S100A9 in inflammation

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Abstract

S100A9 is a small (13 kD) cytosolic calcium-binding protein. The protein is mainly expressed in neutrophils and monocytes in human but can also be expressed in other cell types under inflammatory conditions. S100A9 is normally co-expressed with S100A8 and forms S100A8/S100A9 heterodimers. Previous publications had suggested that S100A9 could be expressed without S100A8 in certain conditions. Various functions for cytosolic S100A8/S100A9 heterodimers have been described. The heterodimer can promote NADPH oxidase activation, is involved in reactions mediated by the iNOS enzyme and assists in tubulin polymerization. Extracellular S100A9 can bind to TLR4 and RAGE. Upon binding TLR4, S100A9 functions as a DAMP molecule inducing a pro-inflammatory cellular response. This thesis mainly focuses on the pro-inflammatory function of hS100A9.

In paper I, the main questions we asked were: in what condition hS100A9 could exist in cells without hS100A8? We found that the half-life of hS100A9 protein was short and degraded fast in cells, but can be stabilized by co-expressing with hS100A8 or by proteasome inhibitor. We also found that inflammatory stimuli could also stabilized hS100A9 protein and promoted the formation of proteolytically-resistant homodimer. This paper suggests that during inflammatory condition, hS100A9 protein might be able to exist as homodimer without hS100A8 and that it therefore may function as DAMP molecule after it has been released out of the cell.

In paper II, we showed that a chemical probe oxyclozanide could inhibit S100A9, S100A4 and S100A4/S100A9 binding to TLR4 and RAGE and inhibiting tumor progression in a mouse model. We also showed that hS100A4 and hS100A9 can form heterodimers. Further, we showed that S100A4 and S100A9 are differentially expressed in mouse myeloid cell populations.

In paper III, we wanted to identify co-receptor(s) involved in S100A9-mediated stimulation of TLR4. We show that CD14 is an essential co-receptor of this stimulation. Our data also suggest that some other co-receptor(s) might exist that help in S100A9 binding to cell membrane. However CD14 was crucial both for S100A9 signaling and internalization.

In summary, in this thesis we discuss the relation between S100A9 and inflammation. We show that during inflammation, the pro-inflammatory cytokine could stabilize the unstable hS100A9 by promoting it forming proteolytically-resistant homodimer. After S100A9 released out of the cell, they could function as DAMP molecule activating TLR4 in a CD14 dependent way. By blocking the interaction between the S100s with their receptors using OX, we propose a compound with clinical potential for treating inflammation and cancer.

Key words: S100A9, inflammation, oxyclozanide, CD14

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Paper I

Human S100A9 protein is stabilized by inflammatory stimuli via the formation of proteolytically-resistant homodimers

Matteo Riva, ***Zhifei He***, Eva Källberg, Fredrik Ivars, Tomas Leanderson.

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Paper II

Common interactions between S100A4 and S100A9 defined by a novel chemical probe

Per Björk, Eva Källberg, Ulf Wellmar, Matteo Riva, Anders Olsson, ***Zhifei He***, Marie Törngren, David Liberg, Fredrik Ivars, Tomas Leanderson.

PLoS ONE 8(5): e63012. doi:10. 1371/journal.pone.0063012

Paper III

CD14 is a co-receptor for TLR4 in the S100A9-induced pro-inflammatory response in monocytes

Zhifei He, Matteo Riva, Per Björk, Karl Swärd, Matthias Mörgelin, Tomas Leanderson, Fredrik Ivars.

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Abbreviations

AA: arachidonic acid

APC: antigen presenting cell

Cav1: caveolin-1

CLR: C-type lectin receptor

DAMP: damage-associated molecular pattern

DC: dendritic cell

ECM: extracellular matrix

ER: endoplasmic reticulum

G-CSF: granulocyte-colony-stimulate factor

LBP: lipopolysaccharide-binding protein

LPS: lipopolysaccharide

Mal: MyD88 adaptor-like

MAPK: mitogen-activated protein kinase

MDSC: myeloid-derived suppressor cell

MD2: myeloid differentiation factor 2

MHC: major histocompatibility complex

MHC-IIA: myosin heavy chain IIA

MMP: matrix metalloproteinase

MRP: myeloid-related protein

MyD88: myeloid differentiation factor 88

MβCD: methyl-β-cyclodextrin

NADPH oxidase: nicotinamide adenine dinucleotide phosphate-oxidase

NLR: NOD-like receptor

OX: oxyclozanide

PAMP: pathogen-associated molecular pattern

PRR: pattern recognition receptor

RAGE: receptor for advanced glycation end products

RLR: retinoic acid-inducible gene (RIG)-I-like receptor

ROS: reactive oxygen species

SAA: serum amyloid A

SPR: surface plasmon resonance

TEM: transmission electron microscopy

TIR domain: Toll-IL-1-resistence domain

TLR: Toll-like receptor

TRIF: TIR domain-containing adaptor-inducing IFN-β

Introduction

Overview of the immune system

The task of immune system is to distinguish self and non-self, healthy and damaged tissue, and eliminate the non-self invaders and abnormal self-tissues to maintain tissue homeostasis. Innate and adaptive immunity are two lines of defense that collaborate to achieve this goal.

All the multicellular organisms including vertebrates, invertebrates and even plants can defend themselves against microbial infection by recognizing molecular features, which are shared by many pathogens. This mechanism is called innate immunity. Our innate immune system includes epithelial barriers, antimicrobial proteins, complement system and innate immune cells. Epithelial cells as the fist line of defense sets up a physical barrier between the external world and us. It can also produce several antimicrobial enzymes or antimicrobial peptides to kill pathogens when they contact the epithelial surface. The complement system can recruit phagocytes to the site of infection, can recognize pathogens and mark them so that phagocytes can recognize and destroy them later. The complement system can also form membrane-attack complex, which lead to the death of certain pathogens directly. The cells of the innate immune system can recognize structures of pathogens. These molecules are called pathogen-associated molecular patterns (PAMPs) [1]. PAMPs are normally crucial for the survival or infectivity of the pathogen, and so the pathogens cannot escape the innate immune system by mutating such molecules, which is what they do when evading the adaptive immune system. The innate immune system can also recognize host cell stress or necrotic cells by recognizing some molecules, which are usually cytosolic or nuclear proteins and expressed intracellularly but released after cell damage. These molecules are called damage-associated molecular patterns (DAMPs) [2]. Host innate cells express receptors, which recognize PAMPs and DAMPs. These receptors are called pattern recognition receptors (PRRs).

The innate immunity provides a fast response to invading pathogens. However, if the pathogens cannot be eliminated in a short while, the adaptive immunity will be activated and provide a more efficient, specific and long-lasting protection for the body. Unlike the innate immune system, which can only recognize a relatively limited number of patterns with their germline encoded PRRs, the adaptive immune system can recognize a much larger number of different antigens. This is because cells of the adaptive immune system can express millions of distinct antigen receptors, which are generated by using mechanisms of somatic gene recombination. B lymphocytes and T lymphocytes are the main cell types of the adaptive immune system. The mature and naïve lymphocytes are resident in peripheral lymphoid organs. Antigen presenting cells (APC) from innate immune system, like dendritic cells, take up pathogens from infection sites, migrate to peripheral lymphoid organs and activate T cells by presenting them the foreign antigens with major histocompatibility complex (MHC) molecules. The APCs also express co-stimulatory molecules and secrete cytokines, which together result in the activation of T cells. The activated T cells then start to proliferate and differentiate into effector T cells. The effector T cells can kill the pathogens directly or assist other immune cells to fight with the pathogens. B cells can recognize and take up antigen with membrane-bound immunoglobulin (also called B cell receptor) and present the degraded peptides on the cell surface with MHC-II molecules. T helper cells, which are activated by the same antigen, recognize these peptides and induce the full activation of B cells. The activated B cells then proliferate and differentiate into plasma cells, which produce the secretory form of the immunoglobulin called antibody. After activation, a small portion of T and B cells become memory cells, which will provide strong and fast protection when next time the body encounter the same pathogens.

This thesis is mainly focused on an endogenous PRR activator named S100A9 and its relation with inflammation. So in this part I will mainly discuss innate immunity and the inflammatory response induced when triggering those receptors.

Inflammation, inflammation related myeloid cells and PRRs

Overview of inflammation

Elimination of foreign invaders and damaged tissue are important for all organisms. Inflammation is a host protective response, which is triggered by non-

self agents or by necrotic cells upon tissue damage. It involves cellular and molecular events, such as activation of blood vessels, local tissue resident cells, recruitment of leucocytes and release of chemical mediators and regulators. By diluting, destroying and neutralizing the harmful agents during inflammation, tissue restores its homeostasis, although sometimes dysregulated inflammation can also cause tissue damage.

