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Tahvili, Sahar

2018

*Document Version:*

Publisher's PDF, also known as Version of record

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*Citation for published version (APA):*

Tahvili, S. (2018). *S100A9 in inflammatory disease: a potential target for amelioration*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Lund University: Faculty of Medicine.

*Total number of authors:*

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# S100A9 in inflammatory disease: a potential target for amelioration

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DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCES | LUND UNIVERSITY





# S100A9 in inflammatory disease: a potential target for amelioration

Sahar Tahvili



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DOCTORAL DISSERTATION

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To be defended on December 13, 2018 at 13.00  
in Belfragesalen, Biomedical Center, Lund, Sweden

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Organization LUND UNIVERSITY		Document name	
Department of experimental medical sciences Section for Immunology		Date of issue	
Author(s) Sahar Tahvili		Sponsoring organization	
Title and subtitle S100A9 in inflammatory disease: a potential target for amelioration			
<p><b>Abstract</b></p> <p>The quinoline-3-carboxamides (Q compounds) are a family of small molecules with immunomodulatory functions that have shown efficacy in various murine models of inflammatory diseases. One such compound has demonstrated anti-tumor effects in murine models. Q compounds bind to S100A9, thereby preventing its ligation to TLR4 and RAGE. S100A9 is a small molecule which has shown to have a role in establishment of tumors.</p> <p>In the first part of this thesis (paper I and II), effects of Q compounds during inflammation and cancer, with the focus on myeloid cells has been investigated. While, the focus of the second part (paper III and IV) was on the molecule S100A9. Given the important role of S100A9 during tumorigenesis, the aim was to evaluate the induction and expression of this molecule <i>in vivo</i> in the context of cancer.</p> <p>In paper I, the effect of the Q compound tasquinimod was evaluated on myeloid cells in a mouse mammary carcinoma tumor. Short-term treatment reduced the accumulation of inflammatory monocytes in the tumors. Depletion of this cell population using an anti-Gr1 antibody resulted in the comparable anti-tumor effect as treatment with tasquinimod during the first few days of tumor growth. Furthermore, long-term tasquinimod treatment reduced myeloid cell expansion in the spleen and made the frequency of precursor cells in spleen of tumor-bearing mice resemble the naïve state.</p> <p>In paper II, the effect of the Q compound paquinimod was studied in the spontaneous mouse model of type 1 diabetes (NOD mouse). Paquinimod was given to the NOD mice in drinking water in two different protocols: short-term and long-term treatment and disease development was monitored weekly. Paquinimod induced a dose-dependent reduction in incidence of diabetes, and delayed the onset of disease in both treatment strategies. Interestingly, the treated mice showed less destructed islets in their pancreas. Moreover, the treatment reduced number of splenic inflammatory monocytes and macrophages.</p> <p>In paper III, the formation of S100A9 homodimer under inflammatory conditions and cancer was investigated. The cellular source of S100A9 homodimer were shown to be CD11b<sup>+</sup>Gr1<sup>+</sup> cells. Given the fact that in order to act as a DAMP, S100A9 should reach extracellular space, presence of S100A9 homodimer in the extracellular milieu was shown. The presence of cells expressing only S100A9, and not both S100A8 and S100A9 was shown in spleens of tumor-bearing animals.</p> <p>In paper IV, the conditions that lead to <i>de novo</i> expression of S100A9 were studied. It was shown that <i>in vivo</i> environment induces S100A9 expression, and this induction is so dependent to this milieu that it was rapidly down-regulated after removal of the cells from <i>in vivo</i>. Hypoxia in tumor microenvironment promotes tumor progression and survival and do so mainly by activity of HIF-1 transcription factor which regulates expression of many genes involved in the process of tumorigenesis. However, providing hypoxic condition was not sufficient for induction of S100A9 expression <i>in vitro</i>. Combination of HIF-1<math>\alpha</math> (one component of the transcription factor HIF-1) stabilizer and cytokines did not induce S100A9 expression either.</p> <p>In summary, in the first part of this thesis we showed that treatment with Q compounds can reduce recruitment of monocytes to the site of inflammation. Given the important role of these cells in promoting development of inflammatory diseases and cancer, this observation may partially explain the ameliorating effects of the Q compounds in a broad range of disease models. Furthermore, the second part of the thesis shows the induction of formation of S100A9 homodimer <i>in vivo</i> under inflammatory conditions and cancer, which may create a positive feedback loop for propagation of inflammatory cascades. Our results also suggest that there is a requirement for a complex interplay of different factors <i>in vivo</i> for induction of S100A9 expression.</p>			
Key words Quinoline-3-carboxamide, myeloid cell, cancer, type 1 diabetes, S100A9			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language English	
ISSN and key title 1652-8220		ISBN 978-91-7619-723-3	
Recipient's notes	Number of pages		Price
	Security classification		

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*Sahar Tahvili*

Date 2018-11-07

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Sahar Tahvili



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Coverphoto features *Janus*, the roman GOD of beginnings, transitions and endings, who is usually depicted as having two faces.

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Lund University, Faculty of Medicine

Department of Experimental Medical Sciences

ISBN 978-91-7619-723-3

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University  
Lund 2018



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*To Mom and Dad*



# Table of Contents

Papers and manuscript included in this thesis .....	8
Abbreviations .....	9
Thesis summary .....	11
General view of the immune system .....	13
Pattern recognition receptors .....	13
Toll-like receptors .....	14
S100 family of proteins .....	15
Overview of inflammation .....	21
Myeloid-innate immune cells .....	22
Monocytes .....	22
Macrophages .....	23
Granulocytes .....	24
Myeloid cells in cancer .....	24
Type 1 diabetes .....	28
Non-diabetic mouse model .....	28
Myeloid cells in islets .....	29
Pathogenesis of T1D .....	30
Treatment of T1D .....	30
Quinoline-3-carboxamides .....	32
Binding targets of the Q compounds .....	32
Q compounds in models of inflammation .....	33
Q compounds in tumor models .....	34
Q compounds in clinical trials .....	35
Aims .....	37

Summaries and discussions of Papers I-IV .....	38
Paper I .....	38
Paper II .....	42
Paper III.....	47
Paper IV .....	50
Acknowledgements .....	54
References .....	56

# Papers and manuscript included in this thesis

## Paper I

The anti-tumor effect of the quinoline-3-carboxamide tasquinimod: blockade of recruitment of CD11b<sup>+</sup>Ly6C<sup>hi</sup> cells to tumor tissue reduces tumor growth

Deronic A, Tahvili S, Leanderson T, Ivars F.

BMC Cancer. 2016 Jul 11;16:440

## Paper II

Paquinimod prevents development of diabetes in the non-obese (NOD) mouse

Tahvili S, Törngren M, Holmberg D, Leanderson T, Ivars F.

PLoS One, 2018 May 9;13(5)

## Paper III

Induction of S100A9 homodimer formation *in vivo*

Källberg E, Tahvili S, Ivars F, Leanderson T.

Biochem Biophys Res Commun. 2018 Jun 7;500:564

## Paper IV

HIF-1 $\alpha$  alone is not sufficient to induce S100A9 expression in cancer cells

Tahvili S, Källberg E, Ivars F, Leanderson T.

*Manuscript in preparation*

Paper III is reprinted with permission from the publisher.

# Abbreviations

**AA** Arachidonic acid

**AP-1** activating protein-1

**Arg-1** arginase-1

**ARNT** aryl hydrocarbon receptor  
nuclear translocator

**APC** antigen presenting cell

**BM** bone marrow

**Ca<sup>2+</sup>** calcium

**CaBP** Ca<sup>2+</sup>-binding protein

**CCR** CC chemokine receptor

**CXCR** CXC chemokine receptor

**CDP** common DC progenitor

**cMoP** common monocyte progenitor

**CNS** central nervous system

**CTLA-4** cytotoxic T lymphocyte  
antigen-4

**DAMP** damage-associated molecular  
pattern

**DC** dendritic cell

**DMOG** dimethyloxalglycine

**DSS** disuccinimidyl suberate

**EAE** experimental autoimmune  
encephalomyelitis

**EHM** extramedullary hematopoiesis

**G-CSF** granulocyte colony-  
stimulating factor

**GM-CSF** granulocyte monocyte  
colony-stimulating factor

**HDAC4** histone deacetylase 4

**HIF-1 $\alpha$**  hypoxia inducible factor 1- $\alpha$

**HMGB1** high mobility group box 1

**HRE** hypoxia response elements

**HSC** hematopoietic stem cell

**HUVEC** human umbilical vein  
endothelial cell

**IBD** inflammatory bowel disease

**IL** interleukin

**iNOS** inducible nitric oxide synthase

**IRAK** IL-1 receptor associated -  
kinase

**IRF** interferon-regulatory factor

**LBP** LPS-binding protein

**LPS** lipopolysaccharide

**M-CSF** monocyte colony-  
stimulating factor

**MD-2** myeloid differentiation-2

**MDP** macrophage and DC progenitor

**MDSC** myeloid-derived suppressor  
cell

**MHC** major histocompatibility  
complex

**MRP-8** myeloid-related protein 8

**MRP-14** myeloid-related protein 14

**MS** multiple sclerosis

**MYD88** myeloid differentiation  
primary response protein 88

**NADPH** nicotinamide adenine  
dinucleotide phosphate

**NCoR** nuclear receptor corepressor  
**NET** neutrophil extracellular trap  
**NOD** non-obese diabetic  
**NOX** NADPH oxidase enzyme complex  
**NF- $\kappa$ B** nuclear factor- $\kappa$ B  
**NK** natural killer  
**NLR** NOD-like receptor  
**NO** nitric oxide  
**PAMP** pathogen-associated molecular pattern  
**panLN** pancreatic lymph node  
**pDC** plasmacytoid dendritic cell  
**PKC** protein kinase C  
**PRR** pattern recognition receptor  
**RAGE** receptor for advanced glycation end products  
**ROS** reactive oxygen species

**SLE** systemic lupus erythematosus  
**SPR** surface plasmon resonance  
**T1D** type 1 diabetes  
**T1DM** type 1 diabetes mellitus  
**TAM** tumor-associated macrophage  
**TGF- $\beta$**  transforming growth factor  $\beta$   
**TIR** Toll/IL-1 receptor  
**TIRAP** TIR domain-containing adaptor protein  
**TLR** Toll-like receptor  
**TNF $\alpha$**  tumor necrosis factor- $\alpha$   
**Treg** regulatory T cell  
**TRIF** TIR-domain containing adaptor protein inducing IFN $\beta$   
**TSP1** thrombospondin-1  
**WB** western blot  
**WT** wild type

# Thesis summary

The quinoline-3-carboxamides (Q compounds) are a family of small molecules with immunomodulatory functions that have shown efficacy in various murine models of inflammatory diseases. One such compound has demonstrated anti-tumor effects in murine models. Q compounds bind to S100A9, thereby preventing its ligation to TLR4 and RAGE. S100A9 is a small molecule which has shown to have a role in establishment of tumors.

In the first part of this thesis (paper I and II), effects of Q compounds during inflammation and cancer, with the focus on myeloid cells has been investigated. While, the focus of the second part (paper III and IV) was on the molecule S100A9. Given the important role of S100A9 during tumorigenesis, the aim was to evaluate the induction and expression of this molecule *in vivo* in the context of cancer.

In paper I, the effect of the Q compound tasquinimod was evaluated on myeloid cells in a mouse mammary carcinoma tumor. Short-term treatment reduced the accumulation of inflammatory monocytes in the tumors. Depletion of this cell population using an anti-Gr1 antibody resulted in the comparable anti-tumor effect as treatment with tasquinimod during the first few days of tumor growth. Furthermore, long-term tasquinimod treatment reduced myeloid cell expansion in the spleen and made the frequency of precursor cells in spleen of tumor-bearing mice resemble the naïve state.

In paper II, the effect of the Q compound paquinimod was studied in the spontaneous mouse model of type 1 diabetes (NOD mouse). Paquinimod was given to the NOD mice in drinking water in two different protocols: short-term and long-term treatment and disease development was monitored weekly. Paquinimod induced a dose-dependent reduction in incidence of diabetes, and delayed the onset of disease in both treatment strategies. Interestingly, the treated mice showed less destructed islets in their pancreas. Moreover, the treatment reduced number of splenic inflammatory monocytes and macrophages.

In paper III, the formation of S100A9 homodimer under inflammatory conditions and cancer was investigated. The cellular source of S100A9 homodimer were shown to be CD11b<sup>+</sup>Gr1<sup>+</sup> cells. Given the fact that in order to act as a DAMP, S100A9 should reach extracellular space, presence of S100A9 homodimer in the

extracellular milieu was shown. The presence of cells expressing only S100A9, and not both S100A8 and S100A9 was shown in spleens of tumor-bearing animals.

In paper IV, the conditions that lead to *de novo* expression of S100A9 were studied. It was shown that *in vivo* environment induces S100A9 expression, and this induction is so dependent to this milieu that it was rapidly down-regulated after removal of the cells from *in vivo*. Hypoxia in tumor microenvironment promotes tumor progression and survival and do so mainly by activity of HIF-1 transcription factor which regulates expression of many genes involved in the process of tumorigenesis. However, providing hypoxic condition was not sufficient for induction of S100A9 expression *in vitro*. Combination of HIF-1 $\alpha$  (one component of the transcription factor HIF-1) stabilizer and cytokines did not induce S100A9 expression either.

In summary, in the first part of this thesis we showed that treatment with Q compounds can reduce recruitment of monocytes to the site of inflammation. Given the important role of these cells in promoting development of inflammatory diseases and cancer, this observation may partially explain the ameliorating effects of the Q compounds in a broad range of disease models. Furthermore, the second part of the thesis shows the induction of formation of S100A9 homodimer *in vivo* under inflammatory conditions and cancer, which may create a positive feedback loop for propagation of inflammatory cascades. Our results also suggest that there is a requirement for a complex interplay of different factors *in vivo* for induction of S100A9 expression.

# General view of the immune system

The first responses towards invading pathogens are provided by the innate immune system. Many biological mechanisms and cell subsets are included in this first line of defense: the epithelial barriers of the skin, respiratory tract, mucosa and complement system, and also several cell subsets such as monocytes, macrophages, granulocytic cells, natural killer (NK) cells, and  $\gamma\delta$  T cells. In addition to that, if necessary, innate immune system can provide the means for activation of the adaptive immune system which provides responses that are more specifically directed against pathogens.

The pluripotent stem cell which is located in the bone marrow (BM) can generate two leukocyte progenitors: the common lymphoid, and common myeloid progenitor. Cells of the innate immune system originate from the common myeloid progenitor, while cells of the adaptive immune system are derived from common lymphoid progenitor [1].

