OF GENE REGULATION OF A NOVEL LPS-INDUCIBLE CDNA*. IMMUNOGENETICS, 1995. **41**(5): P. 263-70.
44. CHEN, B., D. ZHANG, AND J.W. POLLARD, *PROGESTERONE REGULATION OF THE MAMMALIAN ORTHOLOG OF METHYLCITRATE DEHYDRATASE (IMMUNE RESPONSE GENE 1) IN THE UTERINE EPITHELIUM DURING IMPLANTATION THROUGH THE PROTEIN KINASE C PATHWAY*. MOL ENDOCRINOL, 2003. **17**(11): P. 2340-54.
45. PLASSMEIER, J., ET AL., *INVESTIGATION OF CENTRAL CARBON METABOLISM AND THE 2-METHYLCITRATE CYCLE IN CORYNEBACTERIUM GLUTAMICUM BY METABOLIC PROFILING USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY*. J BIOTECHNOL, 2007. **130**(4): P. 354-63.

7 REFERENCES

46. XIAO, W., ET AL., *EXPRESSION PROFILE OF HUMAN IMMUNE-RESPONSIVE GENE 1 AND GENERATION AND CHARACTERIZATION OF POLYCLONAL ANTISERUM*. MOL CELL BIOCHEM, 2011. **353**(1-2): P. 177-87.
47. CHEON, Y.P., ET AL., *IMMUNE-RESPONSIVE GENE 1 IS A NOVEL TARGET OF PROGESTERONE RECEPTOR AND PLAYS A CRITICAL ROLE DURING IMPLANTATION IN THE MOUSE*. ENDOCRINOLOGY, 2003. **144**(12): P. 5623-30.
48. DEGRANDI, D., ET AL., *THE PROINFLAMMATORY CYTOKINE-INDUCED IRG1 PROTEIN ASSOCIATES WITH MITOCHONDRIA*. J INTERFERON CYTOKINE RES, 2009. **29**(1): P. 55-67.
49. MICHELUCCI, A., ET AL., *IMMUNE-RESPONSIVE GENE 1 PROTEIN LINKS METABOLISM TO IMMUNITY BY CATALYZING ITACONIC ACID PRODUCTION*. PROC NATL ACAD SCI U S A, 2013. **110**(19): P. 7820-5.
50. DOHNAL, A.M., ET AL., *CD40 LIGATION RESTORES TYPE 1 POLARIZING CAPACITY IN TLR4-ACTIVATED DENDRITIC CELLS THAT HAVE CEASED INTERLEUKIN-12 EXPRESSION*. J CELL MOL MED, 2009. **13**(8B): P. 1741-50.
51. BASLER, T., ET AL., *MYCOBACTERIUM PARATUBERCULOSIS, MYCOBACTERIUM SMEGMATIS, AND LIPOPOLYSACCHARIDE INDUCE DIFFERENT TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF THE IRG1 GENE IN MURINE MACROPHAGES*. J LEUKOC BIOL, 2006. **79**(3): P. 628-38.
52. HOSHINO, K., ET AL., *DIFFERENTIAL INVOLVEMENT OF IFN-BETA IN TOLL-LIKE RECEPTOR-STIMULATED DENDRITIC CELL ACTIVATION*. INT IMMUNOL, 2002. **14**(10): P. 1225-31.
53. VAINCHENKER, W., A. DUSA, AND S.N. CONSTANTINESCU, *JAKs IN PATHOLOGY: ROLE OF JANUS KINASES IN HEMATOPOIETIC MALIGNANCIES AND IMMUNODEFICIENCIES*. SEMIN CELL DEV BIOL, 2008. **19**(4): P. 385-93.
54. REINHARDT, A. AND T. HUBBARD, *USING NEURAL NETWORKS FOR PREDICTION OF THE SUBCELLULAR LOCATION OF PROTEINS*. NUCLEIC ACIDS RES, 1998. **26**(9): P. 2230-6.