Inflammation can be acute which last from a few minutes to a couple of days. When microbes invade tissue or tissue damage happens, local resident cells, mostly macrophage and also dendritic cells, mast cells and some other cell types will recognize the foreign invaders or necrotic cells, leading to activation of these resident cells and production of inflammatory mediators. These mediators, including cytokines, chemokines and various lipid-derived mediators interact with local blood vessels. This causes vasodilation and induces the increase of blood flow and vascular permeability allowing influx of plasma proteins to the local tissue. The activated endothelial cells of the blood vessels up-regulate selectins, which in combination with the up-regulation of integrin and chemokine receptors on leukocytes leads to the extravasation of neutrophils and other inflammatory cells to sites of infection or damaged tissue to eliminate the noxious stimulation. The activated cells and mediators are short-lived; they become inactivated or are degraded as the harmful stimulation is erased. If the stimulation cannot be eliminated immediately, acute inflammation can turn into chronic inflammation, which can last from days to years. During chronic inflammation, monocytes/macrophages and lymphocytes are recruited, in combination with vascular proliferation and fibrosis, resulting in more serious pathologic consequence.

Inflammation related myeloid cells

Several cell types belonging to the innate immune system, either circulating in blood or resident in tissues, are involved in inflammation. Neutrophils and monocytes are phagocytes that can be recruited to the infection site, recognize and ingest the microbes by phagocytosis. Neutrophils are granulocytes that have the greatest phagocytic activity among the phagocytes. They are the first cell type that is recruited from blood vessels to local tissue upon inflammation. At the inflammation site, they engulf microbes, kill them intracellularly and die after a few hours. Other granulocytes, like eosinophils, mast cells and basophils, play roles in host defence and allergies. Monocytes are another type of phagocyte that is circulating in blood and can enter extravascular tissues upon infections or tissue damage. After entering tissue, monocytes differentiate into macrophages and help

killing off the invaders. Tissue resident macrophages are among the first cells to encounter invading microbes. Dendritic cells (DCs) are also phagocytic cells, and they work as a bridge between innate and adaptive immune system by presenting microbe antigen to T cells as was mentioned above. Some DCs can produce interferon and play an antiviral role. Some microbes, such as viruses and intracellular bacteria, can induce the down regulation of MHC I on cell surface to escape from recognition by adaptive immune system. However the missing MHC I on host cell surface can induce activation of natural killer cells, which kill the infected cells and so eradicate infections.

PRRs and TLRs

In the first step of acute inflammation, invading microbes or tissue damages are recognized by PRRs expressed by the innate immune cells through the identificaiton of PAMPs and DAMPs. Examples of such receptors are Toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and C-type lectin receptors (CLRs). CLRs can lead to internalization of pathogens. However, the more powerful and important function of PRRs are the activation of signaling cascades, which lead to expression of inflammatory mediators and so assist the elimination of pathogens or tissue damages [3]. In this thesis we mainly focus on TLRs.

In 1989 Charles Janeway predicted that PRRs could recognize microbial molecules and so connect innate and adaptive immunity [4]. At that time, IL-1 was known as a pro-inflammatory cytokine but signal mechanism of its receptor IL-1R1 was unknown. In 1991, cytosolic domain of IL-1R1 was found to be homologous to the cytosolic domain of a Drosophila melanogaster protein named Toll [5] and a similar domain was found in a plant protein which played a role in virus resistance [6]. This conserved cytosolic domain, which exists in both plant and animal kingdoms was then named Toll-IL-1-resistence (TIR) domain. Few years later, Toll was found to be involved in production of the antifungal peptide Drosomycin [7, 8]. In 1997 the first mammalian Toll was cloned, which was found to activate NF-κB in monocytes and induced the expression of CD80 [9]. This human Toll was later named TLR4 [10].

Lipopolysaccharide (LPS) is a component of outer membrane of Gram-negative bacteria; it is an endotoxin and can induce septic shock [11, 12]. LPS was shown to induce NF-κB activity [13] and to activate p38-mitogen-activated protein kinase (MAPK) [14]. It took a couple of years before its receptor was found. In 1986,

Lipopolysaccharide-binding protein (LBP) was found to bind to LPS [15]. A few years later, myeloid differentiation factor 2 (MD2) was found to be a co-receptor for LPS [16]. The same year, CD14 was found to strongly enhance LPS signaling [17, 18], but not until 1998 scientists finally found solid evidence that TLR4 was the receptor for LPS [19].

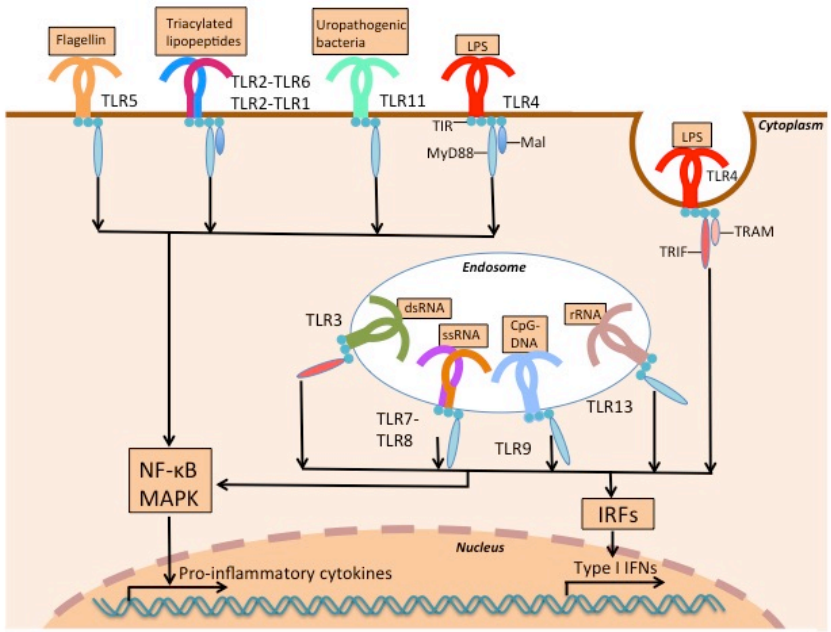
Until now, there are 10 functional TLRs found in human and 13 TLRs are found in mice. They are mainly expressed on innate immune cells, such as monocytes, macrophages, DCs, mast cells, eosinophils, basophils and NK cells. However, they can also be expressed on lymphocytes and tissue cells such as epithelial cells. TLRs can reside in different cellular locations, and they recognize different PAMP or DAMP molecules.

TLR1, TLR2 and TLR6 are expressed on cell surface. TLR2 was shown to form dimers with either TLR1 or TLR6 and to recognize bacterial lipopeptides [20-24]. TLR5 is expressed on cell surface and recognize flagellin, which is a protein component of bacteria flagella [1, 25, 26]. The human TLR11 is non-functional [27]. In mouse, TLR11 with the help of TLR12 has been shown to be activated by profilin from T. Gondii [28-31] and to be involved in preventing infections by uropathogenic bacteria [32]. TLRs can also recognize nucleic acids and this kind of TLRs are normally expressed in endosomal membranes. TLR3 recognizes double stranded RNA [33, 34], which does not exists in mammalian cells but in certain viruses. TLR7 may together with TLR8 be able to recognize single stranded RNA, which is expressed both in viruses and mammalian cells. RNA from mammalian cells normally does not activate TLR7, since in healthy mammalian cells RNA is found outside endosomes [35-38]. TLR9 can be activated by unmethylated CpG dinucleotides from bacteria or viruses [39-42]. The ligand of TLR10 is not known.

After binding of ligands to TLR ectodomains, TIR domains form dimers, recruit adaptor proteins and induce downstream signal transduction. One of the adaptor proteins is myeloid differentiation factor 88 (MyD88), which can interact with TLR2-TLR1, TLR2-TLR6, TLR5, TLR11, TLR7-TLR8, TLR9 and TLR13. MyD88 can transmit signals from both cell membrane and endosomal membranes. Another adaptor protein is TIR domain-containing adaptor-inducing IFN-β (TRIF), which interacts with TLR3 and TLR4 in endosomal membranes. The TLR receptors expressed on cell membrane in general detect bacteria related molecules, so the signal induced by these receptors result in transcription of pro-inflammatory cytokines and chemokines. The TLRs associated with endosomes detect abnormal

nucleic acids in the lumen of endosomes. These nucleic acids are normally a result of virus infection and so the signaling induced by these receptors result in transcription of pro-inflammatory factors and also type I IFNs (Fig 1).

Fig 1 TLRs and TLRs signaling



TLR4 is the only TLR that, depending on its cellular location, can interact with both MyD88 and TRIF. On cell membrane, the formation of TLR4 homodimer recruits bridging factor MyD88 adaptor-like [43]. Together with Mal, TLR4 recruits MyD88 binding to the TIR domain of TLR4. The death domain of MyD88 then recruits IRAKs, which is followed by recruitment of the E3 ubiquitin ligase TRAF-6. TRAF-6 then promotes ubiquitination of itself and protein IKKϒ (also known as NEMO). IKKϒ then actives TAK1 and leads to activation of MAPK and NF-κB. After TLR4 is internalized into endosomes, TRIF binds to TLR4 TIR domain and recruit TRAM. The downstream signaling from this pathway activates IRFs and induces the production of type I IFNs.

LPS is a classical TLR4 ligand and its structure has been well studied. There are several types of LPS and each of them consists of an O-antigen, an outer core, an inner core and lipid A. LPS can bind to the TLR4/MD2/CD14 receptor complex

and activate cells from both the cell surface and the endosome. Cell plasma membrane is the barrier between cells and environment and endocytosis is an important way for cells to communicate and exchange material with external environment. An early paper pointed out that although most well characterized receptor-mediated endocytosis was mediated through clathrin-coated pits, most LPS induced endocytosis was mediated through nonclathrin-coated membrane domains. Only a minor part was mediated by clathrin-coated pits [44].