Innate immunity provides a rapid response within a few minutes or hours towards evolutionarily conserved structures of the pathogens. Cells of innate immune system activate the adaptive immune response by presenting the antigens from pathogens, a process called antigen presentation. The adaptive immune system displays specificity towards a broad range of molecules and has the ability to provide long lasting immunity towards pathogens, so called immunological memory.

## Pattern recognition receptors

Cells of the innate immune system are equipped with arrays of receptors which, although are not specific towards particular antigens, make them capable of recognizing patterns that are conserved among a wide group of pathogens. These receptors are called pattern recognition receptors (PRR) and include several classes, such as the cell surface Toll-like receptors (TLR) or the cytoplasmic NOD-like receptors (NLR). PRRs are responsible for sensing danger signals [2], which are composed of pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) or alarmins [3]. PAMPs are highly conserved structures of microorganisms like bacterial lipopolysaccharides (LPS),



glycolipids or viral nucleic acids. DAMPs include several cellular products in the host with physiological roles that are normally found within the cells and when released can acquire additional functions; alerting immune system about danger. Release of DAMPs can happen both as a result of necrosis and active secretion [4]. The DNA from damaged host cells, high mobility group box 1 (HMGB1), uric acid, heat shock proteins (HSP) and S100 proteins can all act as DAMPs [5-8]. Interaction between PRR and DAMP or PAMP leads to a series of signaling cascades which result in production of inflammatory mediators that assist in the elimination of pathogens. This response is sometimes accompanied by tissue damage [9].

## **Toll-like receptors**

Toll-like receptor (TLR) proteins constitute an evolutionarily highly conserved family of PRRs that are named due to their homology to the *Drosophila* Toll protein [9]. They play important roles in host defense against pathogens in organisms from *Drosophila* to humans [9]. TLRs contain extracellular leucine-rich repeats that bind to antigens, the transmembrane domain, and cytoplasmic Toll/IL-1 receptor (TIR) domain which is required for initiating the intracellular signaling [10]. Upon antigen binding to TLR, one or more adaptor proteins are recruited to the cytoplasmic domain of TLRs. This association happens through homophilic interaction between TIR domains present in both TLR and adaptor protein. Examples of adaptor proteins include myeloid differentiation primary response protein 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), and TIR-domain containing adaptor protein inducing IFN $\beta$  (TRIF). MYD88 is a central adaptor recruited by all members of the TLR family, except for TLR3 that recruits TRIF. TRIF recruitment and the following signal cascades results in induction of type I interferon, in particular IFN $\beta$ . Among TLRs, TLR4 is an exception in that it can activate both MYD88- and TRIF-mediated signaling pathways. MyD88 recruits members of the IL-1 receptor associated kinase (IRAK) family [10]. Although the downstream signaling pathways of MYD88 and TRIF differ, activation of both lead to transcriptional activation of inflammation-related genes via nuclear factor- $\kappa$ B (NF- $\kappa$ B), activating protein-1 (AP-1) and interferon-regulatory factors (IRFs) (Reviewed in [10]). By combining different adaptor proteins, TLRs can mediate alternative pathways in order to initiate immune responses.

TLR4 is expressed on the cell surface of both hematopoietic and non-hematopoietic cells, such as endothelial cells, cardiac myocytes, and cells of the central nervous system (CNS) [11]. TLR4, as other TLRs is composed of an extracellular and intracellular domain which is involved in the intracellular signaling cascade [12]. TLR4/MD-2 receptor complex is composed of TLR4 and myeloid differentiation 2 (MD-2) on the cell surface. MD-2 lacks transmembrane and intracellular domains and is non-covalently associated with TLR4. It is required for ligand-induced

activation of TLR4. Other accessory molecules that enhance the ligand sensing by TLR4 are CD14 and LPS-binding protein (LBP) [11].

## **S100 family of proteins**

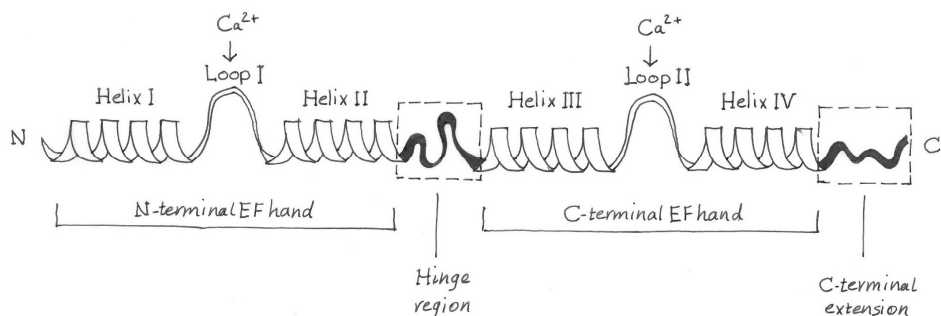
S100 proteins are the largest group of EF-hand signaling  $\text{Ca}^{2+}$ -binding proteins (CaBPs) in humans [13]. These acidic proteins are relatively small (9-14 kDa), and are exclusively expressed in vertebrates [13]. Among all genes in humans, 22 are clustered at the chromosome locus 1q21 [14]. This cluster is close to two important chromosomal regions: the epidermal differentiation complex [15], and the epidermal susceptibility region locus [16]. This proximity indicates the potential involvement of S100 genes in inflammatory and neoplastic disorders. Discovery of S100 proteins goes back to 1965 when B. W. Moore and his colleagues were purifying proteins from bovine CNS and found out that there are some proteins in the mixture that were still soluble in 100% saturated ammonium sulfate at neutral PH, and therefore named them S100 proteins [17].

Calcium ( $\text{Ca}^{2+}$ ) is an intracellular second messenger that controls a broad range of cellular processes such as cell cycle progression, differentiation, and different enzymatic activities, etc. As such, the intracellular concentration of  $\text{Ca}^{2+}$  must be tightly controlled. Some of the regulatory roles of  $\text{Ca}^{2+}$  are done by interaction with CaBPs [18]. One category of CaBPs form membrane-bound channels and keep the intracellular concentrations of  $\text{Ca}^{2+}$  around 100nM which is the case in resting conditions, and achieve this by pumping excess of  $\text{Ca}^{2+}$  to extracellular space or intracellular stores. This prevents formation of  $\text{Ca}^{2+}$  precipitation in cytoplasm or start of unwanted signaling cascades (reviewed in [19]). Another group of CaBPs reside within intracellular stores and have low affinity, yet high avidity for  $\text{Ca}^{2+}$ . Thus, they can quickly release  $\text{Ca}^{2+}$  upon need. Other CaBPs display high  $\text{Ca}^{2+}$ -binding affinity but low avidity, therefore they can either act as buffers of  $\text{Ca}^{2+}$  during the course of  $\text{Ca}^{2+}$  transients or transduce  $\text{Ca}^{2+}$  signals. The latter constitutes of a large fraction of CaBPs, which upon binding to  $\text{Ca}^{2+}$  interact with other protein targets, and therefore as signaling proteins may regulate many cellular functions. Most S100 proteins belong to this group (reviewed in [19]). S100 proteins have a broad range of activities inside and outside the cells, where they can act in autocrine and paracrine manner.

### ***Structure of S100 proteins***

S100 proteins carry two  $\text{Ca}^{2+}$ -binding EF-hand motifs, which are separated by a central hinge (also called linker) region. Each EF-hand is consisted of two  $\alpha$ -helices and a loop with  $\text{Ca}^{2+}$  binding capacity (Figure 1). The C terminal EF-hand is called canonical EF-hand because it is similar to EF-hands found in other  $\text{Ca}^{2+}$  sensors. The N-terminal EF-hand is also called pseudo-canonical or S100 EF-hand. Loop I

is flanked by Helices I and II, while Loop II is flanked by helices III and IV. The amino terminal loop has lower affinity for  $\text{Ca}^{2+}$  than the loop in the C-terminal EF-hand (reviewed in [19]). A string of amino acids, called the C-terminal extension is located at the carboxyl terminus of the protein. The least conserved sequence homology among S100 proteins belongs to hinge region and C-terminal extension, which therefore might contribute to the specificity of S100 proteins. While as expected, the two  $\text{Ca}^{2+}$ -binding loops show the highest sequence identity (reviewed in [13]). The other characteristic of S100 proteins is that they have the ability to form homodimers, heterodimers and higher ranks of oligomers, which also means that their subunits are exchangeable to each other (reviewed in [20]).



**Figure 1.**

Schematic representation of the secondary structure of S100 proteins in a monomer form. Each monomer consists of two EF hands that are connected by hinge region.

Upon binding to  $\text{Ca}^{2+}$ , helix III undergoes reorientation, while helix IV does not move. This conformational change opens the structure and exposes a wide hydrophobic cleft which serves as a site for interaction with target proteins. The cleft is composed of hinge region, helix III and loop II (reviewed in [19]).

The specific cell- and tissue-expression pattern in S100 proteins contribute to their functional diversities. Furthermore, having different ion binding properties, different localization in cellular compartments, and the ability to form oligomers add up to the variations seen in their functions [14].

Among the S100 proteins, S100A8 and S100A9 is of particular interest of this thesis.

### ***S100A8 and S100A9***

S100A8 also known as calgranulin A and myeloid-related protein 8 (MRP8) comprises 93 amino acids [21]. Deletion of the S100A8 gene in the mouse embryo is lethal, which emphasizes on its important and non-redundant function [22].

S100A9 also known as calgranulin B and myeloid-related protein 14 (MRP14) contains 114 amino acids [21]. Targeted deletion of S100A9 in mice results in a loss of both the S100A8 and S100A9 proteins, probably due to a higher turnover of isolated S100A8 in the absence of S100A9 [23, 24]. In models of LPS-induced shock as well as E.coli-induced abdominal sepsis, S100A9 deletion gives the mice a protective phenotype [25].

A truncated form of human S100A9, which lacks 4 amino acids from the N-terminal due to alternative translation, also exists within granulocytes [26]. Both S100A8 and S100A9 proteins can form homodimers, heterodimers and for S100A9 even higher order complexes [26, 27]. However, S100A9 preferentially exists as a heterodimer with S100A8 [28].

Formation of the S100A8/A9 heterodimer is not  $\text{Ca}^{2+}$ -dependent [29]. However, binding of  $\text{Ca}^{2+}$  to the heterodimer opens up the cleft, which makes the heterodimer capable of binding to its targets [19].

The S100A8/A9 complex is also known as calprotectin [30, 31] because of its role in protection against microbial infection such as *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermis* [32]. In human, the S100A8 and S100A9 constitute up to 40% of cytosolic proteins in neutrophils [33, 34] and 5% in monocytes [33], and the expression disappears with monocyte's maturation [34]. It has also been found in dendritic cells (DCs) [35] and platelets [36]. Moreover, under inflammatory conditions, expression of S100A8/A9 can be induced in epithelial cells, keratinocytes [37], microvascular endothelial cells [38] and macrophages [21, 39]. The heterodimer induces an inflammatory and prothrombotic response in endothelial cells *in vitro* [40].

Depending on the conditions, S100A8 and S100A9 can be found in the cytoplasm, nucleus, and plasma membrane or be secreted to the extracellular environment, where they play different functional roles [41].

The S100A8/A9 heterodimer promotes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activation in phagocytes [42]. NOX, a multicomponent enzyme system, is one of the major host defense systems, responsible for the production of reactive oxygen species (ROS). ROS play important role in the anti-microbial activity of neutrophils. NOX constitutes of cytochrome  $b_{558}$ , Rho guanosine triphosphate,  $p40^{phox}$ ,  $p47^{phox}$  and  $p67^{phox}$ . NOX binds to plasma membrane or membrane of phagosomes (Reviewed in [43]). In neutrophils, binding of opsonized particles to their respective receptors leads to activation of downstream signaling pathways and release of  $\text{Ca}^{2+}$  from intracellular stores. This further leads to the activation of  $\text{Ca}^{2+}$  channels in plasma membrane and influx of even more  $\text{Ca}^{2+}$  ion to the cytosol [44]. This elevated  $\text{Ca}^{2+}$  level mediates the recruitment of S100A8/A9 to the phagosomal membrane. The translocation of

S100A8/A9 results in the transfer of arachidonic acid (AA) to S100A9 [45], and later to cytochrome *b*<sub>558</sub>, which triggers NADPH oxidase activation [42]. Subsequent production of superoxide anion radicals (O<sub>2</sub><sup>•-</sup>) leads to an array of enzymatic and non-enzymatic reactions, and production of ROS [43]. In addition to having a role in NOX activity, the cytoplasmic S100A8/A9 complex can bind cytoskeletal filaments and participate in microtubule polymerization, and hence migration [46]. Human S100A9 can be phosphorylated [28] through MAPK-dependent pathway [46, 47], which further leads to translocation of S100A8/A9 to cell matrix, and reduction in microtubule polymerization [46, 47].

Most of the time S100A8/A9 are found within the cytoplasm, however they can also transfer to the nucleus. As an example, in psoriasis mouse model S100A9 has been detected binding to the promoter region of the complement factor C3 gene, suggesting a regulatory role for S100A9 in C3 gene expression [48].

S100A8 and S100A9 proteins can be secreted extracellularly where they can contribute in amplification of pro-inflammatory response. Cytosolic S100A8 and S100A9 lack leader sequences and therefore cannot be secreted via the classical Golgi pathway. Both can be released from necrotic cells, therefore the major source of extracellular S100A8 and S100A9 during inflammation may be dying neutrophils. By binding to metals such as Zn<sup>2+</sup> and Mn<sup>2+</sup>, the heterodimer complex inhibits bacterial growth [49]. In addition to ion chelation, upon release to extracellular environment, S100A8 and S100A9 can bind to different receptors and hence play various biological roles. S100A8/A9 contributes in a feed-forward process during phagocytosis. Ligation of the heterodimer present in the extracellular milieu with the receptor for advanced glycation end products (RAGE) on neutrophils surface, leads to the activation of the transcription factor, NF-κB, and hence production of both S100A8 and S100A9. This interaction also mediates recruitment of leukocytes to inflammatory sites [50]. In addition to TLR4 and RAGE, S100A9, but not S100A8 has been shown to bind to the receptor EMMPRIN (CD147/BASIGIN) [51]. This interaction induces tumor migration as well as expression of matrix metalloproteinases and cytokines [51]. Furthermore, in myelodysplastic syndrome, S100A9 was shown to be the ligand for CD33 on myeloid cells, which induces secretion of IL-10 and TGF-β and contributes to expansion of MDSC [52].