7 REFERENCES

55. MCBRIDE, H.M., M. NEUSPIEL, AND S. WASIAK, *MITOCHONDRIA: MORE THAN JUST A POWERHOUSE*. CURR BIOL, 2006. **16**(14): P. R551-60.
56. JURGENS, B., ET AL., *INTERFERON-GAMMA-TRIGGERED INDOLEAMINE 2,3-DIOXYGENASE COMPETENCE IN HUMAN MONOCYTE-DERIVED DENDRITIC CELLS INDUCES REGULATORY ACTIVITY IN ALLOGENEIC T CELLS*. BLOOD, 2009. **114**(15): P. 3235-43.
57. YU, H., M. KORTYLEWSKI, AND D. PARDOLL, *CROSSTALK BETWEEN CANCER AND IMMUNE CELLS: ROLE OF STAT3 IN THE TUMOUR MICROENVIRONMENT*. NAT REV IMMUNOL, 2007. **7**(1): P. 41-51.
58. PALUDAN, S.R., *SYNERGISTIC ACTION OF PRO-INFLAMMATORY AGENTS: CELLULAR AND MOLECULAR ASPECTS*. J LEUKOC BIOL, 2000. **67**(1): P. 18-25.
59. SCHMIDT, S.K., ET AL., *ANTIMICROBIAL AND IMMUNOREGULATORY PROPERTIES OF HUMAN TRYPTOPHAN 2,3-DIOXYGENASE*. EUR J IMMUNOL, 2009. **39**(10): P. 2755-64.
60. SIOUD, M., *INNATE SENSING OF SELF AND NON-SELF RNAs BY TOLL-LIKE RECEPTORS*. TRENDS MOL MED, 2006. **12**(4): P. 167-76.
61. SLEDZ, C.A., ET AL., *ACTIVATION OF THE INTERFERON SYSTEM BY SHORT-INTERFERING RNAs*. NAT CELL BIOL, 2003. **5**(9): P. 834-9.
62. SUI, H., ET AL., *SIRNA ENHANCES DNA-MEDIATED INTERFERON LAMBDA-1 RESPONSE THROUGH CROSSTALK BETWEEN RIG-I AND IFI16 SIGNALLING PATHWAY*. NUCLEIC ACIDS RES, 2013.
63. BOGUNOVIC, D., ET AL., *TLR4 ENGAGEMENT DURING TLR3-INDUCED PROINFLAMMATORY SIGNALING IN DENDRITIC CELLS PROMOTES IL-10-MEDIATED SUPPRESSION OF ANTITUMOR IMMUNITY*. CANCER RES, 2011. **71**(16): P. 5467-76.
64. REIS E SOUSA, C., A. SHER, AND P. KAYE, *THE ROLE OF DENDRITIC CELLS IN THE INDUCTION AND REGULATION OF IMMUNITY TO MICROBIAL INFECTION*. CURR OPIN IMMUNOL, 1999. **11**(4): P. 392-9.
65. VERHASSELT, V., ET AL., *BACTERIAL LIPOPOLYSACCHARIDE STIMULATES THE PRODUCTION OF CYTOKINES AND THE EXPRESSION OF COSTIMULATORY MOLECULES BY*

7 REFERENCES

HUMAN PERIPHERAL BLOOD DENDRITIC CELLS: EVIDENCE FOR A SOLUBLE CD14-DEPENDENT PATHWAY. J IMMUNOL, 1997. **158**(6): P. 2919-25.

66. KAWAMURA, K., ET AL., *DIFFERENTIATION, MATURATION, AND SURVIVAL OF DENDRITIC CELLS BY OSTEOPONTIN REGULATION.* CLIN DIAGN LAB IMMUNOL, 2005. **12**(1): P. 206-12.

67. SCHRODER, K., ET AL., *INTERFERON-GAMMA: AN OVERVIEW OF SIGNALS, MECHANISMS AND FUNCTIONS.* J LEUKOC BIOL, 2004. **75**(2): P. 163-89.

68. VAN MOURIK, M.S., N.S. MACKLON, AND C.J. HEIJNEN, *EMBRYONIC IMPLANTATION: CYTOKINES, ADHESION MOLECULES, AND IMMUNE CELLS IN ESTABLISHING AN IMPLANTATION ENVIRONMENT.* J LEUKOC BIOL, 2009. **85**(1): P. 4-19.

69. DUMMER, W., ET AL., *T CELL HOMEOSTATIC PROLIFERATION ELICITS EFFECTIVE ANTITUMOR AUTOIMMUNITY.* J CLIN INVEST, 2002. **110**(2): P. 185-92.

70. HOPKEN, U.E., ET AL., *THE RATIO BETWEEN DENDRITIC CELLS AND T CELLS DETERMINES THE OUTCOME OF THEIR ENCOUNTER: PROLIFERATION VERSUS DELETION.* EUR J IMMUNOL, 2005. **35**(10): P. 2851-63.