As we know today, endocytosis includes phagocytosis and pinocytosis. As mentioned above, only certain cells such as neutrophils, monocytes and macrophages can perform phagocytosis. It is a well-regulated, energy consuming process, normally mediated by receptors, such as Fc receptors recognizing antibody-coated bacteria [45]. Pinocytosis includes: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis [46, 47]. Macropinocytosis is an actin-dependent process involving GTPases. During macropinocytosis, cell membrane protrusions collapse and fuse with cell membrane, but little is known about its regulation mechanism [48]. Clathrin-mediated endocytosis involves uptake of material into cells from surface via clathrin-coated vesicles. This is the major route for endocytosis in most mammalian cells. It is a receptor-mediated endocytosis [49, 50]. Some receptors are endocytosed constitutively no matter whether they bind to their ligands or not, while other receptors are internalized after binding their ligands. The endocytosed receptors can traffic back to plasma membrane for another round of trafficking or to lysosome for degradation [51].

Caveolae-mediated endocytosis is a mechanism known to be present in many kinds of cells. A caveolus is a special kind of lipid raft. Lipid rafts are sphingolipid-and cholesterol-enriched subdomains floating in lipid bilayer of cell membranes. There are mainly two kinds of lipid raft domains in mammalian cells: caveolae and planar lipid rafts, both can recruit signaling molecules to its site. Caveolae have flask-shaped membrane invagination and stabilized by caveolin dimers [52, 53], while planar rafts are plane structure continuous with the plasma membrane. Caveolin binds to cholesterol, inserting as a ‘hairpin’ loop into the inner lipid layer of the plasma membrane, leaving both C- and N-terminal domains facing cytoplasm [54]. Caveolae can be disrupted by deleting cholesterol in cell membrane [55], by overexpressing dominant negative mutants or by knocking out the ubiquitously expressed caveolin: caveolin-1 (Cav1) [52, 56]. Cells from $Cav1^{-/-}$ mice don’t have the flask shape caveolae, indicating the importance of Cav1 in maintaining the caveolar structure. $Cav1^{-/-}$ mice have no evident phenotype, although some researchers have shown increased cell proliferation and

tumorigenesis [57, 58] and enhanced endothelial nitric oxide synthase signaling in Cav1$^{-/-}$ mice. Different from clathrin-coated pits, without ligands binding to receptors, caveolae are stable structures on the cell membrane [59, 60], and can be internalized only when the relevant receptors binding to their ligands. After caveolae are internalized, they follow the endocytic pathway [61] or can alternatively be transported back to fuse with plasma membrane without involving the endosome [62-64]. There are also clathrin- and caveolae- independent endocytosis, but the mechanisms are poorly understood.

S100 proteins

In 1965, when scientists were trying to salt out proteins from bovine brain using ammonium sulfate, they found that some proteins in the mixture were still soluble in 100% saturated ammonium sulfate [65]. Because of this, these proteins were named S100 proteins. To date, more than 20 human S100 proteins have been found [66]. Human S100 protein family genes are mainly located in chromosomal region 1q21, while a few others are located on chromosomes 21, 4, 5 and X [67]. Despite their structural similarity, members of S100 protein family are expressed in different tissues and cell types [68] and playing specialized diverse roles in cells such as proliferation, migration and differentiation. Dysregulation of expression of these proteins can induce certain diseases [69].

S100 proteins are low molecular weight (around 10kDa) [70, 71] Ca^{2+} binding proteins, which are only found in vertebrates [72]. In steady state, cytosolic Ca^{2+} concentration is low (50-100nM) [73], while extracellular milieu has higher Ca^{2+} level (1.0-1.5mM) [74]. To maintain this low cytosolic Ca^{2+} concentration, Ca^{2+} is actively pumped out of the cell or pumped into intracellular Ca^{2+} stores, like endoplasmic reticulum (ER). Some stimulation, for example pro-inflammatory cytokines, can trigger to open ion channels on cell membrane or ER, which causes temporarily elevated cytosolic Ca^{2+} concentration (500-1,000nM).

Ca^{2+} binding proteins are a group of proteins that have evolved from a common ancestor and they function as regulators for intracellular Ca^{2+} level or participate in Ca^{2+} signaling pathways. Some Ca^{2+} binding proteins are localized within Ca^{2+} stores and they have high Ca^{2+} binding capacity and low Ca^{2+} binding affinity, which allows them to be able to release Ca^{2+} when it's needed. Other Ca^{2+} binding proteins localized in cytosol, some of them have high Ca^{2+} binding affinity, and so they can function as Ca^{2+} buffer during Ca^{2+} transients. Others can work as Ca^{2+}

sensors and interact with target proteins after they bind to Ca^{2+} and translate cytoplasmic Ca^{2+} concentration changes into cellular activities. Most S100 proteins belong to the last group [69].

S100A8 and S100A9

S100A9 and S100A8 are members of the S100 protein family. S100A9 together with S100A8 can form complex, which is called calprotectin because of its protective anti-microbial effect [75]. S100A9 is also named myeloid-related protein 14 (MRP14) or calgranulin B, because of its Ca^{2+} binding ability and high expression level in granulocytes. In human, the S100A8/S100A9 heterodimer is mainly expressed in neutrophils (constitute up to 40% of human neutrophils cytosolic protein) and in human monocytes (5% of cytosolic protein) [76]; but also have been found in platelets [77] and DCs [78]. S100A8/S100A9 can also be expressed in epithelial cells, keratinocytes [79], microvascular endothelial cells [80] and macrophages in conditions of inflammation [81, 82] as well as in some tumor cells and tumor stromal cells [83, 84].

There are two forms of human S100A9 (hS100A9) in granulocytes. The main form is full-length hS100A9, which is comprised of 114 amino acids and its molecular weight is 13.2kDa. The other form is truncated hS100A9, which is missing the first 4 amino acids in N-terminal and its molecular weight is 12.7kDa [85]. S100A9 can form homodimers [86] and some papers have reported that S100A9 can be expressed without S100A8 [87, 88], but the majority of S100A9 co-expressed with S100A8 forming a non-covalent heterodimer or heterooligomer [85]. hS100A8 comprises of 93 amino acid residues having a molecular weight of 10.8kDa.

S100A8/S100A9 and binding of Ca^{2+} and Zn^{2+}

S100 proteins all have two EF-hand Ca^{2+} binding domains, each EF-hand containing two α-helices and a linker region which is the site of Ca^{2+} binding [72, 89]. In S100 proteins, the two EF-hands have different Ca^{2+} binding affinity: the C-terminal EF-hand has a higher Ca^{2+} binding affinity K_d≈10-50μM and the N-terminal EF-hand has lower Ca^{2+} affinity K_d≈200-500μM[90]. When the cytosolic Ca^{2+} concentration is increased, S100 protein can bind to Ca^{2+} and go through conformational changes. The conformational changes result in exposure of a hydrophobic site on the S100 proteins and allow them to bind their target

proteins which then induce cellular responses [91]. The EF-hands can bind to not only Ca^{2+} but also some other metal ions, like Zn^{2+}, $Mg^{2+}$and Mn^{2+}.

Similarly to other members of the S100 protein family, S100A9 also has two EF-hands, which can bind to Ca^{2+} separately. S100A8/S100A9 heterodimer has also been shown can bind Zn^{2+}. Zn^{2+} cannot only bind to S100A8/S100A9 EF-hands but also can also bind to other Zn^{2+} binding sites such that one S100A8/S100A9 heterotetramer can bind to 12 Zn^{2+}. However, the EF hands have higher affinity for Ca^{2+}, because if Ca^{2+} is added to Zn^{2+} saturated heterotetramer the eight EF-hand binding Zn^{2+} would be replaced by Ca^{2+}. The formation of S100A8/S100A9 heterodimer is Ca^{2+} independent, however the higher molecular weight complex formation needs binding to Ca^{2+} and/or Zn^{2+} [70, 71, 92]

The expression of S100A8, S100A9 and S100A8/S100A9

It is well known, that there is normal expression level of S100A8 mRNA in $S100A9^{-/-}$ mice but S100A8 protein is almost undetectable [93, 94]. $S100A9^{-/-}$ mice can therefore be regarded as S100A9 and S100A8 functional double knockouts. This indicates mS100A8 may not be stable without the present of mS100A9. Our unpublished data showed if incubating $S100A9^{-/-}$ bone marrow derived granulocytes with proteasome inhibitor (MG132), mS100A8 protein expression could be partial restored compared with control sample. Our unpublished data also showed that when mS100A8 or mS100A9 cDNAs were transfected into fibroblast-like monkey kidney cell line COS, which don't express endogenous S100A8 or S100A9, the mS100A8 protein was found to be unstable while mS100A9 was stable. However, mS100A8 could be stabilized by co-transfecting the cells with mS100A9 cDNA. These results are similar yet opposite to the human S100A8 and S100A9 proteins, as shown in paper I. Together, these data indicate that the co-expression of S100A8 and S100A9 are important for stabilization of the unstable proteins (in mouse mS100A8, in human hS100A9), which may explain why in most cases these two proteins are expressed together.