In addition to release from necrotic cells, other investigators suggested that in human monocytes and following the activation of protein kinase C (PKC), S100A8/A9 heterodimers are released through an energy-consuming pathway which also depends on an intact microtubule network [53].

S100A8, S100A9, S100A8/A9 have all been shown to bind to the receptors TLR4 and RAGE [25, 54, 55]. Both TLR4 and RAGE are typical PRRs with the ability to bind to multiple DAMPs. In the study by Vogl. et. al. it was reported that human

S100A8/A9 and also human S100A8, but not S100A9 are the actual DAMPs capable of inducing production of pro-inflammatory cytokines, while human S100A9 possess a regulatory function, by downregulating the activity of S100A8 through dimerization with it [25]. However, the role of mouse and human S100A9 as a bona fide DAMP by induction of cytokine production via TLR4 stimulation has been shown in several studies [56-60]. Whatever the source of these discrepancies be, both S100A8 and S100A9 and also the heterodimeric form can be considered as DAMP molecules through their interaction with TLR4 and RAGE [25, 55]. However, the high serum levels of S100A8/A9 in patients with inflammatory conditions, make it difficult to envision the heterodimer as a potent ligand for TLR4, which otherwise could lead to overt inflammation and septic shock. On the other hand, it has been proposed recently that activity of the S100A8/A9 heterodimer is locally restricted at the sites of inflammation by formation of (S100A8/A9)<sub>2</sub> tetramer which sequesters the TLR4-binding site in the molecule and therefore prevents undesirable effects in non-inflamed organs [61]. Some studies suggest that S100A8 and S100A9 homodimer forms are the biologically active forms and formation of the heterodimer neutralized its activity [25, 62]. The alternative would be that the heterodimer form is only active at the sites of inflammation but not in blood.

There are some differences regarding stability between the mouse and human S100A8 and S100A9. In the S100A9<sup>-/-</sup> mice, the S100A8 mRNA is expressed to the extent that can be seen in the WT mice, however the protein is not detectable, which indicates the need for S100A9 for stabilization of S100A8 protein [23, 24]. Another proof for that is the study showing instability of the mouse S100A8 protein in absence of S100A9 in transfected cells lines which don't express S100A8 and S100A9 endogenously (unpublished data from our group). In the same study it was shown that co-expression of S100A9 with S100A8 can rescue S100A8 protein. However, it is the human S100A9 that actively degrades in the absence of S100A8 [63]. Inhibition of proteosomal degradation and co-expression with S100A8 are known to rescue human S100A9 protein [63]. All in all, we can conclude that presence of S100A8 along with S100A9 is important for stability of both proteins, which could further explain the reason behind their preference towards heterodimer formation. Importantly, it was shown that presence of the pro-inflammatory cytokine, IL-1 $\beta$  not only stabilizes expression of S100A9, but also induces formation of the S100A9 homodimer [63]. The speculation could be that *in vivo* and in presence of pro-inflammatory cytokines, S100A9 homodimer could be produced, which may act as a DAMP molecule and amplify the ongoing pro-inflammatory responses as well as promoting tumor growth which will be discussed further.

The link between S100 proteins and myeloid-derived suppressor cells (MDSC) was first proposed by Cheng et. al. [64]. MDSC are a heterogeneous group of immune cells that accumulates in tumor-bearing hosts and in response to inflammation and

present CD11b and Gr1 on their surface. They are known to play strong immunosuppressive role in cancer. Their mechanism of action will be discussed further in this thesis. By generation of ROS, S100A9 inhibits myeloid cell differentiation, thereby promotes accumulation of MDSC in pathological processes, and contributes to tumor growth [64]. S100A8/A9 inhibits casein kinase I and II [65], which suggests their role in differentiation of myeloid cells [66]. In the EL-4 tumor model in S100A9<sup>-/-</sup> mice, no accumulation of MDSC was detected [64]. In the same study, it was shown that tumor-derived factors could upregulate S100A9 expression in myeloid cells, which led to inhibition of differentiation in myeloid cells, and therefore promoting accumulation of MDSC in the tumor site [64]. Moreover, ligation of extracellular S100A8/A9 to RAGE on the surface of myeloid cells, results in accumulation of MDSC to tumor tissue [55]. S100A8 and S100A9 are potent chemoattractants for MDSC and have been implicated in the promotion of tumor growth and metastasis by MDSC [67-69]. Therefore, MDSC recruitment to tumor sites may represent a vicious circle: tumor-derived chemokines recruit MDSC to tumor site, and the infiltrated MDSC produce more of S100A8/A9, thereby facilitate recruitment of more MDSC.

Expression of S100A8 and S100A9 has for some time been associated with inflammatory diseases and cancer [5, 41, 70]. Differential expression of S100A8 and S100A9 has been shown to contribute to the development and progression of various types of cancer, such as pancreatic adenocarcinoma [71], bladder cancers [72], and breast cancers [73]. Higher degrees of S100A8 and S100A9 expression has been correlated with invasiveness of tumors. For example, while the non-invasive MCF-7 breast cancer cell line is negative for S100A9, its gene expression can be induced by cytokine oncostatin M through the STAT3 signaling [74]. In serum of patients with prostate cancer compared to benign prostate hyperplasia, S100A8 and S100A9 show elevated levels, and therefore have been suggested to serve as a diagnostic marker [75]. Interestingly, there are high correlations between plasma concentrations of S100A8/A9 and clinical as well as laboratory markers of inflammation [76]. Moreover, the levels reach the steady state following clinical improvement. As an example, S100A8 and S100A9 concentration in serum of patients with systemic inflammatory response and sepsis is significantly higher compared with healthy controls [77].

S100A8/A9 has been shown to exert apoptotic activity against tumor cells, and this potential makes it an attractive tool in cancer therapy (reviewed in [78]). However, it bears the risk for immune escape mechanisms by tumors because of the potential effect on promoting the functions of MDSC [69]. Further investigation should consider both side of the same coin when designing therapeutic strategies using these molecules. Among the compounds that target S100A9 protein, I focus on the quinoline-3-carboxamide family of compounds, which is discussed later in this section.

## Overview of inflammation

Inflammation is part of the non-specific immune response, which is triggered by certain non-self-antigens or by self-components released upon tissue damage, the latter is called sterile inflammation [7]. As a result of cellular stimulation, pro-inflammatory cytokines and chemokines are released. In minor and local concentrations, these mediators can have autocrine and paracrine effects, while if released in large quantities, they can exert endocrine effects. The additional supply of immune cells to the site of infection is provided by chemokines [6, 7]. The fundamental signs of inflammation (redness, heat, pain, swelling, and loss of function) happen due to increased blood flow, increase in cellular metabolism, release of soluble mediators from cells, and vasodilation, which in turn leads to influx of immune cells to the site of damage. The innate immune system, by mediating the very first response, plays an essential role in both acute and chronic inflammation [79]. Interaction between PAMP or DAMP with PRRs results in release of various pro-inflammatory cytokines, and also chemokines which attract immune cells to the site of injury. The manifestations of inflammation are the consequences of these interactions. Monocytes and macrophages have essential role in both acute and chronic inflammation, and the hallmark of chronic inflammation, fibrosis, is due to their activity in this process [2].

Under normal conditions, several inhibitory mechanisms are activated that eventually leads to modulation of inflammation and homeostasis. In fact, the resolution of inflammation is not simply due to the elimination of the initial stressor. If the inflammation becomes chronic, inflammatory diseases may develop, and it also may render the host vulnerable to secondary infections [2].



# Myeloid-innate immune cells

As mentioned previously, the inflammatory response is mediated by recruitment of different subsets of immune cells, mostly myeloid cells from bloodstream to the site of inflammation. Myeloid cells, comprised of neutrophils, monocytes, macrophages, DCs, and eosinophils, have important roles from sensing the infection to resolution of inflammation, as well as linking innate and adaptive immunity. The focus of this thesis is on monocytes, neutrophils and macrophages, which will further be discussed below.

## Monocytes

The historic definition of monocytes defines them as a population of mononuclear leukocytes that develop in the BM from dividing monoblasts, and are released to the bloodstream in the state of non-dividing cells [80].

Under normal conditions, maturation of monocytes takes place in the BM from a progenitor that is called MDP (macrophage and DC progenitors). MDP give rise to CDP (common DC progenitor) and cMoPs (common monocyte progenitors) [81]. CDP give rise to plasmacytoid DCs (pDC) and pre-DCs. DCs, but not monocytes, are generated from Pre-DCs. Monocytes which are derived from cMOPs express low levels of CXCR4 (CXC chemokine receptor 4) and eventually egress from the BM and end up in blood or spleen [82, 83].

The blood monocyte population of the mouse (circulating monocytes) is categorized into two main types: classical ( $\text{Ly6C}^+$ ) and non-classical or patrolling ( $\text{Ly6C}^-$ ) monocytes [81]. Several cell biologically active surface molecules are used to distinguish between these two types.  $\text{Ly6C}^+$  monocytes express high levels of CCR2 which is necessary for their egress from the BM and to enter both non-inflamed and inflamed tissue [80]. On the other hand,  $\text{Ly6C}^-$  monocytes are defined by low levels of CCR2 and high levels of CXCR3 and serve as a precursor for resident myeloid cells in tissues such as liver, lung, spleen and brain under non-inflammatory conditions [80]. One study suggests that while patrolling monocytes lack CCR2 expression, sphingosine 1-phosphate receptor 5 might have role in their egress from the BM [84]. Patrolling monocytes only display motility (“crawling”) along endothelial lumen, while classical monocytes in response to signals can transmigrate through endothelium [85, 86]. Another surface marker that is differentially

expressed among these two types of monocytes is CD62L. Classical monocytes express high levels of CD62L which helps them to migrate into tissues and lymph nodes, while non-classical monocytes lack CD62L expression which is in line with their inability to enter tissues [86].

Under inflammation, emigrated monocytes acquire pro-inflammatory properties, and macrophages that develop from these monocytes also exhibit the same function which can later develop into anti-inflammatory functions [87].

During steady-state, monocytes are generated from HSC (hematopoietic stem cell) in the BM. However, under certain disease conditions or due to insufficient BM function or ineffective hematopoiesis, several other organs such as spleen, liver, and lymph nodes can act as an extra source of hematopoiesis; so called EHM (extramedullary hematopoiesis) [88]. Among these organs, especially spleen acts as reservoir of both Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes [89-91]. EHM has been shown to be actively involved in myocardial infarction [92], atherosclerosis [93], colitis [94], and cancer [95].

## **Macrophages**

Macrophages are phagocytic cells with the ability to clear cellular debris and responding to injury and infection and are present in many different tissues throughout the body. Due to expression of many PRRs that can recognize both DAMPs and PAMPs, macrophages have the ability to produce inflammatory cytokines and chemokines, and thus contribute to the inflammatory processes [96].

It has long been thought that tissue-resident macrophages are developed from blood-recruited monocytes that recently came from BM. However, recent studies have suggested that many of these macrophages are in fact derived during embryogenesis from primitive hematopoiesis in the yolk sac and also hematopoiesis in the fetal liver, and maintained mostly by local proliferation rather than replenishment from circulating monocytes [97]. This prenatal origin contributes to tissue macrophages in different organs such as brain and spinal cord (microglia) [98-100], skin (Langerhans cells), liver (Kupffer cells), and peritoneal macrophages [101]. However, as mentioned previously, under inflammatory conditions recruited monocytes contribute in restoring the reservoir of macrophages [85]. There have been many attempts to classify specific stages of macrophage maturation. Hence, a number of terms to this end exist in literature which include pro-inflammatory vs pro-resolving, classically activated vs alternatively activated, and M1-like vs M2-like [97]. However, during ongoing inflammation, macrophages are highly heterogeneous with regard to function and maturation stages [102].

## Granulocytes

Granulocytes are comprised of neutrophils, eosinophils, basophils and mast cells and are key effectors of the innate immune system's rapid response against pathogens. The name, granulocytes is given to them due to presence of various granules in their cytoplasm. Thanks to growing body of evidence, the classical view of these cells as terminally differentiated and short-lived phagocytes has recently been modified, and we now know that they are in fact a heterogeneous population of cells that also has a potent ability in presenting antigen [103].

Neutrophils are the dominant granulocytic cell population in blood and has diverse roles during immune defense. They are normally generated in the BM, identified with the phenotype CD11b<sup>+</sup>Ly6G<sup>hi</sup> cells, and are among the first cells that arrive at damaged tissue [104]. They display two major mechanisms in clearance of tissues from infection: Extracellularly by release of granules, and intracellularly by phagocytosis of pathogens. Their cytoplasmic granules are of three main types: azurophilic, specific and gelatinase granules [104]. Another important mechanism of neutrophil function is performed through a process called NETosis that leads to release of neutrophil extracellular traps (NET) [104, 105]. NETs are extracellular neutrophil-derived DNA that harness pathogens and potentially prevent them from spreading and therefore facilitates phagocytosis. NETosis always lead to neutrophil death [104, 105] which also results in the release of S100A8/A9 reservoir within cells. Furthermore, the ability of neutrophils to present antigens to effector T cells and contribute in adaptive immunity has been recently acknowledged [106].

Eosinophils derive from eosinophil precursor cell, which is recognized by the expression of CD34 and IL-5R $\alpha$  [107]. IL-5 is required for the development of eosinophils from their progenitors [108]. A marker that is commonly used to identify eosinophils in the mouse is the cell surface lectin SiglecF [107]. Like neutrophils, the cytoplasm of eosinophils is equipped with granules full of proteolytic enzymes and other inflammatory mediators, e.g. major basic protein, eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin and lipid bodies (containing prostaglandins and leukotrienes) which are released upon inflammatory stimulation and cell activation [107].

## Myeloid cells in cancer

This part of the thesis will focus on the relationship between inflammation and cancer and how cells of the immune system can either promote or inhibit tumor growth.

In a review article which published on 2000, Hanahan and Weinberg summarized six fundamental genetic alterations required for tumorigenesis, and named them as hallmarks of cancer. The authors mentioned sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis as the necessary characteristics of malignancy [109]. On 2011, the same authors published another review in which they added up two elements to the previously-reported hallmarks [89]. Reprogramming of energy metabolism and evading immune destruction, which also considers the important role of stromal cells and tumor microenvironment in tumorigenesis [89].