71. ZINKERNAGEL, R.M. AND H. HENGARTNER, *REGULATION OF THE IMMUNE RESPONSE BY ANTIGEN.* SCIENCE, 2001. **293**(5528): P. 251-3.

72. SATO, K., ET AL., *NITRIC OXIDE PLAYS A CRITICAL ROLE IN SUPPRESSION OF T-CELL PROLIFERATION BY MESENCHYMAL STEM CELLS.* BLOOD, 2007. **109**(1): P. 228-34.

73. STUEHR, D.J., *MAMMALIAN NITRIC OXIDE SYNTHASES.* BIOCHIM BIOPHYS ACTA, 1999. **1411**(2-3): P. 217-30.

74. GROHMANN, U., F. FALLARINO, AND P. PUCCETTI, *TOLERANCE, DCs AND TRYPTOPHAN: MUCH ADO ABOUT IDO.* TRENDS IMMUNOL, 2003. **24**(5): P. 242-8.

75. ZEECK, A., ET AL., *CHEMIE FÜR MEDIZINER.* 6., VÖLLIG ÜBERARB. AUFL. ED. 2005, MÜNCHEN ; JENA: ELSEVIER, URBAN UND FISCHER. XIV, 434 S.

76. STRELKO, C.L., ET AL., *ITACONIC ACID IS A MAMMALIAN METABOLITE INDUCED DURING MACROPHAGE ACTIVATION.* J AM CHEM SOC, 2011. **133**(41): P. 16386-9.

7 REFERENCES

77. TELANG, S., ET AL., *SMALL MOLECULE INHIBITION OF 6-PHOSPHOFRUCTO-2-KINASE SUPPRESSES T CELL ACTIVATION*. J TRANSL MED, 2012. **10**: P. 95.
78. PERNIS, A.B., *RHO GTPASE-MEDIATED PATHWAYS IN MATURE CD4+ T CELLS*. AUTOIMMUN REV, 2009. **8**(3): P. 199-203.
79. ZHENG, L.M., ET AL., *EXTRACELLULAR ATP AS A TRIGGER FOR APOPTOSIS OR PROGRAMMED CELL DEATH*. J CELL BIOL, 1991. **112**(2): P. 279-88.
80. TRAUTMANN, A., *EXTRACELLULAR ATP IN THE IMMUNE SYSTEM: MORE THAN JUST A "DANGER SIGNAL"*. SCI SIGNAL, 2009. **2**(56): P. PE6.
81. GORINI, S., ET AL., *REGULATION OF INNATE IMMUNITY BY EXTRACELLULAR NUCLEOTIDES*. AM J BLOOD RES, 2013. **3**(1): P. 14-28.
82. WELLER, M., M. HAAG, AND W. LAING, *GTP, AS WELL AS ATP, CAN ACT AS A SUBSTRATE FOR THE INTRINSIC PROTEIN KINASE ACTIVITY OF SYNAPTIC PLASMA MEMBRANES*. MOL CELL BIOCHEM, 1981. **40**(2): P. 75-85.
83. GYSBERS, J.W., ET AL., *EXTRACELLULAR GUANOSINE 5' TRIPHOSPHATE ENHANCES NERVE GROWTH FACTOR-INDUCED NEURITE OUTGROWTH VIA INCREASES IN INTRACELLULAR CALCIUM*. NEUROSCIENCE, 2000. **96**(4): P. 817-24.