But some data have indicated that these two proteins are not co-expressed and forming heterodimers in all situations. An in vitro experiment had shown that mS100A8 could be oxidized and form covalent dimer by incubating with activated promyelocytic leukemia cell HL-60. In vivo mS100A8 homodimer has been found in bronchoalveolar lavage fluid in LPS-induced pulmonary mouse model [95]. In human glomerulonephritis, high amount of hS100A8/S100A9 is found in

glomerulus during active inflammation and the heterodimer can be seen as an indicator of acute inflammation. However, researchers have reported the existence of $27E10^{-}/S100A8^{+}/S100A9^{+}$ cells (27E10 is an antibody can recognize hS100A8/S100A9 heterodimer) in SLE-glomerulonephritis patients' interstitium [96].

Also, by inserting coverslips in to subcutaneous tissues to induce chronic lesions in mice, scientists found expression of mS100A9 but not mS100A8 in epithelioid cells on the surface of coverslips [88]. hS100A9 also been found strongly expressed in human tuberculous lymph node granuloma without hS100A8 [87]. In human psoriasis, hS100A8 was found expressed in basal cells, but hS100A9 was found in all epidermal layers [97]. In invasive ductal carcinoma, hS100A9 was found strongly expressed and hS100A8 was barely detected [84].

In conclusion, S100A8 and S100A9 are co-regulated and expressed together in most cases, but under some circumstances like inflammation and cancer, they may be expressed without their partner protein. This thesis is mainly focusing on hS100A9, and in paper I we attempted to find conditions that would enable the expression of the unstable hS100A9 protein in the cells without hS100A8.

S100A8/S100A9 function

S100A8/S100A9 has both intracellular an extracellular functions. Inside cells, S100A8/S100A9 is mainly involved in reactive oxygen species (ROS) production in neutrophils and cell migration by interacting with cytoskeleton. After S100A8/S100A9 released from cells, it can function as fatty acid transporter, mediate neutrophil migration, play an antimicrobial role, promote tumor growth etc. One of the most well studied and although still under bate extracellular role of these proteins is working as DAMP molecule that can activate TLR4 and thereby inducing release of pro-inflammatory factors, which I will discuss later.

Intracellular functions of S100A8/S100A9

S100A8/S100A9 is mainly expressed in cytosol of myeloid cells, especially neutrophils. Neutrophils as a member of the innate immune system, one of their functions is to recognize and be activated by antibody or complement coated

particles. After activation, neutrophils can produce ROS. ROS is a group of reactive molecules and free radicals derived from oxygen. They play important roles in cell signaling and homoeostasis [98] and they are also toxic for pathogens [99]. The production of ROS is through NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase). NADPH oxidase is an enzyme complex including b_{558}, Rho guanosine triphosphatase, p40phox, p47phox and p67phox. The complex bind to plasma or phagosome membranes. In neutrophils, binding of opsonized particles triggers downstream signaling pathways and induce release of Ca^{2+} from ER. The released Ca^{2+} in ER triggers the activation of Ca^{2+} channel on plasma membrane, resulting in influx of Ca^{2+} and Ca^{2+} concentration elevation in cytosol [100]. This elevation of Ca^{2+} leads to binding of S100A8/S100A9 to arachidonic acid (AA) [101]. S100A8/S100A9-AA was then translocated to the NADPH oxidase complex located on cell membrane or phagosome. S100A8/S100A9-AA complex binds to cytochrome b_{558} and triggers NADPH oxidase activation [102]. Mutation experiment shown that Thr113 phosphorylation is important for S100A8/S1009-AA induced NADPH activation [103]. S100A8/S100A9 can be oxidized by ROS [104-106]. The relationship between S100A8/S100A9 and AA is also a good example of S100A8/S100A9 binding to different ions would result in different biological consequence. Binding to Ca^{2+} can induce S100A8/S100A9 binding to AA; however after S100A8/S100A9-AA is released from the cell, physiological serum concentration of Zn^{2+} can inhibit AA binding induced by Ca^{2+}. As a consequence, S100A8/S100A9 can carry AA at local inflammatory site but not in the blood.

Except transporting AA, S100A8/S100A9 has also been shown having some other intracellular functions. Investigators have shown that Ca^{2+} can induce S100A8/S100A9 tetramer formation and the tetramer can bind to tubulin [85]; it is also shown that Ca^{2+} can induce S100A8/S100A9 binding to intermediate filament and so mediate cellular migration [107]. S100A8/S100A9 has been reported to direct target-selective S-nitrosylation by transferring NO moiety from iNOS to S100A9 and then to the target protein. S100A8 together with S100A9 direct the site selection during the transfer procedure [108]. S100A9 has also been reported to regulate TLR3 trafficking and activation by promoting TLR3 containing early endosome maturation after virus engulfment [109]. Most of the time S100A8/S100A9 is located in the cytosol or at the cell membrane but they have also been found in nucleus. S100A9 has been detected binding to the promoter region of the complement factor C3 and to regulate its expression, thereby promoting development of psoriasis in a mouse model [110].

Extracellular functions of S100A8/S100A9

S100A8/S100A9 are not only found in cytoplasm or nucleus. During some inflammatory diseases or cancers, serum and/or local level of S100A8/S100A9 can be up regulated and the high level S100A8/S100A9 may be related to the disease development. Cytosolic proteins S100A8 and S100A9 lack leader sequences and cannot be exported via the classical Golgi pathway. How the proteins are released from cells is still unclear. Some investigators have suggested that these proteins are passively released from dead neutrophils during the disease development. In contrast, other investigators have suggested that these proteins can be secreted out in an energy-dependent, tubulin-dependent, protein kinase C related alternative pathway [111, 112]. After they are released from cells they can bind to various receptors on cell surface and play different biological roles.

Receptors for S100A8/S100A9

TLR4

In 2007, Vogl *et al* for the first time reported that S100A8 is an endogenous TLR4 ligand. In the paper, the author claimed that S100A8 is the active part of the S100A8/S100A9 heterodimer and neither S100A9 nor S100A8/S100A9 could stimulate bone marrow cells to produce TNFα [113]. Since then, many studies have been done to uncover the nature of S100A8, S100A9 and S100A8/S100A9 as DAMP molecules. Data from a recently published paper supported the idea that S100A8 could activate cells via TLR4 [114], and another paper pointed out that the C-terminal region of S100A8 is crucial for S100A8 binding to TLR4/MD2 [115]. Other data showed that S100A8/S100A9 heterodimer couldn't activate human PBMC to produce proinflammatory cytokines [116], which supported the conclusion of the paper by Vogl *et al*.

Although may be contrary to Vogl *et al*'s results, some other groups have shown that S100A9 itself can also activate TLR4. Thus, S100A9 was shown to trigger periodontal ligament cells to release of IL-6 and IL-8 through the NF-κB and p38 pathways in a TLR4 dependent way [117]. Our laboratory similarly showed that S100A9 can activate NF-κB in BM-DCs, leading to release of IL-6, IL-1β and TNFα in a TLR4 dependent way [118]. Researchers have also reported that both S100A8 and S100A9 could activate autoreactive $CD8^+$ T cells via TLR4 [119]. S100A8 and S100A9 alone have been shown could activate PBMC to produce pro-inflammatory cytokine in TLR4 dependent way

[116]. Extracellular S100A8/S100A9 heterodimer have also been shown having biologically function in ventilator lung injury by amplifying the damage in TLR4 dependent way [120].

Which of the S100A8, S100A9 and S100A8/S100A9 actually possess the biological activity to activate TLR4 is still under debate. However, from the fact that serum level of S100A8/S100A9 in patients with inflammatory diseases could reach a quite high level without leading to symptoms similar to 'septic shock', we can speculate that the S100A8/S100A9 proteins in patients' serum are most likely not in a form that can stimulate TLR4 properly. Indeed, some papers mentioned above [113, 116] suggest that S100A8 and/or S100A9 are biologically active for the stimulation of TLR4 and the formation of the heterodimer neutralizes that biological activity. Also, researchers tend to believe that in serum the majority of S100A8 and S100A9 exist in the form of heterotetrameric calprotectin which might not be stimulatory. Together, these may be able to explain why patients don't get 'septic shock' from S100 proteins. Alternatively, S100A8/S100A9 heterodimer might be able to activate TLR4 only at local inflammation sites but not in the blood stream. Maybe ions, proteins or other factors in serum may cause some conformational changes of S100A8/S100A9, so that they can no longer properly bind to TLR4 and so may weaken or eliminate the biological activity of the heterodimer. These questions are not fully understood at present. My purpose is to review the field and future research will hopefully clarify these discrepancies.