As mentioned in the previous section, under normal homeostatic conditions, myeloid cells originate from a precursor cell in the BM, migrate towards periphery where they mature and acquire their optimal function in activating the immune system [1]. A variety of pathological conditions can result in a substantial need of leukocytes, which in turn calls for “emergency myelopoiesis”, that aims to eliminate the trigger of such disturbance, e. g. cancer, infection, or tissue damage [110]. Under these circumstances, granulocyte monocyte colony-stimulating factor (GM-CSF) drives myelopoiesis, and granulocyte colony-stimulating factor (G-CSF) and monocyte colony-stimulating factor (M-CSF) induce granulocyte and macrophage differentiation from precursor cells [110]. However, under chronic inflammatory conditions associated with various types of cancer and autoimmune diseases, and because of high concentration of pro-inflammatory mediators, these factors favor the production of cells with immune suppressive functions [111]. Early studies in the mouse highlighted the presence of two markers that are shared between these myeloid cells: CD11b and Gr1 [112, 113]. This population of cells can further be subdivided into  $\text{Ly6C}^{\text{hi}}$   $\text{Ly6G}^{\text{low}}$  and  $\text{Ly6C}^{\text{int}}$   $\text{Ly6G}^{\text{hi}}$  cells, which identifies monocytic- and granulocytic- populations, respectively [114, 115]. Given the heterogeneity of these suppressor cells, and in order to codify them, on 2007, Gabrilovich et. al. suggested to utilize the term “myeloid derived suppressor cells” (MDSC) for them in the context of cancer [116]. Furthermore, for the proper identification of MDSC, functional assessment of the cells is also required. To this end, the enzymes inducible nitric oxide synthase (iNOS) which is highly expressed in  $\text{Ly6C}^{\text{hi}}$  cells and arginase 1 (Arg1) which is expressed in  $\text{Ly6G}^{\text{hi}}$  MDSC are associated with this immunosuppressive capacity [117, 118].

### ***Immunosuppressive activity of MDSC***

Upon encounter with antigens, neutrophils and monocytes utilize different mechanisms in order to protect the host from invading pathogens, as well as providing means for tissue repair after resolution of inflammation. Phagocytosis, respiratory burst activity and release of pro-inflammatory cytokines constitute mechanisms which these cells utilize in order to achieve these goals. On the

contrary, MDSC are considered to be in the pathological state, which result from continuous exposure of them to weak signals coming from tumors or the sites of chronic inflammation. At pathological state cells are defined with their potential in suppression of immune system, which include their weak-phagocytic ability, production of high levels of ROS and nitric oxide (NO), and pro-inflammatory cytokines [119]. The hypothesis behind the existence of MDSC is that their activity in the final stages of inflammation helps to avoid uncontrolled immune response and therefore extensive tissue damage. However, tumors can hijack and amplify MDSC activity in order to evade the immune system surveillance [119].

As mentioned previously, iNOS and Arg1 are the two enzymes necessary for the immune-regulatory activity of MDSC. Moreover, MDSC are high producers of ROS, which are produced via the activity of NOX complex. Both iNOS and Arg1 metabolize the amino acid arginine. iNOS oxidizes arginine to NO and L-citrulline, while Arg1 converts arginine to urea and L-ornithine [117, 118]. These two metabolites induce apoptosis and inhibit activation and proliferation of T cells. MDSC also control the availability of the amino acid cysteine, and do so by competing with APCs for cystine, convert it to cysteine but do not export it, hence rendering T cells devoid of this amino acid. The amino acid Cysteine is converted from cystine in APCs and these cells are responsible for transferring of this amino acid to T cells. [120]. MDSC are further capable of suppression of the immune system indirectly by induction of regulatory T (Treg) cells. It was proposed that MDSC cross-present tumor-derived antigens to Tregs and induce their expansion [121].

The role of MDSC in promoting of metastasis has been shown. Several studies suggest that organs such as the lungs and brain can be sensitized by primary tumors which makes them more susceptible to invasion by tumor cells [122]. Moreover, myeloid cells have been shown to accumulate at sites of metastasis prior to the arrival of tumor cells. It has been shown that tumor cells secrete TNF $\alpha$ , VEGF and TGF- $\beta$  which promote the upregulation of S100A8 and S100A9 by myeloid cells and lung endothelial cells [67]. S100A8 and S100A9 can recruit tumor cells and MDSC to the site of metastasis. Ly6G<sup>hi</sup> MDSC accumulate at metastatic sites and express S100A8 and S100A9 [123]. Depletion of Ly6G<sup>hi</sup> MDSC by antibodies or blocking of G-CSF reduces lung metastasis of various subcutaneous tumors [123]. Ly6C<sup>hi</sup> cells has also been linked to metastasis, as depletion of them by blockade of CCL2/CCR2 axis has been shown to result in reduced recruitment of this population to the lungs, thereby reduction in metastasis [124, 125].

### ***Hypoxia and cancer***

When the oxygen levels which is necessary for cells to perform their activities drops, hypoxia occurs. In solid malignant tumors, due to high growth rate non-functional blood vessels form which fail to transfer sufficient oxygen to the growing tissue.

Therefore, these kind of tumors contain many areas with hypoxic characteristics (reviewed in [126]). The hypoxia-inducible factor (HIF) family of transcription factors are activated following formation of hypoxic conditions and mediate the expression of numerous genes that derive adaptation and progression of cancer cells [127]. Each HIF transcription factor is constituted of two subunits: HIF- $\alpha$  and HIF- $\beta$ . The  $\alpha$ -subunit is sensitive to the presence of oxygen, while the  $\beta$ -subunit or aryl hydrocarbon receptor nuclear translocator (ARNT) is ubiquitously expressed regardless of oxygen tension (reviewed in [126]). In sufficient oxygen tension, proline residues in HIF-1 $\alpha$  subunit are hydroxylated which leads to its degradation [128]. However, in low oxygen concentration, the HIF-1 $\alpha$  is stabilized, translocated to the nucleus where it dimerized with the  $\beta$  subunit and forms the HIF transcription factor. In the nucleus, HIFs bind to the hypoxia-responsive elements (HREs), which are A/GCGTG consensus motif in target gene promoter regions. Thereafter this binding leads to recruitments of transcriptional co-activators, and regulation of the expression of numerous genes involved in different processes including angiogenesis, metabolism, erythropoiesis, apoptosis, pH regulation, metastasis, and cellular differentiation [129]. Given this central role of hypoxia in adaptation of tumors, it is not surprising that the HIF-1 $\alpha$  protein is overexpressed in various common solid malignant tumors such as breast, colon, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas compared to their respective normal tissues [127, 130, 131]. In contrast, many benign tumors show very little or no HIF-1 $\alpha$  expression. Thus, tumor hypoxia is associated with aggressive tumor behavior such as treatment resistance, metastasis and poor clinical outcome [127]. Given the prominent role of hypoxia in regulation of expression of a vast number of genes, as well as the role of S100A8 and S100A9 in progression of tumors, it is reasonable to assume that the expression of S100A8 and S100A9 genes may be under the control of hypoxia. In this regard, Grebhardt et. al. have shown regulatory functions of HIF-1 $\alpha$  in S100A8/A9 expression in prostate cancer cell lines *in vitro* and *in vivo* [132].

# Type 1 diabetes

Type 1 diabetes mellitus (T1DM), is a chronic autoimmune disorder characterized by severe loss of pancreatic  $\beta$ -cells that leads to insulin deficiency (reviewed in [133]). It is one of the most common endocrine disorders affecting both children and adults worldwide. The classic symptoms associated with disease onset are excessive thirst, eating and urine production along with overt hyperglycemia that serve as diagnostic markers in children, adolescents and to lesser extent adults [134].

## Non-obese diabetic mouse model

The Non-obese diabetic (NOD) mouse model is an extensively used model in studying T1D. Although, compared to human T1D, a more severe inflammation of islets (insulitis) occurs in this model, NOD mice display several characteristics in common with human T1D; for example, restriction to susceptible MHC haplotypes. There are also some common genetic mutations between human T1D and the NOD model such as the cytotoxic T lymphocyte antigen (CTLA)-4, IL-2 and insulin [135-137]. The NOD model is a spontaneous model and highly T cell-dependent. The first inflammatory phase in NOD mice denoted as peri-insulitis takes place at around 3-4 weeks of age [138] when infiltration of islets with macrophages and DCs occurs [139-141]. This is followed by recruitment of self-reactive  $CD4^+$  and  $CD8^+$  T cells and this invasion ultimately leads to  $\beta$ -cell destruction and severe insulitis at around 10 weeks of age [142, 143]. The initial immune responses against  $\beta$ -cells antigen take place in draining pancreatic lymph nodes (panLN) and by DCs which present islets antigens to autoreactive T cells [138, 144, 145]. The phase of hyperglycemia and overt diabetes occurs at around 14-25 weeks of age [135-137]. Interestingly, it has been shown that insulitis can affect different islets in a given animal to a different extent, resulting in coexistence of heavily infiltrated and intact islets [146].

## Myeloid cells in islets

### *Macrophages*

The pancreatic islets of Langerhans of all species contain macrophages, and very few or no DC. Until the inflammation begins, macrophages are the only CD45<sup>+</sup> cells detectable in the islets [147]. Moreover, macrophages with the full capacity of antigen presentation are present even in the islets of non-diabetogenic strains of mice [147]. Resident islet macrophages are not derived from blood monocytes and instead are maintained by replication in place [148]. They display M1-like activation pattern, with expression of high levels of surface MHCII, TNF $\alpha$  and IL-1 $\beta$  transcripts [149]. It was shown that depletion of macrophages and circulating monocytes in NOD mice delay both onset and incidence of diabetes [150]. Even these cells play important role in the acute model of diabetes which involves transfer of diabetogenic T cells [151]. This shows the crucial role of macrophages and monocytes not only in initiation, but also effector phase of T cell pathogenesis. Interestingly, macrophages in NOD mice possess a profile of TNF $\alpha$ , IL-10, and IL-12 production which is different to their counterparts in WT mice, that reflects intrinsic defects of the innate immune system with the potential to initiate and further amplify the pathogenic T cell response [152].

### *DCs*

It has been shown that CD103<sup>+</sup> DCs are critical for development of diabetes in NOD mice and in their absence islets never gets infiltrated [144]. These DCs which are present even in uninflamed islets [153] can activate CD4<sup>+</sup> T cells, which in turn promotes killing of  $\beta$ -cells through mechanisms exerted by macrophages [154]. Another key function of CD103<sup>+</sup> DCs is cross-presentation of  $\beta$ -cell antigens to CD8<sup>+</sup> T cells, thereby rescuing these cells from peripheral anergy and deletion [154], which therefore leads to production of potent CD8<sup>+</sup> T cells with the ability of contribution in autoimmune processes in NOD mice. DCs are shown to be critical for maintaining the T cell response [155]. DCs in NOD mice display NF- $\kappa$ B hyperactivation as compared to DCs in WT strain which leads to increased IL-12 production [156]. Hence, they are more potent in breaking the peripheral tolerance to autoantigens [157]. Purified CD103<sup>+</sup> DCs from overtly diabetic NOD mice can confer diabetes to the young NOD mice by breaking peripheral T cell tolerance to  $\beta$ -cell antigens [157].



## Pathogenesis of T1D

What triggers the autoimmune processes that leads to  $\beta$ -cells destruction is still unknown, but probably includes a combination of genetic and environmental factors [158]. The pathogenesis of T1D is not completely understood, however in both human and NOD mouse, T1D it is mostly attributed to T cells, given the genetic loci that are functioning in T cell activation and the strong link to T1D susceptibility [126]. T1D is also associated with the appearance of autoantibodies long before the onset of symptoms [133]. It is generally believed, however not proven, that the production of these autoantibodies is the result of continuous exposure of B cells to  $\beta$ -cell autoantigens [133]. One important step in initiating autoimmunity towards  $\beta$ -cells is the transfer of  $\beta$ -cell granules to islet APCs. Through the homeostatic pathway that  $\beta$ -cells utilize to remove the excess of hormone and secretory granule materials, these metabolites are delivered to islet-resident macrophages and CD103<sup>+</sup> DCs [159]. The clearance of these granules is generally non-inflammatory, rather tolerogenic [159]. However, the unwanted consequence of this delivery could be the access of immunogenic peptides to the immune system [160]. Both islet-resident macrophages and CD103<sup>+</sup> DCs capture dense granules from  $\beta$ -cells [144]. Moreover,  $\beta$ -cell death, either non-physiological or physiologically-induced, result in the release of an even broader range of autoantigens [161]. The released autoantigens are presented to autoreactive CD4<sup>+</sup> T cells [153]. Many of the early T cells recognize insulin-derived peptides and are found in tight contact with islet APCs [162]. CD4<sup>+</sup> T cells with low affinity to  $\beta$ -cell autoantigens that has escaped thymic negative selection can respond to both autoantigens and neo-antigens which are absent in thymus [163]. The main function of these T cells is to provide help for autoreactive CD8<sup>+</sup> T cells and B cells. Once CD8<sup>+</sup> T cells are primed, any  $\beta$ -cell presenting self-antigen on its surface MHCI will be eliminated [154].

## Treatment of T1D

Until now, no cure is available for patients with T1D, and the most common treatment offered is providing them with the exogenous insulin. Although this treatment came as a revolution in treatment of T1D [164], still majority of patients develop severe complications. A series of therapeutic approaches have been tested in the NOD mouse model which targeted T cells [165-167] or B cells [168, 169] by specific antibodies. Some of these studies led to clinical trials that have also shown promising effects on humans with T1D [170-172]. However, none of these successful preclinical studies led to establishment of a therapy which provides diabetic patients with life-long independence of exogenous insulin [172]. Therefore, there is a high demand for novel treatments in T1D. Targeting myeloid cells which

are known to have key roles in pathogenesis of T1D sounds like an ideal approach. Because the disease is diagnosed when the immune system has already initiated its destructive processes, a therapy that combines suppression of innate immune inflammation and providing  $\beta$ -cells with regenerative agents such as betatrophin [173] and exenatide [174] at the same might be an ideal therapy for the disease.

# Quinoline-3-carboxamides

The quinoline-3-carboxamides or Q compounds are a group of small molecule compounds that first were introduced over 35 years ago. The family of Q compounds comprises a large number of molecules, among them this part of the thesis will focus on two of the compounds: tasquinimod (ABR-215050) and paquinimod (ABR-215757).