8 LIST OF FIGURES

Figure 1.1: Life cycle of a dendritic cell.....	10
Figure 1.2: Toll-like receptor mediated immune responses.....	12
Figure 1.3: The JAK/STAT pathway.. ..	13
Figure 1.4: Differentiation of a pro- into an anti-inflammatory DC phenotype.....	15
Figure 1.5: Formation of an immunological synapse leads to T cell activation.....	16
Figure 1.6: Polarization of naïve CD4 ⁺ T cells.. ..	17
Figure 1.7: Schematic representation of oxidative phosphorylation, anaerobic and aerobic (Warburg effect) glycolysis.....	21
Figure 1.8: Suppressive tumor microenvironments affect proper T cell functionality.	22
Figure 1.9: Metabolic map of the methylcitrate cycle and tricarboxylic acid cycle.	23
Figure 1.10: siRNA-mediated silencing of selected candidate genes in human DCs.....	25
Figure 4.1: Localization of IRG1 in LPS stimulated DCs.	42
Figure 4.2: IRG1 expression kinetics in DCs stimulated with different TLR ligands and cytokines.	43
Figure 4.3: Production of cytokines upon TLR ligation.	45
Figure 4.4: Effect of JAK1/JAK2 inhibition on IRG1 expression.. ..	47
Figure 4.5: Phenotype of activated DCs targeted withIRG1 specific siRNA.....	49
Figure 4.6: Assessment of CD8 ⁺ T cell proliferation.. ..	51
Figure 4.7: Proliferation of CD8 ⁺ T cells upon co-culture with LPS activated IRG1-silenced DCs at different ratios.	52
Figure 4.8: Proliferation of CD8 ⁺ T cells upon treatment with cell-free supernatants of IRG1 deficient DCs.. ..	53
Figure 4.9: Annexin V staining of CD8 ⁺ T cells after treatment with supernatant of IRG1 deficient DCs.....	55
Figure 4.10: Differentiation of CD4 ⁺ T cells after treatment with supernatant of IRG1 deficient DCs.....	56
Figure 4.11: Analysis of differentially secreted molecules in the supernatants of IRG1 silenced DCs by HPLC.	58
Figure 4.12: Mass spectrometric analyzes of HPLC fractions (IRG1 ⁺ supernatant).....	59

9 LIST OF TABLES

Table 3.1 Erylysis buffer composition.....	27
Table 3.2: siRNAs used for the transfection procedure of DCs.....	28
Table 3.3: TLR ligands and cytokines used as stimuli for DC activation.....	30
Table 3.4: Co-culture conditions of DCs and T cells	31
Table 3.5: Supernatant dosages used for T cell incubation	32
Table 3.6: Co-culture setup for CD4+ T cell phenotyping	32
Table 3.7: Antibodies used for DC phenotyping	33
Table 3.8: Antibodies used for T cell phenotyping	33
Table 3.9: Master mix for cDNA reverse transcription.....	35
Table 3.10: Program for Thermocycler.....	35
Table 3.11: Master Mix for gene expression analysis	35
Table 3.12: Gene expression assays used for RT-PCR	36
Table 3.13: Reagents used for sample preparation	37
Table 3.14: Composition of stacking and separating gel for SDS-PAGE.....	37
Table 3.15: 10 x Running Buffer composition	37
Table 3.16: 1 x Transfer Buffer composition	38
Table 3.17: 1 x TBS composition	38
Table 3.18: Antibodies used for Western Blot.....	39

10 ABBREVIATIONS

Ann V	Annexin V
AP-1	activator protein 1
APC	antigen-presenting cell
ARG	arginase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CCL2	CC-chemokine ligand 2
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
DAPI	4', 6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethyl sulfoxide
ECMDB	E.coli Metabolome Database
FLA	flagellin
FoxP3	forkhead box P3
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HMDB	Human Metabolome Database
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IKK	I-kappa-B-kinase
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IRF3	interferon-regulatory factor 3
IRG1	immune-responsive gene 1
iTreg	induced T-regulatory cells
JAK/STAT	Janus kinase/ signal transducer and activator of transcription
JAKAFI	inhibitor of Janus kinases 1 and 2
JNK	c-Jun N-terminal kinase
LAG-3	lymphocyte-activation gene 3
LN	lymh node
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MS	mass spectrometry

MSCs	myeloid suppressor cells
MyD88	myeloid differentiation primary response protein 88
NAD	nicotinamide adenine dinucleotide
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
NOS	nitric-oxide synthase
NTC	non target control
o/n	over night
OVA	ovalbumin
PAMPs	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKR	serine–threonine protein kinase R
Poly (I:C)	polyinosinic:polycytidylic acid
R848	resiquimod
RNAi	RNA interference
ROR γ t	RAR-related orphan receptor gamma T
RT	room temperature
RT-PCR	realtime-polymerase chain reaction
SDS	sodium dodecyl sulfate
sIL-2RA	soluble IL-2 receptor alpha molecules
siRNA	small interfering RNA
SN	supernatant
SPF	specific pathogen free
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TCA	tricarboxylic acid cycle
TCR	T cell receptor
Tfh	follicular helper T cell
TGF- β	transforming growth factor β
Th	T helper cell
TIR	Toll-IL-1 R
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1	regulatory type 1 cells
Treg	regulatory T cells
	toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing
TRIF	interferon- β
UT	untransfected

11 ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my advisor Prof. Erhard Hofer at the University of Vienna, who agreed to supervise my master thesis and invited me to hold a presentation on my scientific work within his own researcher's group to give me input how to proceed during the last year.