RAGE

Heterodimeric S100A8/S100A9 has been shown to bind to receptor for advanced glycation endproducts (RAGE) [121]. By using surface plasmon resonance (SPR), our group has also shown that hS100A9 can bind to RAGE. However, the binding between RAGE and hS100A8/S100A9 was weaker compared to the hS100A9 binding and no binding of hS100A8 to RAGE was detectable [122]. RAGE is 35kDa transmembrane multifunctional receptor belonging to immunoglobulin superfamily and can bind to a broad range of ligands. RAGE is expressed by many cell types such as hepatocytes, neurons, smooth muscle cells, endothelial cells, myeloid cells, myeloid-derived suppressor cells (MDSCs), lymphocytes, and tumor cells [123]. RAGE regulates a variety of cellular processes through different intracellular signaling pathways and plays an important role in inflammation and cancer. Tumor and tumor stromal cells can secrete pro-inflammatory mediators, such as TNFα, IL-6 and IL-1, and RAGE ligands. These RAGE ligands can bind

to RAGE receptor on endothelium and myeloid cells, thereby activating these cells and resulting in the recruitment of myeloid cells to the tumor site. Further, RAGE has been shown to be involved in S100 mediated migration of monocytes [116]. Also RAGE expressed on endothelium can mediate myeloid cell recruitment though myeloid cell surface molecule Mac-1 [123, 124]. The RAGE ligands, such as AGE, HMGB1 and S100 proteins, can promote the secretion of themselves by autocrine and paracrine feedback loops [125]. The important role of RAGE in promoting tumor growth has been supported by studies using the $RAGE^{-/-}$ mouse. For example, it has been shown that in comparison with WT mice, at least in DMBA/TPA-induced skin carcinogenesis, $RAGE^{-/-}$ mice have much lower tumor incidence rate and lower number of tumors per mouse [126]. In relation to these data, S100A8/S100A9 was also shown to promote tumor growth by activating NF-κB in a RAGE dependent way [127]. Other research groups have also reached similar conclusions by showing that colon tumor cells, which express RAGE modified by carboxylated glycans, can bind to S100A8/S100A9. This binding activated NF-κB, which led to tumor cell proliferation [128].

CD36

It has reported that elevated plasma concentration of S100A8/S100A9 is associated with higher risk for acute ST-segment elevation myocardial infarction (STEMI) in apparently healthy individuals [77]. Further, plasma concentration is associated with recurrent myocardial infarction (MI) or cardiovascular death [129]. The authors show that CD36 together with S100A8/S100A9 play a role in regulating thrombosis. In vitro experiment has shown that S100A8/S100A9 can directly interact with CD36 [130]. CD36, also known as fatty acid translocase (FAT), is an integral membrane protein. It can be expressed in many different cell types such as monocyte/macrophage, endothelium, epithelium, muscle cells, adipocytes, and platelets [131, 132]. S100A8/S100A9 may bind to CD36 on platelet surface causing platelet activation. The authors have shown a prolonged carotid artery occlusion time in $S100A9^{-/-}$, which can be shortened by infusion WT platelets (which can be the source of S100A8/S100A9) or purified S100A9 protein, but not in $S100A9^{-/-}$ $CD36^{-/-}$ double knockout [133]. They also showed that S100A9 could bind to CD36 and activate platelets in a CD36 dependent way. These data indicate, that CD36 could be a binding and signaling receptor for S100A9 that may activate platelets and promote thrombus formation.

Other receptors for S100A8/S100A9

S100A8/S100A9 was shown to bind to endothelial cell surface heparan sulfate proteoglycan in a Ca^{2+} and Zn^{2+} dependent way. S100A9 but not S100A8 is the

subunit binding to the endothelial cell [134]. EMMPRINE, also called Basigin or CD147, is a transmembrane glycoprotein belonging to immunoglobulin superfamily. S100A9 can bind to EMMPRIN and inducing expression of cytokines and matrix metalloproteinase (MMP), which may promote tumor metastasis [135].

Other extracellular functions of S100A8/S100A9

Chemotaxis and role in leukocyte migration

mS100A8 have been shown to have chemotactic biological function for neutrophils and mononuclear cells both in vitro and in vivo [136, 137]. Also it has been shown that injection of mS100A8, mS100A9 or mS100A8/S100A9 intravenously could increase neutrophil number in blood and reduce the number in bone marrow. This indicated that both mS100A8 and mS100A9 might play a role in neutrophils migration [138]. Except myeloid cells, many tumor cells can also express S100A8 and S100A9 [84, 139-141]. Using in vitro cell migration settings, it has been shown that supernatant from culture of mouse breast cancer cell line 4T1, was chemotactic for myeloid-derived suppressor cells (MDSCs), and this chemotactic effect can be reduced in the presence of S100A8 or S100A9 blocking antibody [142]. This suggests that by releasing mS100A8 and mS100A9 protein, tumor cells might attract MDSCs to tumor site and facilitate tumor growth. However, another group has shown that in LPS induced lung inflammation, blocking mS100A8 but not mS100A9 with blocking antibody can reduce neutrophil infiltration to lung in some degree [143] and this may challenge the idea that mS100A9 can regulate neutrophils migrating to inflammatory site.

Which among hS100A8, hS100A9 and hS100A8/S100A9 has chemotactic function is still under debate [144, 145]. But scientists seems agreed on that hS100A9 is able to increase Mac-1 affinity in neutrophils [144, 145]. During normal conditions, endothelial cells barely express S100A8 and S100A9, however it has been reported that during atherogenesis, endothelial cells start to express S100A9 mRNA and different forms of hS100A9 (hS100A9, $hS100A9_2$, hS100A8/S100A9, $hS100A9_3$) were detect by western blot [80]. Also, other publications have shown that hS100A9 and hS100A8/S100A9 can bind to LPS activated human microvascular endothelium cell line HMEC-1 [146]. These data indicate that at least under certain conditions, endothelial cells can present S100A9 and/or S100A8/S100A9 on cell surface. hS100A9 on endothelial surface then maybe be able to activate Mac-1 and facilitate neutrophils binding to endothelial cells.

Antimicrobial function

Since Zn^{2+} is necessary for bacterial and fungal growth, chelation of Zn^{2+} by extracellular S100A8/S100A9 heterodimer may play an antimicrobial role [147]. S100A8 and S100A9 alone cannot bind to Zn^{2+}, which was supported by the fact that S100A8/S100A9 heterodimer but not S100A8 or S100A9 alone can prevent growth of *Klebsiella* in in vitro [148]. Except the chelation of Zn^{2+}, human S100A9 but not human S100A8 can also play antimicrobial function by inducing neutrophils degranulation in a MAPK-dependent way [149] and enhancing bactericidal activity by increase neutrophils phagocytosis [150].

S100A8/S100A9 and diseases

S100A8/S100A9 and inflammatory diseases

Because of the serum level of S100A8/S100A9 correlating with the extent of inflammation in the ongoing diseases, monitoring serum level of these S100 proteins has become a helpful approach to detect disease activity and patients' response to treatments in different diseases, such as multiple sclerosis [151], Crohn's disease [152], giant cell arteritis [153], rheumatoid arthritis, systemic lupus erythematosus and progressive systemic sclerosis [154]. Except monitoring S100A8/S100A9 serum level, a recent paper showed monitoring local S100A8/S100A9 level, may be able to predict disease development in a more sensitive and reliable way [155].

Not just as biomarker, S100A8/S100A9 may actually contribute to the development of some inflammatory related diseases. $S100A9^{-/-}$ mice have reduction in inflammatory response towards LPS, therefore are protected from endotoxin-induced septic shock [113]. In $S100A9^{-/-}$ psoriasis model, the skin inflammation was strongly reduced [110]. By using an antigen-induced arthritis model, researchers have shown joint swelling and cartilage destruction were significantly lower in $S100A9^{-/-}$ mice compare with wt [156]. Another paper has shown that hS100A8/S100A9 may be involved in the development of juvenile idiopathic arthritis [157], the promotion of the disease by S100A8/S100A9 may partically because of S100A8/S100A9 can mediate recruitment of more $CD11b^{+}$ cell to the local site [144, 158, 159].

S100A8/S100A9 and cancer

During decades of study in cancer biology, it has been realized that the development and progression of cancer is not only just the biological events that happen to cancer cells themselves, but also the establishment of tumor environment by non-tumor stromal cells play active role in tumorigenesis. Also in recent years, the important connection between inflammation and cancer has become a central theme of cancer biology [160]. S100A8/S100A9 has been reported to be overexpressed in different cancers [84] and high expression of S100A9 could relate to poor outcome [161], suggesting that these S100 proteins might play a role in cancer development.

MDSCs

MDSCs are a heterogeneous group of myeloid cells with a phenotype Gr-1^{+}CD11b^{+} in mice. MDSCs include immature macrophages, granulocytes, DCs, and myeloid progenitors. MDSCs were found accumulating in different kinds of cancer [162-164]. MDSCs play strong immunosuppressive role in cancer by inhibiting T [165, 166] and NK cells activation. S100A9 plays an important role in MDSC formation and recruitment at least in some tumor models. Thus no accumulation of MDSC was detected in S100A9$^{-/-}$ tumor bearing mice in the EL-4 tumor model [167]. In physiological conditions, S100A9 expression level is down-regulated during myeloid cell maturation [168]. Tumor derived factors can up-regulate S100A9 in myeloid cells, which in turn inhibits myeloid cell differentiation [167]. Except inhibiting myeloid cell differentiation, it is also shown that S100A8/S100A9 can bind to RAGE on MDSCs and signal through NF-κB, resulting the migration and recruitment of MDSCs to the tumor site [142, 169]. These together result in the accumulation of MDSC that facilitates tumor growth.