## Binding targets of the Q compounds

### *S100A9*

The first paper that described S100A9 as a target of Q compounds was published on 2009 [54], long after these compounds entered clinical trials. By using a FITC-labeled Q compound in a sample of human peripheral blood mononuclear cells, CD14<sup>+</sup> human monocytes were proved to be the cells with the affinity for Q compounds. As the next step, CD14<sup>+</sup> human monocytes were purified and shown that can interact with <sup>14</sup>C-labeled compound. In an attempt to define the actual molecule that binds to Q compounds, membrane fraction of the same cells was isolated and subjected to 2D gel electrophoresis. By using autoradiography and mass spectrometry, the authors identified S100A9 as the major target on the surface of this cell population. In order to confirm binding of Q compounds to S100A9, surface plasmon resonance (SPR) was performed. These *in vitro* studies showed that the *in vitro* binding of Q compounds to S100A8 homodimer or S100A8/A9 heterodimer is significantly weaker than for the S100A9 homodimer [54]. Interestingly, it was also shown that Q compounds, TLR4/MD-2 [25] and RAGE [50] bind to the same site on human S100A9 homodimer. In another study it was shown that the effect of the Q compounds in mice resembles the phenotype seen in the S100A9<sup>-/-</sup> mouse model; reduction in TNF $\alpha$  response after LPS challenge [25].

As mentioned previously, MDSC have an important role during tumor progression [64]. These cells express S100A8, S100A9, TLR4 and RAGE and can secrete S100A8 and S100A9 *in vitro* [69, 175]. The chemotaxis effect of S100A8 and S100A9 on MDSC has been shown previously [69, 175]. It has been shown that several types of tumor cells also express S100A8 and S100A9 [176]. Furthermore, in solid tumors, S100A9 recruits MDSC to the tumor site, resulting in immune

suppression, increased tumor growth, and metastasis [69]. Therefore, it is reasonable to postulate that Q compounds, by binding to S100A9, inhibit migration of MDSC to the tumor site, hence affecting the whole tumor microenvironment and immune response against tumors. This hypothesis is supported by studies showing reduced spontaneous tumor growth in S100A9<sup>-/-</sup>, TLR4<sup>-/-</sup> [177] and RAGE<sup>-/-</sup> mice compared to WT controls [178].

### ***HDAC4***

In an attempt to discover another possible target molecules of Q compounds, a study was performed which based on the available crystal structure data identified histone deacetylase HDAC4 as a candidate [179]. Using SPR, binding of tasquinimod to HDAC4 was confirmed. This binding prevents the formation of a complex of HDAC4, nuclear receptor corepressors (NCoR) and HDAC3 which in turn renders HDAC3 unable of deacetylation of its target genes, like HIF-1 $\alpha$  [179]. Given the important role of HIF-1 $\alpha$  in response to hypoxia [180] and the crucial role of hypoxic condition in tumor progression [126], it was assumed that by binding to HDAC4 tasquinimod may interfere with tumor progression.

### ***AhR***

Laquinimod, which belongs to the family of Q compounds, has shown beneficial effects in clinical trials for multiple sclerosis (MS) [181, 182]. Kaye et. al. has shown that laquinimod treatment in a model of experimental autoimmune encephalomyelitis (EAE) induces genes that are known to be associated with the aryl hydrocarbon receptor (AhR) pathway [183]. AhR is an important regulator of development as well as function of both innate and adaptive immune cells [184]. The authors showed the induction of one gene that is related to the AhR pathway *in vitro*, and further verified the induction of the same gene in the liver of mice treated with laquinimod. By using the AhR<sup>-/-</sup> mice, and monitoring the clinical score and analysis of samples from spinal cord, they could show that the effect of laquinimod treatment on the course of EAE was completely lost in the absence of AhR. Hence, they concluded that AhR is a pathway that is modulated by laquinimod treatment.

## **Q compounds in models of inflammation**

Q compounds have been evaluated in several *in vivo* models of inflammation and has shown potential benefits to the host. The first compound that was introduced was linomide or LS-2616. Linomide was shown to reduce the development of type II collagen-induced arthritis [56] and T1D in the NOD model [185]. However, after linomide showed adverse effects in clinical trials [186, 187], second generation compounds were developed. Among Q compounds that belong to the next

generation, paquinimod and laquinimod were evaluated in models of inflammation. Paquinimod reduces the development of collagenase-induced osteoarthritis [188], skin fibrosis [189], SLE (systemic lupus erythematosus) [190], and also development of atherosclerotic plaques in a S10012 transgenic ApoE<sup>-/-</sup> mouse model [191]. Paquinimod demonstrated beneficial effect in EAE [192]. In a model of sterile acute inflammation, it was shown that paquinimod treatment reduced accumulation of Ly6C<sup>hi</sup> cells and SiglecF<sup>+</sup> eosinophils in peritoneum [194]. In a model of chronic liver inflammation, treatment with paquinimod reduced inflammation which led to regression of fibrosis in liver [195].

## Q compounds in tumor models

Tasquinimod has been evaluated in several non-transplantable (spontaneous) and transplantable tumor models like lymphoma [177], melanoma [193], colon carcinoma [194] and prostate tumors [90, 91, 193]. It has been shown that treatment of tumor-bearing mice with tasquinimod can reduce tumor growth and metastasis [90, 91, 177, 193, 194]. In prostate cancer models it was shown that the treatment reduced formation of lung and lymph node metastases [91].

Two main mechanism of action has been attributed to tasquinimod. First, inhibition of angiogenesis and therefore metastasis and second, possessing immunomodulatory properties.

*In vitro* studies in human umbilical vein endothelial cells (HUVEC) showed that tasquinimod reduced tube formation [90]. Moreover, it was shown that tasquinimod can decrease blood vessel density in different prostate cancer tumors [90]. In an attempt to elucidate the mode of action of tasquinimod, Olsson et. al. performed *in vitro* and *in vivo* experiments with gene microarray analysis in LNCaP prostate tumor cells [195]. It was shown that one of the most significant differentially expressed genes after tasquinimod treatment was thrombospondin-1 (TSP1). The authors concluded that tasquinimod-induced upregulation of TSP1 which is associated with downregulation of HIF-1 $\alpha$  is part of the mechanism by which reduction in angiogenesis occurs [195], which can therefore result in suppression of angiogenic switch.

In a study in mouse model of melanoma, when used in combination with two other immunotherapeutic agents, tasquinimod inhibited distinct MDSC populations and tumor-associated macrophages (TAM) with the M2 phenotype (CD206<sup>+</sup>), while displayed no effect on B or T cells and their functions [193]. CD11b<sup>+</sup> cells that were isolated from treated mice expressed lower levels of Arg-1 and higher levels of iNOS, suggesting a shift from immunosuppressive M2-type TAM towards pro-inflammatory M1-type. Indeed, these cells were less immunosuppressive compared

to the cells isolated from untreated tumor-bearing mice [193]. The results were confirmed in the MC38-C215 colon carcinoma tumor model, where tasquinimod treatment induces a rapid shift (already 1 day after start of treatment) in TAM population: M2 expressing CD206 versus M1 expressing MHCII and CD86 [194]. An increase in IL-12 production and a decrease in tumor neovascularization were also observed. The same observation was made in the 4T1 breast cancer model, in which tasquinimod treatment induced a prolonged inhibition on tumor growth and a reduction in lung metastasis development [194]. Given the role of M2 TAMs in promotion of angiogenesis, it seems reasonable to assume that the immunomodulatory and anti-angiogenic functions of tasquinimod at least in part is due to its effect on this population. In the preclinical models of bladder cancer, tasquinimod treatment reduced the immunosuppressive properties of myeloid cells [196]. The authors further used tasquinimod in a combination therapy with anti-PD-L1 antibody and detected enhanced anti-tumor immune response. Based on their observations, Nakhle et. al. suggested further using of tasquinimod in combination therapies against cancers [196].

## Q compounds in clinical trials

The first Q compound that reached clinical trial was linomide. It was first evaluated for treatment of relapsing-remitting and secondary progressive MS. It even reached the phase III of clinical trial, however due to severe side effects the compound was withdrawn [186, 187]. In an attempt to generate safer compounds, some alterations in the original structure of linomide was made which led to the development of the second generation of Q compounds. From this second generation, Q compounds showed efficacy in reducing development of EAE [197] and inhibition of angiogenesis, metastasis and growth of prostate tumors [90]. From these *in vivo* studies, two compounds were generated and entered clinical trials.

Tasquinimod has successfully reached the phase III of clinical trial for metastatic castration-resistant prostate cancer with promising effects [198-200]. However, the report from the last trial showed that while tasquinimod can significantly reduce the progression-free survival among patients, it cannot significantly affect the overall survival [201].

Two phase III clinical trials were performed for evaluation of laquinimod for relapsing-remitting MS patients. In the first study, a significant reduction in annualized relapses rates, reduced disability progression and brain atrophy were recorded [181]. In the second study as well, the annualized relapses rate and disability progression were reduced, however, the results didn't reach statistical significance [182]. Laquinimod has also shown potential therapeutic effects in

treatment of Huntington's disease [202] and lupus nephritis [203]. Furthermore, it has been tested in clinical trials for inflammatory bowel disease (IBD) [204].

Paquinimod has been evaluated in phase I clinical trial in SLE patients. The results showed that the compound possess some disease-modifying effects and was well tolerated in SLE patients [190].

# Aims

The aims of this thesis are included in the following four papers:

## **Paper I:**

To evaluate the effect of the Q compound tasquinimod on:

- recruitment of myeloid cells in a transplantable tumor model
- the resulting anti-tumor effect
- hematopoiesis in spleen in the course of tumor burden

## **Paper II:**

To evaluate the effect of the Q compound paquinimod on:

- incidence and onset of diabetes in NOD mouse model
- diabetes development in short and long-term treatment
- myeloid cells in spleen and pancreatic lymph nodes

## **Paper III:**

To study the formation of homodimeric form of S100A9 under inflammatory condition and tumor burden.

## **Paper IV:**

To study the conditions needed for induction of S100A9 expression in cell lines with or without endogenous expression.



# Summaries and discussions of Papers I-IV

## Paper I

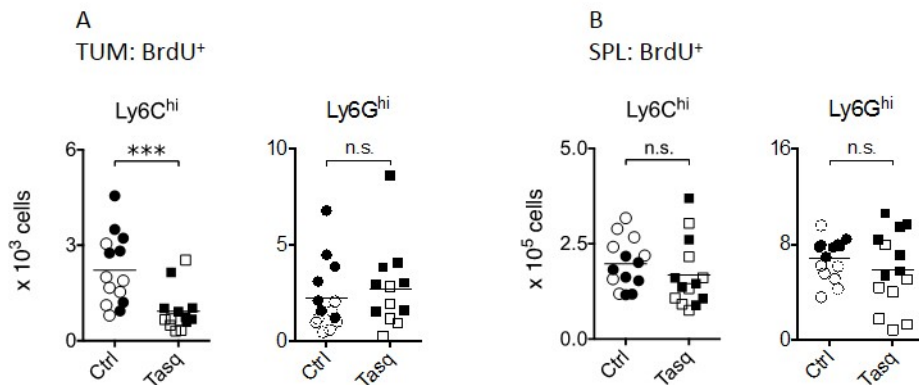
### *The quinoline-3-carboxamide tasquinimod reduces the recruitment of Ly6C<sup>hi</sup> MDSC to tumors and their proliferation in the spleen*

The tumor microenvironment is characterized by presence of a population of myeloid cells, under the name of MDSC [114]. MDSC are a crucial element of the immunosuppressive milieu in cancer with the ability to suppress both the innate and adaptive immune responses [114, 115, 205]. The impact of quinoline-3-carboxamides (Q compounds) on myeloid cells has been studied previously. In a model of inflammation induced by CFA injection, the Q compound paquinimod, could reduce expansion of myeloid cells [192, 206, 207], and particularly Ly6C<sup>hi</sup> and SiglecF<sup>+</sup> cells in the spleen [193]. In a model of necrotic cell-induced peritonitis, paquinimod reduced the accumulation of Ly6C<sup>hi</sup> and SiglecF<sup>+</sup> cells in the peritoneum [208]. Furthermore, it was shown that the ameliorating effect of paquinimod treatment mainly operates during the induction phase of acute EAE, and the treatment also reduced the immunization-induced splenic myelopoiesis [192].

Given the effect of Q compounds on reducing recruitment of myeloid cells to the site of inflammation [208, 209], and also the anti-tumor effect of tasquinimod [210], another member of the Q compound family, we decided to assess if part of this effect is mediated through reducing recruitment of MDSC to the tumor tissue. Moreover, because of the appreciated role of the spleen as a reservoir of myeloid cells during tumor growth [83, 211], we investigated the effect of tasquinimod on extramedullary expansion of myeloid cells.

In tumor-bearing mice, Ly6C<sup>hi</sup> and Ly6G<sup>hi</sup> MDSC within tumor are in a non-proliferative state and instead they mainly proliferate either in the spleen or bone marrow [115], from where they migrate towards tumor tissue. Younos et al. has demonstrated this phenomenon for the 4T1 mammary carcinoma tumor model [212]. We therefore utilized the BrdU pulse-labelling method to enable the detection

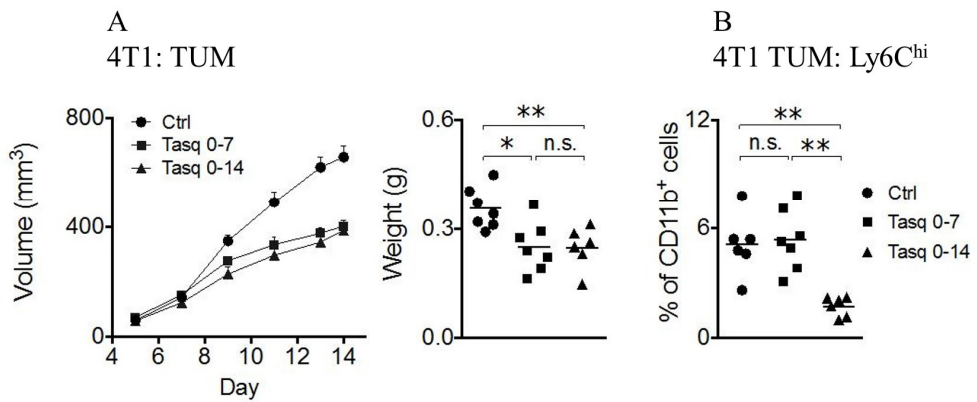
of recently divided cells that have been newly recruited to the tumor tissue. According to mentioned studies [115, 212], the cells that were in the tumor tissue at the time of BrdU-labelling wouldn't incorporate BrdU. Tumor-bearing mice were injected three times with BrdU starting from day 5 post tumor inoculation, and with 14h of interval. Mice were sacrificed at day 7 post-inoculation. This results in BrdU-labeling of the proliferating Ly6C<sup>hi</sup> and Ly6G<sup>hi</sup> cells in the spleen, but not the non-proliferative cells in the tumor. Therefore, we could study the effect of tasquinimod on the recruitment of the BrdU-labeled cells from the spleen to the tumor. Treatment of tumor-bearing mice with tasquinimod between day 4 and day 7 reduced the absolute number of BrdU<sup>+</sup>Ly6C<sup>hi</sup>, but not BrdU<sup>+</sup>Ly6G<sup>hi</sup> cells (Fig. 2A). However, tasquinimod treatment did not reduce the total number of the two mentioned cell populations in the spleen (Fig. 2B), which supports the hypothesis that tasquinimod inhibits cell recruitment and not proliferation. This treatment strategy neither affect the tumor growth nor the spleen weight.