I would like to thank Prof. Thomas Felzmann at the St. Anna Children's Cancer Research Institute (CCRI), for inviting me to join his research group about three years ago and for giving me advice or comfort whenever I had a problem.

I also want to thank my supervisor within my research group, Dr. Alexander M. Dohnal who gave me this exciting research topic for my master thesis. Thank you for giving me the possibility to work and think independently and to share and discuss my ideas with you and other scientists.

Furthermore, I want to thank all members of my research group, as well as the team members of Activartis and all other people at the CCRI for their support, friendship and patience during the last years.

A special thanks to my family and my friends especially Ulrich, Jacqueline, Nadia and Philipp who always helped and motivated me during my study. My particular thanks go to my boyfriend Christian, who always encouraged me especially in difficult times but also shared scientific knowledge with me.

12 CURRICULUM VITAE

Personal Information

Name: **Natalie Knapp**
Address: Rennbahnweg 46/6/32, 1220 Wien
Telephone: 0699/19434824
E-mail: netelie_k@yahoo.de
Date of Birth: 27.08.1989
Place of Birth: Vienna



Education

since APRIL 2011

MASTER STUDY – Molecular Biology – UNIVERSITY of VIENNA

Master position at the Laboratory of Tumor Immunology at the St. Anna Children's Cancer Research Institute, Zimmermannplatz 10, 1090 Vienna

Master Thesis: "Proposed metabolic function of Immune Responsive Gene 1 (IRG1) in terms of dendritic cell mediated immune suppression"

Supervisor: Prof. FELZMANN

Study areas: Immunology, Molecular medicine

MAY 2012

Internship at medical University of Vienna Department of Neuroimmunology

Supervisor: Prof. LASSMANN

Study areas: Iron in models for inflammation of the CNS

OKT 2007 – APR 2011

BACHELOR STUDY – Biology – UNIVERSITY of VIENNA

Bachelor position at the Laboratory of Tumor Immunology at the St. Anna Children's Research Institute, Zimmermannplatz 10, 1090 Vienna

Bachelor Thesis: "Intracellular staining of IL-10/IL-12 expressing human dendritic cells"

Supervisor: Prof. FELZMANN

Study areas: Immunology, Molecular medicine

AUG 2009

Internship at veterinary University of Vienna at Vetomics Technology Center

Supervisor: PROF. KLEIN

SEPT 1999 –JUN 2007

Bundesrealgymnasium with scientific focus
at Theodor Kramer Strasse 3 – Degree with distinction

Working Experience

JAN – FEB 2014

FH Campus Wien

Tutor within the Bachelor programme (cell biology course)

DEZ 2013

University of Vienna

Tutor within the Bachelor programme Nutrition Science and Biology
(general chemical course)

SEPT 2010 – AUG 2013

St. Anna Children's Cancer Research Institute, VIENNA

Bachelor student 09/2010 – 01/2011

part time job 06/2011 – 08/2012
working on dendritic cell-based cancer immunotherapy

Master student 09/2012 – 08/2013

MAY 2013 – JUN 2013

University of Vienna

Tutor within the Bachelor programme Nutrition Science and Biology
(general chemical course)

Skills

Foreign languages

German	native
Polish	excellent
English	excellent
Russian	basic

Computer skills

Knowledge in MS Office (Word, Excel, Power Point)
FlowJo (Treestar)
GraphPad Prism
Adobe Illustrator

Interests/Hobbies

travelling, painting (Acryl), music (piano, guitar), dancing (Salsa), foreign language

Publications

Romana Luger, Sneha Valookaran, **Natalie Knapp**, Caterina Vizzardelli, Alexander M. Dohnal, Thomas Felzmann **(2013)**: Toll-like receptor 4 engagement drives differentiation of human and murine dendritic cells from pro- into an anti-inflammatory mode. PLOS ONE. Volume 2, Issue 2 e54879

Congresses

07-08 JUN 2013	CCRI at the Interface: symposium and 25th anniversary Poster presentation: "Proposed metabolic function of Immune Responsive Gene 1 (IRG1) in terms of dendritic cell mediated immune suppression"
19- 20 JUN 2013	YSA – PHD Symposium at the Medical University of Vienna Poster presentation: "Proposed metabolic function of Immune Responsive Gene 1 (IRG1) in terms of dendritic cell mediated immune suppression"