Other mechanisms in cancer

Except promoting the accumulation of MDSC, S100A8/S100A9 can also assist tumor growth and metastasis by inducing cytokine production. It was shown that S100A8/S100A9 from myeloid cells could bind to RAGE on colon cancer cells activating MAPK and NF-κB resulting the up-regulation of genes relating to leukocyte recruitment, tumor migration, angiogenesis and premetastatic niches formation [170]. Similar result was shown with TLR4 in EL-4 tumor model [171] and EMMPRINE in melanoma model [172]. Also the primary tumors released factors that can affect distant organs, like brain, liver and lung, to up-regulate

S100A8/S100A9, which will then facilitate the formation of pre-metastatic niche[91] in these distant organs.

S100A4

S100A4 (also known as placental calcium binding protein, mts1, FSP1, 18A2, 42A, CAPL, metastatin-1, p9Ka, PEL98, calvasculin) is another member of the S100 protein family. S100A4 is expressed in monocytes and macrophages but can also be expressed in other cells, such as endothelial cells and fibroblasts [173]. In 1993, S100A4 was reported for the first time to induce a metastatic phenotype in a rat epithelial cell line [174]. Later on another study showed that a transgenic mouse strain, which overexpressed S100A4 in lactating mammary gland had a normal phenotype. However, after crossing these mice to the GRS/A strain, a strain having high incidence of mammary tumors but seldom metastasizing, nearly half of the hybrid females developed metastasis in lungs [101]. These results indicated that S100A4 itself was not tumorigenic but it may enhance tumor metastasis. The connection between S100A4 and metastasis has also been proven in S100A4$^{-/-}$ mice, since in these mice less metastasis was found [175-177]. Since then many studies have been done trying to figure out the mechanism behind the pro-metastatic function of S100A4.

Intracellular function of S100A4

Intracellular S100A4 is related to tumor cell mobility. S100A4 has been found to bind non-muscle myosin heavy chain IIA (MHC-IIA) [178, 179]. Non-muscle myosin molecules can form bipolar filaments, and interact with actin, thereby producing a contractile force. It has been shown that S100A4 co-localizes with MHC-IIA in lamellipodia structures in the leading edge of breast cancer cell line MDA-MB-231 [180]. MHC-IIA and S100A4 were also shown to interact in vitro in a Ca^{2+} dependent way [179]. Also binding between S100A4 and myosin can suppress myosin assembly by increasing its solubility in vitro [179]. It has been shown that S100A4 can affect cell polarization. With the help of S100A4, cells can form forward protrusions towards a chemotaxtic source [181]. Cell migration normally includes three steps: the extension of lamellipodium, attaching leading lamella to extracellular matrix and forming focal contact, and retracting the tail towards the leading edge with contractile force. During cell migration, stress fibers and membrane protrusions are important dynamic structures that are regulated by Rho GTPases, and RhoA is one of them. RhoA have been shown being active at the front edge of migration cells [182] and mediating membrane ruffling and stress

fiber formation. For many years investigators had wondered, how does RhoA switch role from promoting membrane ruffling to stress fiber formation? A recent study showed that this change needed cooperation between RhoA, S100A4 and Rhotekin. Rhotekin is a scaffold protein, which can interact with RhoA [183]. S100A4 can bind Rhotekin in a Ca^{2+} dependent way. After S100A4 binding to Rhotekin, S100A4-Rhotekin together can form complex with active RhoA. The S100A4-Rhotekin-Rho complex can promote membrane ruffles and suppress stress fiber formation in response to epidermal growth factor. This can result in invasive tumor growth. In the absence of S100A4 binding, opposite to S100A4-Rhotekin-Rho, Rho-Rhotekin mediates myosin-IIA oligomerization and inhibits formation of membrane ruffles. So S100A4 may work as a regulator switching Rho's role between membrane ruffles and stress fiber formation [184].

S100A4 has also been shown to bind to other molecules, such as liprin β1, F-actin and non-muscle tropomyosin. However the role of S100A4 in these interactions is not clear [91]. Interestingly, upon IL-1β stimulation the cytosolic S100A4 has been reported to be post-translationally modified by the sumo-1 protein and translocated to nucleus. This translocation correlated with the production of MMP13 [185], which may facilitates matrix remodeling during cell migration.

Extracellular function of S100A4

S100A4 can be released from tumor or stromal cells and serve as an autocrine and/or paracrine factor to promote tumor progression [186]. Extracellular S100A4 was shown to stimulate tumor cells to secrete pro-inflammatory cytokines and so to facilitate tumor growth. Scientists have shown that extracellular S100A4 can induce tumor cells to up-regulate and release inflammatory factors such as CCL2, acute-phase response proteins serum amyloid A (SAA) and IL-8. CCL2 and SAA can contribute to monocyte recruitment and may also help in polarization of these monocytes to M2 phenotype, while IL-8 can facilitate vascular destabilization and angiogenesis [187]. In their work, melanoma cell lines Melmet1 and Melmet5 were stimulated with rS100A4 and they found that Melmet5, which had higher expression of RAGE, responded better [187]. This indicates that S100A4 may be a RAGE ligand. S100A4 has also been shown to activate NF-κB through TLR4 and induce expression of SAA1 and SAA3. SAA proteins then can induce expression of granulocyte-colony-stimulate factor (G-CSF), which can induce angiogenesis and promote tumor growth [188]. SAA proteins can also stimulate expression of MMP2, MMP3, MMP9 and

MMP13. MMPs are important for tumor invasion. MMPs can break down extracellular matrix (ECM) and so facilitate invasive tumor growth. Such activity is also important for metastasis formation. MMPs can process pro-angiogenic factors and release them from their inactive form. MMPs can also impair epithelial cell adhesion by cutting off the extracellular domain of E-cadherin and thereby promoting tumor invasion [189, 190].

Pharmacological potential for S100 proteins

Because of all the biological function described above, inhibiting the biological activity of S100 proteins could potentially be use as a treatment in inflammatory diseases and cancers. The idea of blocking S100 proteins binding to their receptors has been pursued. Nowadays there are already some compounds under testing.

One group of such blocking compounds are quinoline-3-carboxamides, also called Q-compounds. Q-compounds have been found can bind to S100A9 and block their binding to TLR4 and RAGE [122, 171]. Q-compounds have been shown to reduce inflammation in different mouse models. Paquinimod, a member of Q compounds, has been shown to reduce collagenase-induced osteoarthritis [191], can inhibit disease in a murine lupus model [192]. Also, by using acute peritonitis mouse model, our group showed paquinimod could reduce influx of $Ly6C^{hi}$ inflammatory monocytes to a site acute inflammation [193]. Laquinimod, another Q-compound, have been reported to inhibit the development of murine EAE (acute experimental autoimmune encephalomyelitis) [194]. Tasquinimod, also a member of Q-compounds, has been shown to reduce tumor growth in different mouse models. By using a 4T1 tumor bearing mice model, our group have shown tasquinimod could reduce influx of $Ly6C^{hi}$ cells into the tumor site and that it may at least partially contribute to the inhibition of tumor growth [195]. Tasquinimod has also been shown can inhibit tumor growth in the EL4 lymphoma model [171], prostate cancer [196, 197] and colon carcinoma tumor model [198]. Some Q-compounds have been tested in clinical trials. Laquinimod has been tested in phase 3 clinical trials for relapsing-remitting multiple sclerosis and the fist study showed the reduction of relapse rate and slowing of the progression of the disease [199], but the second study showed no difference [200]. Now the third clinical trail is on going. Tasquinimod has pass phase 1 [201] and phase 2 clinical trails [202] in metastatic castration-resistant prostate cancer. However, a recently phase 3 clinical trail showed tasquinimod did not significantly improve overall survival rate [203].

Salicylic amides are another group of compounds that have been described that can interfere with S100s. Niclosamide, a member of salicylic amides, have been reported to inhibit S100A4 transcription and reduce metastasis in a mouse colon cancer model [204]. Oxyclozanide [108] is another member of salicylic amides. In paper II, we show that this compound can bind to S100A9 and S100A4 and inhibit their binding to TLR4 and RAGE. This will be further discussed below.

Results and discussion of the papers

Paper I

Human S100A9 protein is stabilized by inflammatory stimuli via the formation of proteolytically-resistant homodimers

Aim:

To investigate whether the stability of intracellular hS100A9 could be increased by inflammatory stimuli.

Questions addressed in paper I:

1) What is the half-life of hS100A8, hS100A9 and hS100A8/S100A9 and in what form do they exist in cells?

It is well known that S100A8 and S100A9 tend to form S100A8/S100A9 heterodimer but not homodimers in cells expressing both proteins. To confirm that observation, we used the human monocytic cell line THP1, which expresses both hS100A8 and hS100A9. To investigate the formation of dimers of these S100 proteins, we used a cell permeable cross-linker DSS. Using this tool, dimeric forms could still be detected after running protein gels under reducing conditions. By doing so, we found only heterodimer form of the proteins in THP1 cells, which support the previous observations. We could also detect hS100A8 and hS100A9 monomers in the western blot, which may due to that the cross-linker is not fully efficient.

By using the translation inhibitor cycloheximide we showed that in THP1 cells, the half-life of hS100A8 and hS100A9 were both longer than 24hrs. To determine the half-life of hS100A8 and hS100A9 when they expressed alone, we transfected COS cells with hS100A8 and hS100A9 expression constructs

separately. We found out that the half-life of hS100A9 is very short and that the half-life of hS100A8 is around 4hrs. We further show that the level of hS100A9 protein was increased in cells incubated with proteasome inhibitor MG132 or co-transfected with hS100A8. These results indicated that the hS100A9 protein, when expressed in the absence of hS100A8, is degraded by the proteasome. These results also explained the short half-life of the hS100A9 protein. Similar results were detected in experiments using LEP cells, a human fibroblast cell line. We also performed co-immunoprecipitation experiments to prove that hS100A8 and hS100A9 proteins formed heterodimers in the transfected COS cells.