**Figure 2.** Tasquinimod reduces recruitment of Ly6C<sup>hi</sup> cells to 4T1 tumors. Absolute number of BrdU<sup>+</sup> Ly6C<sup>hi</sup> and BrdU<sup>+</sup> Ly6G<sup>hi</sup> cells in 4T1 tumors (A) and spleens (B) of control and tasquinimod-treated mice.

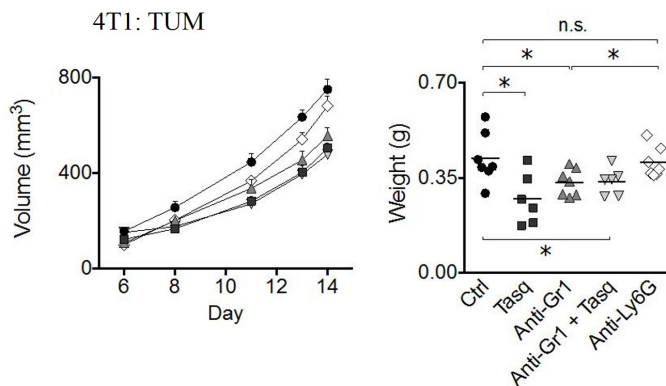
As the reduced recruitment of Ly6C<sup>hi</sup> cells following treatment with tasquinimod was observed already at day 7 of tumor growth, we further analyzed if short-term treatment in the early phase of tumor development would display the anti-tumor effect. Indeed, tasquinimod treatment provided in only the first 7 days inhibited the tumor growth equally effectively as treatment for throughout the whole study (14 days) (Fig. 3A). The frequency of Ly6C<sup>hi</sup> cells within tumor were significantly reduced after 14 days of treatment, however, in the group of mice treated during just the first 7 days of the experiment, the population of Ly6C<sup>hi</sup> cells were restored (Fig. 3B). Overall, this data shows that the inhibitory effect of tasquinimod is mediated during the initial phase of tumor growth, and further indicates that in this model, the pro-tumorigenic properties of Ly6C<sup>hi</sup> cells is especially important during the first

few days of tumor development. To confirm that tasquinimod mainly operates on the initial phase of tumor development, we performed another experiment in which we started the treatment in a group of mice at day 7 after tumor inoculation until end of the study (day 14). We could show that although the treatment significantly reduced the frequency of Ly6C<sup>hi</sup> cells in the tumor, it did not reduce the tumor growth (data not shown in Figure 2). In another set of experiments, we started the treatment from day 3 and again detected a significant anti-tumor effect of tasquinimod. Therefore, we conclude that the presence of the compound at the time of tumor inoculation is not necessary for its anti-tumor effect.



**Figure 3.** The anti-tumor effect of tasquinimod operates during the first week of tumor development. A) Tumor growth curve and weight and B) frequency of Ly6C<sup>hi</sup> cells in the control and tasquinimod-treated mice.

In order to verify the important role of Ly6C<sup>hi</sup> cells in the early phase of tumor growth, we injected anti-Gr1 antibody one day prior to tumor inoculation. This injection removed Ly6C<sup>hi</sup> cells from spleen, bone marrow and blood, which resulted in a reduction in tumor weight, with the same efficiency as 7 days of tasquinimod treatment. Combination of anti-Gr1 antibody and tasquinimod treatment for the first 7 days of tumor growth had no additional anti-tumor effect (Fig. 4) which further supports the hypothesis that one major anti-tumor mechanism of tasquinimod is mediated through the reduction of influx of Ly6C<sup>hi</sup> cells to the tumor site. To rule out that anti-Gr1 antibody performs its anti-tumor effects by targeting Ly6G<sup>hi</sup> cells, we depleted these cells by injecting anti-Ly6G antibody and detected no significant impact on tumor growth (Fig. 4). Given the fact that Ly6C<sup>hi</sup> cells contribute to tumor progression via promotion of angiogenesis and metastasis and suppression of immune effector functions [213, 214], we assume that reduced recruitment of these cells during the early phase of tumor development may result in a milieu with less immunosuppressive characteristics, and therefore reduced tumor growth.



**Figure 4.** Ly6C<sup>hi</sup> cells are required for 4T1 tumor growth. Tumor growth curve and weight in the five groups of mice treated with tasquinimod and/or different antibodies.

Since the spleen acts as a reservoir of myeloid cells [95] and their progenitors during tumor growth, we evaluated the effect of tasquinimod on 4T1-induced splenic myelopoiesis. There is a huge increase in the size of spleen (so-called splenomegaly) in 4T1-tumor bearing mice due to extramedullary hematopoiesis [215]. Tumor-bearing mice treated with tasquinimod from day 0 until the end of the study (day 14) demonstrated a significant decrease in the weight of the spleen and in the frequency of splenic Ly6C<sup>hi</sup> cells, as shown in paper I.

Considering the effect of long-term tasquinimod treatment on myeloid cells in the spleen, we next investigated the influence on their hematopoietic progenitors (Lin<sup>-</sup> c-kit<sup>+</sup> Sca1<sup>-</sup>). Spleen of 4T1-tumor bearing mice at a later phase of tumor growth contains increased numbers of these progenitors due to a huge production of cytokines by tumor and stromal cells [114, 213]. Long-term treatment with tasquinimod did not influence the total frequency of these cells, however, it clearly decreased the frequency of progenitor cells within this population that give rise to erythrocytes and megakaryocytes. The fact that tasquinimod treatment did not affect the progenitors of granulocytes and macrophages, further suggests that the impact on Ly6C<sup>hi</sup> cells is not simply due to reduction in the generation of these cells in the spleen. Overall, tasquinimod treatment made the frequency of precursor cells in the spleen of tumor-bearing mice resemble the naïve state. One speculation could be that the effect of tasquinimod treatment on spleen could be a result of its anti-tumor effect. However, treatment of tumor-bearing mice with tasquinimod starting from day 7 of tumor growth, induced similar effects on spleen without affecting tumor development. Instead, the effect of the treatment on splenic Ly6C<sup>hi</sup> cells is most likely due to the reduced recruitment to spleen and also reduced containment of them within this organ. Several studies with other members of the Q compound family support this theory [216-218]. Therefore, we can speculate that the

extravasation of cells from blood vessels in the tumor and retention of cells in spleen might both involve adhesion to endothelium and that tasquinimod potentially interfere with certain cell-endothelial interactions at these two sites.

In summary, our results indicate that short term treatment with tasquinimod during the induction phase of tumor growth can reduce recruitment of Ly6C<sup>hi</sup> MDSC to the tumor site. Moreover, long-term treatment provided from the first day of tumor growth until end of the study leads to reduction in the frequency of splenic myeloid cells and some specific populations of myeloid precursor cells.

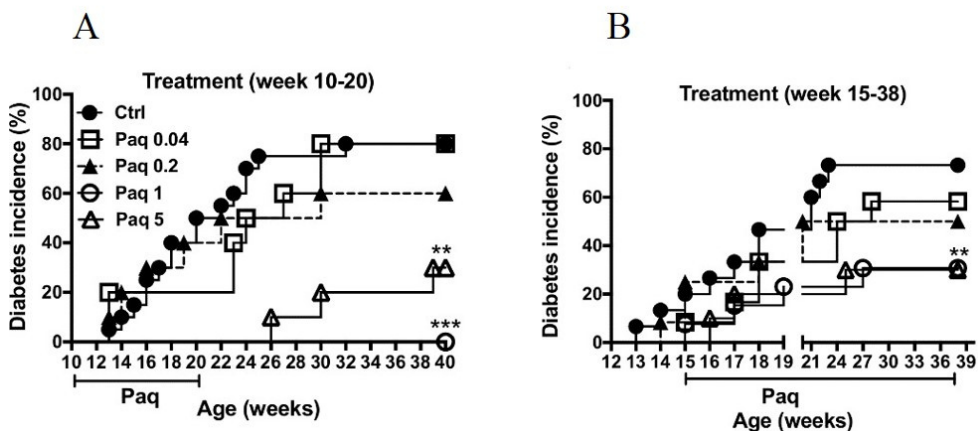
## Paper II

### ***The quinoline-3-carboxamide paquinimod prevents development of diabetes in the NOD mice***

T1D is an autoimmune disorder that causes severe loss of pancreatic  $\beta$ -cells and insulin production [133]. Although T cells are known to be the central effector cells in the pathogenesis of diabetes [219], the underlying mechanisms regulating functions of these cells are limited. The NOD mouse model is a good model for T1D. While a more severe form of insulinitis occurs in NOD mice, still there are several characteristics that are common between this model and human T1D [135-137]. Currently administration of exogenous insulin is the most common form of therapy in humans with T1D. However, due to severe complications that eventually happens even in treated patients [220], there is an urgent demand for better choices of therapy. Previous studies have shown efficacy of Q compounds in several models of inflammatory diseases [190, 192, 208, 221] and cancer [222, 223]. Q compounds are known to have selective effects on inflammatory monocytes [192, 208, 224], eosinophils [208, 224] and myeloid DCs [225]. Therefore, we tested whether one such compound, paquinimod have beneficial effects on the development of T1D in the NOD mouse.

In the first step, we assessed the efficacy of paquinimod as a preventive agent on development of diabetes. We treated NOD mouse with increasing doses of paquinimod from week 10 until week 20 of age, and monitored them for glycosuria as a sign of disease development. We observed delay in onset of disease and also a clear dose-dependent reduction in diabetes development in the treated mice (Fig. 5A, significant for the doses: 1 and 5 mg/kg/day). We further decided to investigate the effect of paquinimod on the later stages of inflammation in the islets (insulinitis). Although the time of onset of diabetes is variable in this model [137], the development of insulinitis is more homogenous and we know that at week 15 of age, NOD mice generally display extensive insulinitis in pancreatic islets [135-137]. Therefore, we started treatment from 15 weeks of age which lasted until the end of

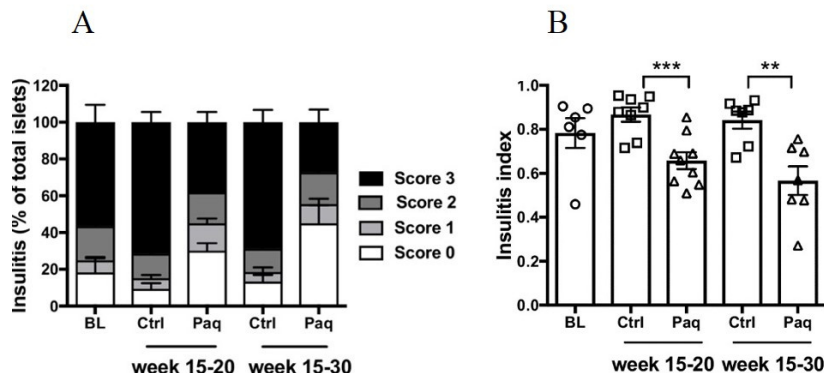
the study (week 38 of age) using the same doses of paquinimod as the previous experiment. Likewise, paquinimod treatment significantly reduced disease development in a dose-dependent manner, and also delayed the average week of disease onset in the treated mice compared to the control group (Fig. 5B, significant for the doses: 1 and 5 mg/kg/day). These results further support the initial hypothesis that paquinimod treatment delays the progression of insulinitis.



**Figure 5.** Delayed onset and reduced susceptibility to diabetes in paquinimod-treated NOD mice starting from 10 (A) or 15 (B) weeks of age.

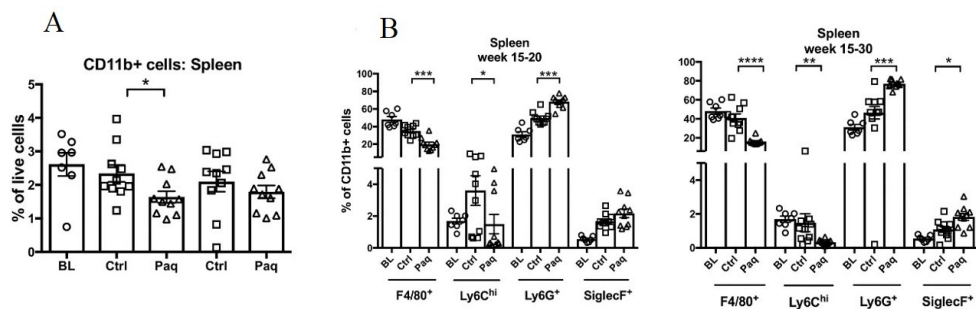
Development of diabetes in the NOD model is spontaneous and highly T cell dependent [219]. Insulinitis is the key factor in the disease course which causes  $\beta$  cell death [135-137]. To analyze the impact of paquinimod on the established insulinitis we started treatment of NOD mice with paquinimod from 15 weeks of age with the dose 1mg/kg/day which showed the similar efficacy as the dose 5mg/kg/day in the experiment shown in Figure 3. At the weeks 15, 20 and 30 of age, mice were sacrificed and incidence and onset of diabetes were calculated. As expected, incidence was reduced, and the onset was delayed significantly (as shown in paper 2). Pancreatic tissue sections from sacrificed mice were stained with H&E and scored from 0 towards 3 based on the severity of leukocyte infiltration. In both treatment groups, paquinimod-treated mice showed significantly fewer score 3 of islets and elevated number of score 0 islets compared to the control group (Fig. 6A). We also calculated insulinitis index, and as shown in fig. 6B, mice treated for both 5 and 15 weeks with paquinimod, showed significantly reduced insulinitis index. Therefore, we concluded that paquinimod treatment delays the progression of insulinitis in the NOD mice. Regeneration of  $\beta$  cells might be one mechanism by which more non-infiltrated islets were detected in the treated mice. This

phenomenon has previously been reported in NOD mice treated with different therapeutic regimens [226-229].



**Figure 6.** Reduced insulinitis in paquinimod treated NOD mice. A) Insulinitis score and B) insulinitis index in the same group of mice which were treated for 5 or 15 weeks.

Previous studies have shown immunomodulatory effects of Q compounds in various conditions such as inflammatory diseases and cancer. In some of these studies, monocytes and DCs were proposed to be target populations for the compounds [192, 224, 230, 231]. The inhibitory effects of these compounds on the recruitment of inflammatory monocytes and eosinophils to the site of inflammation [208, 221] and cancer [224] has been shown previously. Therefore, we also checked the effect of paquinimod on myeloid cells in spleen and panLN. The frequency of total splenic CD11b<sup>+</sup> cells were significantly reduced after 5 weeks of treatment and the trend can still be seen after 15 weeks of treatment although not significant (Fig. 7A). However, paquinimod did not show any impact on the same cell population in panLN. Moreover, paquinimod induced a significant decrease in the frequency of splenic F4/80<sup>+</sup> macrophages and Ly6C<sup>hi</sup> inflammatory monocytes (Fig. 7B). This effect was selective since the absolute numbers of SiglecF<sup>+</sup> and Ly6G<sup>+</sup> cells did not change. Overall, the effect of paquinimod on different splenic myeloid cells shown in paper 2, follows the similar trend that were reported on studies in other disease models using either paquinimod or other compounds belonging to the family of Q compounds [221, 224].



**Figure 7.** Paquinimod treatment reduces the frequency of CD11b+ (A) and F4/80+ and Ly6Chi cells (B) in the spleen of NOD mice.

As early as 3 weeks after birth, pathogenic events start in the islets of NOD mice with the appearance of the first antigen presenting cells [138]. These APCs, which are known to be closely related to the initiation and progression of disease are comprised of macrophages and DCs and present self-antigens to auto-reactive T cells [232]. At 4-5 weeks of age, there is a significant accumulation of these cells around islets which precedes infiltration of destructive T cells within the islets [140, 233]. It was shown that depletion of these two subsets delays onset and incidence of disease [151]. Recruitment of circulating monocytes from BM to the islets by overexpression of CCL2 chemokine was proven to cause insulinitis [234] and even development of diabetes [235]. Spleen is one of the major reservoirs of monocytes during inflammation [92], and in addition to that, NOD mice are reported to contain more splenic DCs and Macrophages compared to other mouse strains such as C57BL/6 [236]. Another phenomenon in NOD strain is that blood monocytes demonstrate an increase tendency to mature into macrophage with no specific stimulation. Therefore, it is reasonable to assume that reduction in the number of splenic monocytes following paquinimod treatment might contribute to the ameliorating effect of this compound [92]. Moreover, members of the Q compound family; paquinimod and tasquinimod have been shown to interfere with recruitment and accumulation of myeloid cells to sites of inflammation [208, 221, 224], which again may be one of the ways in which paquinimod influence the course of diabetes in this current study. It would be interesting to perform a study in which treatment with paquinimod would start as early as 3 weeks of age to see its potential inhibitory effects on development of insulinitis in NOD mice.

In summary, our data shows that paquinimod treatment can provide a long-term protection from diabetes development which makes it an attractive non-cytotoxic agent for treatment of T1D in humans.



### ***Are the immunomodulatory effects of Q compounds attributed to their binding to S100A9?***

Previous studies have identified S100A9 as a target for Q compounds, which prevents its binding to the two pro-inflammatory receptors, TLR4 and RAGE [54]. S100A9 promotes accumulation of MDSC in tumor microenvironment by acting as a chemoattractant [69, 175] and also by inhibition of MDSC maturation to mature DCs [64, 237]. It has been proposed that one mode of the action of tasquinimod in inhibition of tumor development is mediated through prevention of S100A9 interaction with TLR4 [177, 193, 194]. In addition to S100A9, Q compounds can bind to HDAC4 and thereby prevent the complex formation of HDAC4 with N-CoR and HDAC3 [179]. This binding was shown to inhibit deacetylation of HIF-1 $\alpha$  by MDSC suppressing their differentiation into TAMs, and thus prevention of transcription of various proangiogenic factors which are necessary for survival of tumor cells [179]. Therefore, it is reasonable to assume that binding of tasquinimod to S100A9 and HDAC4 would mediate its anti-tumor effects reported in Paper I. So, it can reduce accumulation of MDSC to tumor site and spleen and in addition to that, suppress proangiogenic properties of TAMs. However, the study needs to be done in S100A9<sup>-/-</sup> mice in order to assess if the inhibitory effects of tasquinimod is due to its binding to S100A9. In this case if any anti-tumor effect would be seen in tumor-bearing mice, it can likely be related to tasquinimod binding to HDAC4 or another potential targets. It'd also be interesting to analyze the effect of S100A9 deficiency on recruitment of MDSC to both tumor tissue and spleen.

S100A9<sup>-/-</sup> mice develops a more severe form of EAE compared to WT controls, however, paquinimod maintains its ameliorating effects in these mice [54]. Therefore, we can assume that there is a biological redundancy that can play a substitution role of S100A9 in this context. In addition to that, there might be another molecular targets for paquinimod that mediate its ameliorating effects. Moreover, in necrotic cell-induced peritonitis model in S100A9<sup>-/-</sup> mice, paquinimod can reduce recruitment of Ly6C<sup>hi</sup> and SiglecF<sup>+</sup> cells to the peritoneum to the same extent observed in WT mice. In a model of immune mediated glomerulonephritis, S100A9<sup>-/-</sup> mice are significantly protected from disease [238]. Paquinimod treatment failed to display any impact on disease, neither on prevention nor in treatment studies, however authors argued that using paquinimod in a longer duration of studies may be necessary in order to fully investigate paquinimod effects in this context [239]. In a recent study by Pelletier et. al. using tasquinimod and laquinimod in culture of human neutrophil and THP-1 cells, they argue that these treatments could not inhibit the activities that are related to the binding of S100A9 to its receptors [240]. Furthermore, they showed that laquinimod can inhibit the activation of TLR1/TLR2 receptors, a binding activity that was not reported for Q compound [240].

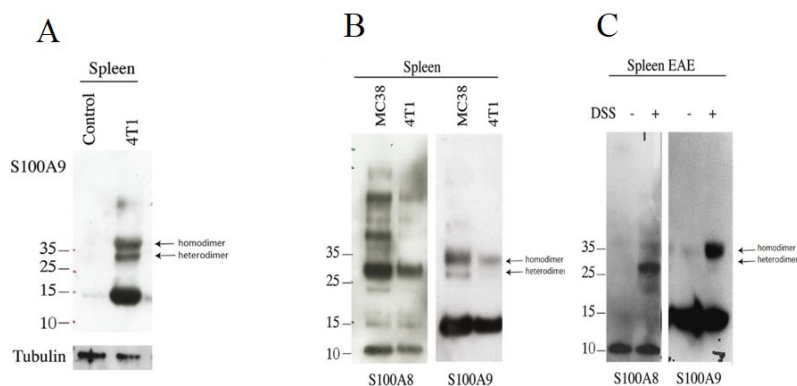
## Paper III

### ***S100A9 homodimer formation is induced during inflammation or tumor burden***

The important role of S100A9 as a DAMP with the ability to bind to TLR4 [54, 58] and RAGE [50] has been shown previously [58]. S100A9 is known to have prognostic value in many types of cancers e.g. colorectal, prostate and breast malignancies [73, 241]. S100A9 can form both homodimers and heterodimers with another family member of the S100 proteins; S100A8. The majority of S100A9 protein is in the heterodimeric form [242], and many inflammatory conditions correlate with elevated serum levels of S100A8/A9 [5, 243]. Moreover, it has been shown that compared to the homodimer, the heterodimeric form binds to TLR4 and RAGE rather poorly [54]. It has been shown that human S100A9 protein is relatively unstable in S100A9-expressing cells, and its stability can be increased by stimulating cells with the pro-inflammatory cytokine IL-1 $\beta$ . More importantly, S100A9 formed homodimers upon that stimulation [63]. Therefore, it is reasonable to assume that under inflammatory conditions and in the presence of pro-inflammatory cytokines, S100A9 can be stabilized, exposed to the cell surface and released to the extracellular space where it can act as a potent DAMP. Therefore, we sought to investigate this hypothesis under inflammatory conditions and tumor challenge.

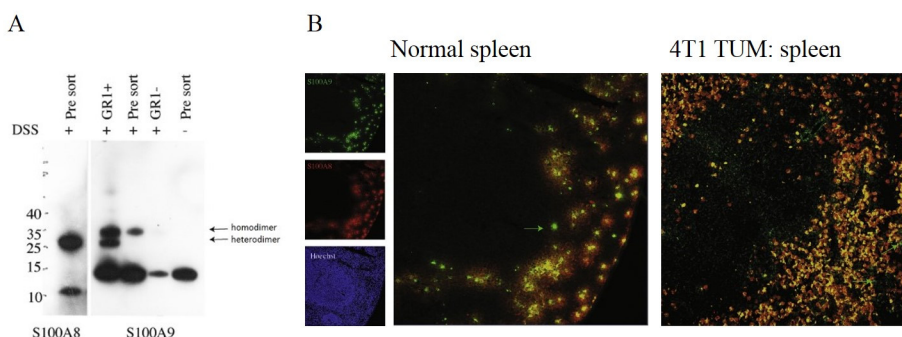
We provided two settings in the mice that we assumed can support formation of the S100A9 homodimer: challenge with the 4T1 mammary carcinoma tumor cells and EAE induction. Moreover, we focused our analysis on tissue rather than circulation. Due to the more pronounced induction of both S100A8 and S100A9 proteins in the spleens of mice challenged with tumor rather than EAE-induced mice, we focused our analysis on the tumor-bearing mice (data shown in paper III).

In order to investigate formation of S100A9 homodimers, we isolated spleen cells from 4T1-tumor-bearing mice and treated them with the membrane-permeable chemical cross-linker DSS, which allowed us to detect the presence of proteins in the form of multimers. Western blot (WB) analysis using anti-S100A9 antibody revealed the presence of S100A9 in the form of monomer, heterodimer, homodimer as well as higher ranks of this protein in the spleen (Fig. 8A). We repeated this experiment in mice with 4T1 and MC38 tumor (Fig. 8B) and also ongoing EAE (Fig. 9C), and formation of the S100A9 homodimer was confirmed in those settings as well. Using anti-S100A8 antibody confirmed presence of S100A9 homodimers. Therefore, we concluded that this phenomenon is not particular to 4T1 tumor-bearing mice.



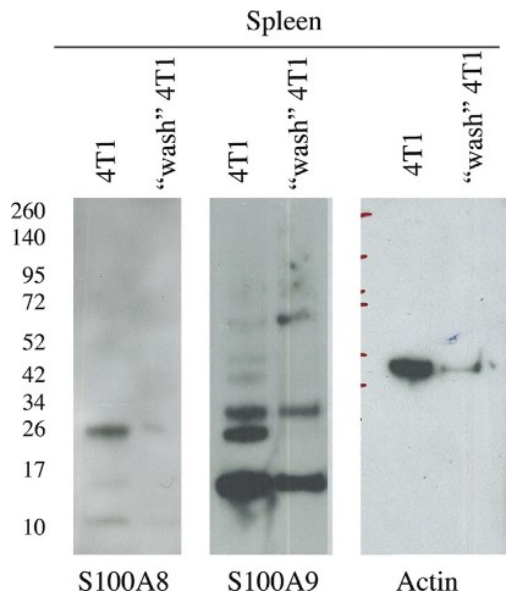
**Figure 8.** Formation of S100A9 homodimer in the spleen cells of 4T1 (A) and MC38 (B) tumor-bearing mice, and mice with ongoing EAE (C)

Previous studies have shown that myeloid cells are the major producers of S100A9 [5, 242]. Moreover, it has been shown that in 4T1 tumor model, a significant accumulation of Ly6G<sup>hi</sup> cells happens due to production of G-CSF cytokine from tumor cells [123]. To further investigate the source of S100A9 homodimers, we sorted CD11b<sup>+</sup>Gr1<sup>+</sup> cells from spleen of normal and tumor-bearing mice, treated them with DSS and performed WB analysis. We used anti-Gr1 antibody because it is known to detect both Ly6G<sup>hi</sup> and Ly6C<sup>hi</sup> cells [244]. As expected, we detected that the major source of S100A9 homodimer are Gr1 expressing cells (Fig. 9A). To investigate the presence of the S100A9 homodimer *in vivo*, we prepared tissue sections from spleens of 4T1 tumor-bearing mice and indeed detected cells which only express S100A9 but not both S100A8 and S100A9 (Fig. 9B). This further support the formation of S100A9 homodimer in tumor setting.



**Figure 9.** CD11b<sup>+</sup>Gr1<sup>+</sup> cells proven to be the major source of S100A9 homodimer in the spleen. A) S100A8 and S100A9 expression analyzed by WB of the FACS-sorted cells from tumor bearing mice. B) Spleen sections of naïve vs 4T1-tumor carrying mice showing presence of S100A9 homodimer in addition to S100A8/A9 heterodimer.

To check whether S100A9 homodimers are actively exported to the extracellular space where they can act as a DAMP, we investigated the presence of the homodimers in the supernatants prepared from spleen cells of 4T1 tumor-bearing mice. Extracts from spleens were centrifuged and cell pellet and supernatant were separated, treated with DSS and checked with WB for the presence of S100A9 homodimer. As shown in Fig. 10, all forms of S100A9 were detected in the cell pellet fraction, while only the S100A9 monomer and homodimers were in the suspension (wash). LDH assay was performed and confirmed a low level of cell lysis. Furthermore, the low amount of actin in the “wash” part compared to the cells in addition to the imbalance between the heterodimer and homodimer forms of S100A9 intracellular- and extracellularly indicates that an active transportation of the S100A9 homodimer is involved in the secretion.



**Figure. 10.**  
Secretion of S100A9 homodimers to the extracellular space.

In summary, our results show that in animals that are challenged with inflammatory stimulus or tumor, formation of S100A9 homodimer increases, and it can be secreted to the extracellular space. Moreover, the major source of this homodimer is the population of CD11b<sup>+</sup>Gr1<sup>+</sup> cells. All these factors encourage the theory that S100A9 homodimer formation may act as a feed-forward signal in inflammation and tumor burden.