2) Would inflammatory stimuli influence the stability of hS100A9?

To address this question, we stimulated COS cells, which had been transfected with either hS100A9 or hS100A8, with IL-1β, LPS or TNFα. We found that these inflammatory stimuli could increase the level of hS100A9 protein but didn't influence hS100A8 expression much. We also showed that in cells treated with MG132 or IL-1β, hS100A9 could form homodimers when transfected alone. These data supported the hypothesis that inflammatory stimuli might influence the stability of the hS100A9 protein. However, in this paper we did not formally show that the hS100A9 protein had a longer half-life in the stimulated cells. Thus, there may be alternative explanations to the increased hS100A9 protein level seen in the stimulated cells.

Discussion:

It is well known that in most situations S100A9 proteins tend to form heterodimer with S100A8. However, data from our laboratory [118] as well as studies from other laboratories showed that S100A9 alone could activate TLR4 [116, 117, 205]. Since hS100A9 have been shown to interact with TLR4 and RAGE [122] and S100A9 expressed without S100A8 have been reported [87, 88], it is possible that under some conditions, S100A9 may be expressed alone and might play a biological role as a pro-inflammatory factor.

This paper is based on the thought that in physiological conditions hS100A9 is not stable inside the cell and needs to bind hS100A8 to be stabilized. However, under inflammatory conditions, possibly due to some post-

translational modification(s), hS100A9 might form intracellularly stable homodimer. This homodimer could potentially be released and play a pro-inflammatory function.

After this paper was published, we have done some follow up experiments trying to further understand the relationship of hS100A9 stability and inflammatory stimulation. Since papers from other laboratories have shown that S100A9 can be post-translationally modified in different ways [104, 206], the first question we asked was could hS100A9 be modified under inflammatory conditions and if so would that change the stability of the hS100A9 protein? It has been shown that hS100A9 can be phosphorylated on Thr113 [206] and that the phosphorylated hS100A9 can be translocated to the cytoskeleton [207]. This may result in S100A9 binding to a new partner protein and be stabilized thereby. To address whether the Thr113 residue would influence protein stability, we mutated this amino acid residue. First, Thr113 was mutated to either Glu or Asp to mimic the negative charge of phosphorylation. Further, we mutated Thr113 to Ala to generate a hS100A9 protein that could not be phosphorylated at this site. However, the three mutants behaved the same as wt-hS100A9 in transfection experiments, which means phosphorylation of Thr113 most likely does not influence the stability of the hS100A9 protein (Fig2 A).

As shown in the paper, the half-life of hS100A9 is quite short, but we did not determine its half-life more precisely. In follow-up experiments, we found out that the half-life of hS100A9 was about 5-10min (Fig2 B). As mentioned above, hS100A9 accumulated in transfected cells exposed to a proteasome inhibitor. We also tested whether a lysosomal inhibitor chloroquine would have a similar effect but that was not the case (Fig2 C), suggesting that the main site of degradation is the proteasome.

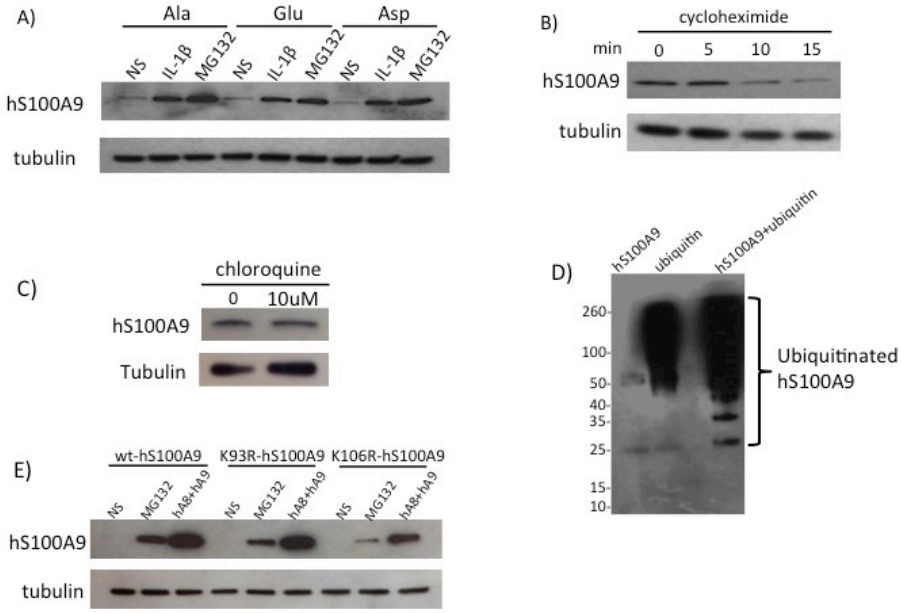
Using immune-precipitation we could formally show that hS100A9 can be ubiquitinated (Fig2 D). We therefore tried to identify the ubiquitin site(s) of hS100A9 protein. Because hS100A9/S100A9 is unstable and hS100A8/S100A9 is stable, we thought that an ubiqutination site(s) might be exposed in the homodimer, but inaccessible in the heterodimer. In collaboration with Guenter Fritz, we compared the 3D structure of hS100A8/S100A9 with hS100A9/S100A9 (unpublished results from Guenter Fritz). This analysis predicted Lys93 in hS100A9/S100A9 to be accessible while less so in hS100A8/S100A9. Based on analyses using software

predicting ubiquitination sites, we also identified Lys106 a potential site. We therefore independently mutated Lys93 and Lys106 in hS100A9. However, the stability of the mutant proteins was not changed by mutating either of these two Lysines to Arginins (Fig2 E). To directly determine which Lysine residues are ubiquitinated in hS100A9 we use nano LC-MS/MS to analyze hS100A9 protein isolated from transfected cells treated with proteasome inhibitor. The results from MS/MS indicated Lys50, Lys51, Lys54 and Lys57 might be ubiquitinated. Since the samples were digested by trypsin before running for MS/MS, and some of the digestion products of hS100A9 could be only few amino acid long, these small peptides could not be detected by MS/MS, which means the actual ubiquitinated Lysines might more than the ones listed above. Based on these observations, we predicted that hS100A9 could be broadly ubiquitinated on many Lysine sites. It is possible that when hS100A9 is expressed alone in the cell, the majority of hS100A9 maintain as monomer, which cannot fold properly without hS100A8 and so easily be recognized by ubiquitin and degraded in proteasome in a non-specific way.

Conclusions:

The hS100A9 protein is unstable and can be ubiquitinated and degraded in the proteasome in the absence of hS100A8. But the expression level of hS100A9 protein is increased under inflammatory stimuli. Together with the fact that S100A9 is a DAMP molecule, the increased expression of hS100A9 maybe involved in the development of inflammation disease.

Fig 2



A) Similar expression pattern of T113-hS100A9 mutants and wt-hS100A9. COS cells were transfected with hS100A9 mutants T113A, T113E or T113D. 24hrs after the transfection, cells were treated with IL-1β or MG132 for 6hrs. After incubation, cells were harvested for WB and analysed for S100A9 and tubulin expression. The three mutants were all unstable and the expressions were increased in the presence of IL-1β and MG132, which was similar to wt-hS100A9 as shown in the paper. B) Half life of wt-hS100A9. COS cells were transfected with wt-hS100A9. 24hrs after transfection, cells were treated with cycloheximide for different time period. Samples were analyzed in WB, staining for hS100A9 and tubulin. Half life of hS100A9 was between 5-10min. C) Lysosomal inhibitor could not increase hS100A9 expression level. COS cells were transfected with wt-hS100A9. 24hrs after transfection, cells were treated with lysosomal inhibitor chloroquine for 6hrs. After the 6hrs incubation, cells were harvested for WB and stained for hS100A9 and tubulin. NO significant difference was observed between the samples with and without chloroquine, suggesting that inhibiting lysosome cannot increase hS100A9 level in the cells. D) hS100A9 can be ubiquitinated. COS cells were transfected with hS100A9-flag, ubiquitin-HA, or hS100A9-flag and ubiquitin-HA together. 24hrs after transfection, cells were incubated with MG132 for 6hrs. After 6hrs incubation, cells were lysed and hS100A9-flag were pulled down using anti-flag M2 affinity gel. Protiens were eluded from beads and run in SDS gel and stained for HA. A size ladder can be seen in the double transfected sample, which indicated hS100A9 was modified with varible number of ubiquitin molecules. E) The stability of hS100A9 was not changed by mutating wt to K93R or K106R. COS cells were transfected with wt, K93R, or K106R alone or together with hS100A8. 24hrs after transfection, single transfected cells were treated with or without MG132 for 6 hrs. Cells were harvest for WB, staining for hS100A9 and tubulin. Mutant proteins were not more stable than wt.

Paper II

Common interactions between S100A4 and S100A9 defined by a novel chemical probe

Aim: To identify novel chemical probes, which can block the interaction of S100 proteins with their receptors.