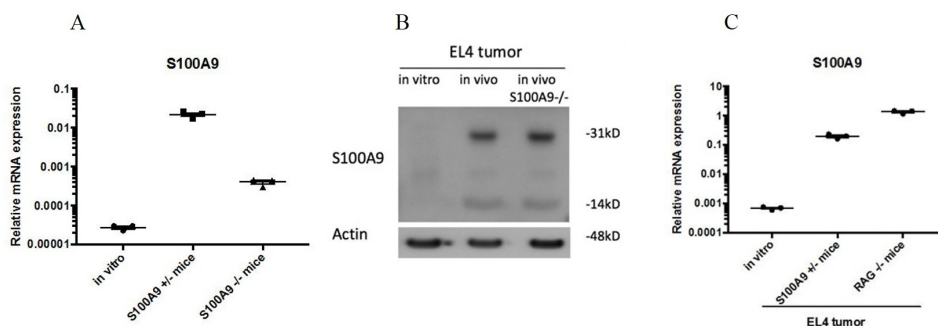
## Paper IV

### ***HIF-1 $\alpha$ alone is not sufficient to induce S100A9 expression in cancer cells***

The crucial role of S100A8 and S100A9 proteins in the process of tumorigenesis has been studied extensively [73, 241]. S100A9 is known to be important in the suppression of immune system's effector functions towards tumor cells [64, 69]. In mice, neutrophils display constitutive expression of both S100A8 and S100A9 proteins and their expression can be induced in other cell types such as epithelial and certain cancer cells [73, 245]. Given the prominent role of S100A9 in tumor microenvironment, it is important to understand the triggers of its expression.

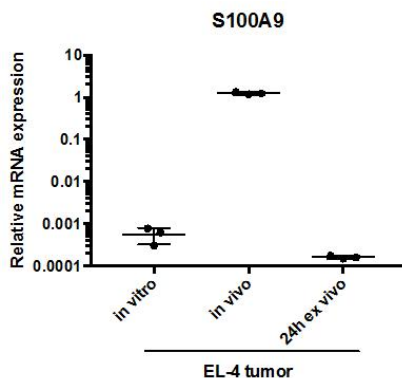
Insufficient tissue oxygenation, or hypoxia, contributes to aggressive and metastatic nature of cancer [126]. Downstream effects of hypoxia are mediated through the transcription factor; hypoxia-inducible factor 1 (HIF-1) [180]. HIF-1 is composed of two subunits HIF-1 $\alpha$  and HIF-1 $\beta$  [246]. Under hypoxic conditions the HIF-1 $\alpha$  subunit is stabilized, which leads to the expression of many genes involved in different processes including angiogenesis, metabolism and metastasis (reviewed in [126]). HIF-1 $\alpha$  expression is demonstrated in the majority of human cancers and associated with poor prognoses and relapse on treatment [127, 247]. Thus, we tested whether stabilization of HIF-1 $\alpha$  would induce expression of S100A9 in cell lines with or without intrinsic S100A9 expression.

From our previous studies, we knew that EL4 and B16 cell lines show no expression of S100A9 *in vitro*. However, at the end of *in vivo* growth, the expression could be detected both at mRNA and protein level in both cell lines (Fig. 11A, 11B) (data not shown for B16 cells). In order to rule out the effect of lymphocytes as the source of S100A9 expression, we inoculated RAG<sup>-/-</sup> mice with EL4 tumors and again could detect the induction of expression to the same extent as in S100A9<sup>+/-</sup> mice (fig. 11C).



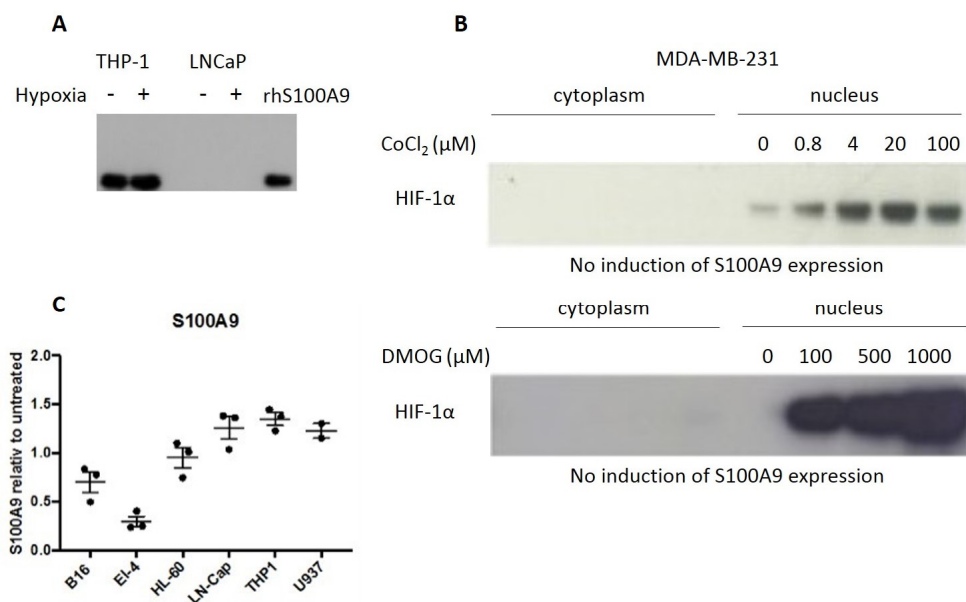
**Figure. 11.** Induction of expression of S100A9 in vivo. S100A9 mRNA (A) and protein (B) levels of EL4 cells *in vitro*, S100A9<sup>+/-</sup> and S100A9<sup>-/-</sup> mice. C) S100A9 mRNA expression *in vitro*, S100A9<sup>+/-</sup> and RAG<sup>-/-</sup> mice.

To ask whether the phenotype of S100A9 expression is dependent on the *in vivo* milieu, we grew EL4 cells in normal mice for 14 days and analyzed the S100A9 expression after 24h of culture *ex vivo*. As can be seen in Fig. 12, the expression is lost already after 24h hours of not being in contact with *in vivo* environment.



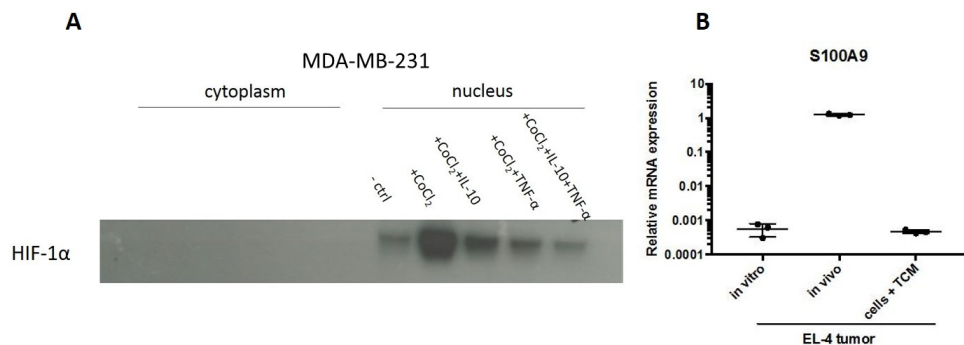
**Figure. 12.**  
Loss of S100A9 expression after 24h of culture *ex vivo*.

To assess the potential role of HIF-1 $\alpha$  on inducing expression of S100A9, we cultured THP-1 and LNCaP cell lines under hypoxic condition. THP-1 shows intrinsic S100A9 expression while LNCaP is negative for it. As it is shown in Fig. 13A, hypoxia treatment failed to induce S100A9 protein expression in both cell lines. To evaluate the potential role of HIF-1 $\alpha$  on induction of S100A9 expression, we treated MDA-MB-231 cells with two stabilizers of HIF-1 $\alpha$  protein: Cobalt chloride (CoCl<sub>2</sub>) and Dimethyloxalylglycine (DMOG). Although stabilization of HIF-1 $\alpha$  could clearly be detected at the protein level, both of the stabilizers failed to induce S100A9 expression (Fig. 13B). Furthermore, we evaluated a panel of cell lines for their S100A9 expression after CoCl<sub>2</sub> treatment and again detected no induction of expression at mRNA level (Fig. 13C).



**Figure. 13.** HIF-1α expression does not induce S100A9 expression *in vitro*. S100A9 protein levels in THP-1 and LNCaP cells after 24 hr of normoxic or hypoxic treatment. (B) Western blot analysis of HIF-1α and S100A9 protein levels in MDA-MB-231 cells after treatment with CoCl<sub>2</sub> and DMOG. C) mRNA expression of S100A9 in the indicated cell lines after treatment with CoCl<sub>2</sub>.

In addition to hypoxia, tumor microenvironment is a mixture of mediators provided by the skewed inflammatory milieu that could potentially act as a trigger of S100A9 expression. Therefore, we exposed MDA-MB-231 cells with a combination of CoCl<sub>2</sub> and IL-10 and/or TNFα, which are important cytokines in the tumor microenvironment. Again, we detected no induction of S100A9 expression (Fig. 14A). To provide culture conditions which might better resemble *in vivo* conditions, we prepared tissue culture medium (TCM) from EL4 cells that had been grown *in vivo*. *In vitro*-expanded EL4 cells were thereafter cultured in the presence of the TCM. There was no detectable expression of S100A9 mRNA in those EL4 cells either (Figure 14B).



**Figure. 14.**

Combination of CoCl<sub>2</sub> and cytokines and TCM fails to induce S100A9 expression *in vitro*. A) Western blot analysis of HIF-1α and S100A9 protein levels in MDA-MB-231 cell line cultured in presence of CoCl<sub>2</sub>, CoCl<sub>2</sub> + IL-10, CoCl<sub>2</sub> + TNFα and CoCl<sub>2</sub> + IL-10 + TNFα. B) S100A9 mRNA expression in EL4 cells grown *in vitro*, after 14 days of growth *in vivo* and EL4 cells cultured in presence of TCM.

In summary, we could show that *in vivo* growth induce a reversible S100A9 expression in cell lines with no intrinsic expression of this protein. This induction was not due to hypoxia or HIF-1α expression. Even culture of the cells with TCM or a combination of hypoxia condition with selected cytokines failed to induce expression of S100A9. Therefore, we conclude that the complex interplay of cells and mediators in the tumor microenvironment may control S100A9 expression *in vivo*.



# Acknowledgements

Choosing to do a Ph.D. is not an easy decision. Would I recommend others to go for it? Of course yes, it's not just about getting introduced to the infinite world of science, but to learn to push the limits, grow and become a totally different person. I would like to express my gratitude to all people whose presence made these years full of memories and learnings, especially:

My supervisor, **Fredrik Ivars**: Seeing your passion towards science has been so inspirational to me. Thank you for sharing your knowledge and most importantly, for all the support and understandings during these years. I've learned and achieved a lot through all those ups and downs. You indeed have a heart of gold.

My co-supervisor, **Tomas Leanderson**: You taught me to look at world from a totally different perspective. You brought up the concept of "*Copernicus*" at the best time possible. I appreciate your honesty and caring to give feedbacks, which although sometimes was hard to take, was necessary for my development.

My co-supervisor, **Dan Holmberg**, thank you for your support in paper II.

**Eva**: You taught me way beyond science. Your presence is a "must" in the TL/FI group. Just talking with you seems like everyday solution for all issues. I enjoyed your company so much. **Ansa**: Performing all animal experiments would be impossible without you. Thank you for sharing your exciting knowledge about dogs.

**Adnan**: Thank you for all the laughter, for trying your best to teach me all you knew, and your patience in the animal facility. **Zhifei**: you always inspired me how to be free and live in the moment.

**Veronika** and **Duoja**: for our mommy-gossips and relatable experiences. **Mo**: for listening to me, and always being there for me. **Kasia L.**: I like your easy going and caring nature, **Malin Samuelsson**: for being so soft, nice and caring. **Kasia P.** and **Janne**: for being my nice office-mates during the last stage of my PhD, **Kasia Krawczyk**: for our cozy lunch times.

**Gudrun, Jenny, Katha, Knut, Thorsten, Aymeric, Lieneke, Mimoza, Christine V., Lena Svensson, Andrzej, Yaxiong, Elin, Dora, Elsa, Una, Ester, Fatemeh, Shiva, Joy, Konjit, Kadir, Manar, Lena Meyer**, and members of **Pontus**

**Nordenfelt and Alex Evilevitvh's** group: for creating a relaxed and nice atmosphere in D14 to work at.

And, many thanks to all previous collaborators at Active Biotech: **Anders Olsson**: I enjoyed those few months that we worked together. You taught me how to keep up even in the toughest moments of life. **Marie Törngren** and **Helena Ericsson**: Thank you for all your support during the projects.

**Karin Leandersson**: for your quick responses whenever I needed help, although you were not directly involved in the projects. **Dr. Erik Berntorp**: for showing me that in the huge world of science, one can be both successful and generous.

**Zebbie, Björn**: For welcoming me to Sweden. **Khanum Tabrizi**: Thank you for making the Immunology Section at Shiraz University the best place to work at. Those memories will keep shining forever in my mind, **Kurosh** and **Farzaneh**: for being my cool friend since many years back. **Shima** and **Zahra**: for being my besties, **Sepideh**: for showing me how to manage life in the best way possible, **Maryam M.** and **Hossein**: Thank you for all the talking and all the things we have in common, **Naghmeh, Hossein H., Gelareh** and **Behzad**: for being our lovely and caring friends, **Mohammad Zakaria**, for your support and kindness, **Farideh, Behrouz, Saida, Fredrik, Mana** and **Salar**: For all happy gatherings and sharing your experiences about parenthood. **Naghmeh** and **Ali**: for being whom you are **Hossein, Parand, Faramarz, Pardis, Ehsan, Samira, Andisheh**: for staying as my lovely friends although we live far from each other. **Angela, Tibor, Mina** and **Tahir**: for always being there for us.

**Dr. Tamaddon, Mrs. Riazi, Dan** and **Ingela**: for being our nice neighbors. Dan: the first thing I'll do after Ph.D.? lära mig Svenska, Jag lovar ☺

**Mehdi**: Thank you for always being there for me, for being my loyal friend and man. You helped me mature into what I'm proud of today. **Melody** and **Lilly**: you little girls are my dream came true.

**Saeed, Saeedeh, Alla** and **Ali**: for all the memories that we share. I love you to the moon and back.

**Maryam** and **Maman joon**: for sharing all the laughter and the potato fest ;)

**Khale, Amu Reza, Kia, Daei, Zandaei** and **Amir**: Thank you for all your valuable support. **Amoo Siavash**: Thank you for being my lovely uncle. You are one in a million.

And last but not least to **Dr. Farhang Holakouee**: Listening to your speeches and conversations was an enjoyable learning tool for me during the past few years. Thank you for all your efforts.

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