Questions addressed in paper II:

1) Can a chemical probe, the salicylic amide oxyclozanide [108], block S100A9 interaction with RAGE and TLR4 and play a biological role?

In this paper, by using SPR we showed that OX could bind S100A9 and block S100A9 binding to RAGE and TLR4 in a dose-dependent and also Ca^{2+} and Zn^{2+} dependent manner. The strongest binding was detected in the presence of both Ca^{2+} and Zn^{2+}.

We knew from our previous work that binding of S100A9 to TLR4 might promote tumor growth [171, 196]. Further, Q-compounds blocking S100A9 binding to its receptors can inhibit tumor growth [195, 196, 198]. We wondered if OX would have similar function. We tested OX in EL4 tumor model and observed inhibitory effect on tumor size and tumor weight.

2) Can the OX compound also bind to S100A4?

We showed in this paper, that S100A4 could also bind to TLR4 and RAGE and that this binding could be inhibited by OX in a dose dependent way. We also showed that S100A4 could bind to OX and that this binding is Ca^{2+} and Zn^{2+} dependent. Further, S100A4 and S100A9 can form heterodimers in a Zn^{2+} dependent way in vitro. Importantly, these proteins could also form heterodimers in transfected cells. We could also show that both TLR4 and RAGE can interact with S100A4/S100A9 heterodimer in vitro, and that this interaction even stronger than with S100A9 or S100A4 alone.

3) Does expression of S100A4 and S100A9 overlap in mouse myeloid cells?

We found that in mouse cells, S100A4 is mainly expressed $Ly6C^{++}$ cells while S100A9 is mainly expressed in $Ly6G^{+}$ cells both in steady state and in inflammation and cancer models. This conclusion was also supported by the fact using fluorescence microscopy no S100A4-S100A9 co-staining was found in tissues from diseased mice in inflammation and tumor models.

Discussion:

S100A9 may play an important role in pro-inflammation and in promoting tumor growth, as I summarized in the Introduction. Blocking S100A9 binding to its receptor would potentially have therapeutic effects in inflammation and cancer diseases, just as what have been seen using Q compounds [171, 199, 202, 208]. In this paper, we wanted to identify new compounds, which can block S100A9 binding to its receptors.

We found that the OX compound could efficiently bind to hS100A9 and also hS100A4 in a Ca^{2+} and Zn^{2+} dependent way. In the paper, we show that the hS100A9 and hS100A4 binding affinity to OX was stronger when both Ca^{2+} and Zn^{2+} were available as compared to Ca^{2+} alone. This may be because binding of the two ions could lead to a distinct conformational change of the proteins. Indeed, it is known that metal-binding properties play important roles in modulating the conformation of S100 proteins. As I mentioned above, for most S100 proteins binding to Ca^{2+} can induce a conformational change, which leads the exposure of a hydrophobic cleft and recognition of theirs targets [209]. However, S100 proteins can also bind to other ions and induce different conformations and biological functions. For example, while Ca^{2+} can induce heterotetramerization of S100A8/S100A9 and extra Zn^{2+} enable the proteins to resist degradation by certain proteases [210] and forming amyloid structures, which may lead to the amyloid formation in the ageing prostate [209, 211]. Ca^{2+} and Zn^{2+} also influence folding and function of other S100 proteins [212, 213]. These data may be able to explain the differential binding properties of S100A9 and S100A4 to OX, in the presence of different ions. Further, this may also be the reason why homo- and hetero-complex formation of S100A4 and S100A9 is Zn^{2+} dependent.

In this paper, we for the first time show that S100A4 and S100A9 can form heterodimer and bind to RAGE and TLR4. This may not be surprising, because although S100 proteins have diverging primary sequence, they have very similar three-dimensional structures [214]. In our paper we also show that expression of S100A9 and S100A4 did not overlap in mouse spleen cells, BM cells, EL4 tumor model and EAE model. This suggested that if the S100A4/S100A9 would exist in vivo, they would not form intracellularly but potentially outside the cell after these proteins have been passively released or secreted out of the cell. For example, a recently published paper has shown extracellular S100A4 can up-regulate the expression of SAA via active TLR4 in mouse adenocarcinoma cell line. The SAA proteins would then stimulate the transcription of S100A8 and S100A9 [215]. This study indicated the possibility that S100A4 could indirectly induce expression of S100A9 which, if can be released from the cells, might facilitate the S100A4/S100A9 formation. However, although mouse Ly6C^{++} cells mainly express mS100A4 without mS100A9, human monocytes may express both hS100A4 and hS100A9 [76, 173] and in this paper we showed human monocyte cell line THP1 could express both hS100A4 and hS100A9. Combining with the double transfection-crosslinking data, we speculate that hS100A4/S100A9 may potentially also be able to form heterodimer intracellularly.

It is known that S100A4 can activate TLR4 [215] and RAGE [216]. In this paper we showed S100A4/S100A9 might have higher binding affinity to TLR4 and RAGE comparing with S100A4 and S100A9 alone. This suggests that if S100A4/S100A9 would exist in vivo they maybe have more pro-inflammatory potency than S100A4 and S100A9 alone. Since OX can block S100A4, S100A9, S100A4/S100A9 binding to their receptors, it may have a stronger anti-inflammation and anti-tumor effect than the compounds that only block S100A9-binding.

Conclusion:

The small molecular weight compound OX, which binds S100A9 also binds S100A4. The finding that OX-binding blocks the interaction of both these proteins, as well as a heterodimer thereof, with TLR4 and RAGE receptors, suggests that these S100 proteins may share a common binding site for OX and that this binding site overlaps with their binding site to the receptors.

Paper III

CD14 is a co-receptor for TLR4 in the S100A9-induced pro-inflammatory response in monocytes

Aim:

To identify possible co-receptors for S100A9-mediated TLR4 simulation

Questions addressed in paper III:

1) S100A9 as a TLR4 ligand, what cellular structure is associated with its pro-inflammatory role?

In previous work from our laboratory [118] and in paper III, we have shown that S100A9 can activate monocytes and induce a cytokine response in these cells. We knew from previous publications, that in the case of LPS-induced activation of TLR4, TLR4 would form homodimers upon LPS-binding, translocate to lipid rafts and be internalized into endosomes. The first question we asked was if hS100A9-induced stimulation would follow the same general pathway.

Using transmission electron microscopy (TEM), we found that gold-labeled hS100A9 (hS100A9-Au) displays focal binding, most probably to some cell membrane subdomains and can be internalized into vesicles. By staining with gold-labeled antibody, we found that hS100A9-Au co-localized with TLR4 on cell membrane subdomains and in intracellular vesicles, which supports the previous results that hS100A9 is a TLR4 ligand.

We found that hS100A9-Au co-localized with Cav1 and Rab5, indicating hS100A9 binds to caveolae on cell surface and is thereafter internalized into early endosomes. We also showed that S100A9 could still bind to cell surface in $Cav1^{-/-}$ BM-DC. We used methyl-β-cyclodextrin (MβCD) to disintegrate lipid rafts of THP1 cells before incubating the cells with hS100A9-Au. We found that instead of focal binding on cell surface, only some occasional binding was seen, indicating that lipid raft structures are important for focal hS100A9 binding. We also found hS100A9-Au in Golgi.

2) What role does CD14 play during S100A9 binding to TLR4, signaling and endocytosis?

As we show in this paper, in TLR4$^{-/-}$ BM-DC, hS100A9-Au can still focally bind to the cell membrane and be internalized, and also co-localize with Cav1. This observation indicates that there is some co-receptor assisting hS100A9 binding and internalization. Since CD14 plays an important role in LPS binding, we then wondered whether CD14 would also be involved in the binding of hS100A9 and its internalization. First, we found in TLR4$^{-/-}$ BM-DC, that CD14 co-localized with hS100A9-Au both on cell surface subdomains and also in intracellular vesicles. Using SPR analysis, we showed CD14 could bind hS100A9 but not hS100A8. By blocking THP1 or peritoneal wash cells with a CD14 blocking antibody or using BM-DCs derived from CD14$^{-/-}$ mice, we showed CD14 was crucial for the hS100A9 induced cytokine response. Interestingly, in CD14$^{-/-}$ BM-DC, we still could observe hS100A9-Au binding on cell surface but no hS100A9-Au was internalized into the cells. This finding indicated that CD14 is crucial for hS100A9 internalization, but is not the only receptor for hS100A9 binding to the surface of monocytoid cells.

Discussion:

During inflammation and cancer, myeloid cells will be recruited to the lesion site and activated by the inflammatory mediators in local environment. These inflammatory mediators include pro-inflammatory cytokines and also some DAMP molecules that can be released from damaged cells and tissue. This paper is focused on the mechanism of DAMP molecule hS100A9-mediated activation of TLR4 in monocytes.

It has been shown, that upon LPS stimulation, TLR4, CD14 would migrate to lipid rafts and that the integrity of lipid raft is important for LPS induced TNFα secretion [217]. After translocation to lipid rafts, TLR4 can be taken up into the cell by endocytosis and can transmit signals also from the endosome. It is well know that TLR4 is important for LPS signaling and our previous work showed that TLR4 was also important for S100A9 signaling, since TLR4$^{-/-}$ BM-DC could not be activated by S100A9 [118]. In this paper, we show that hS100A9 can bind focally to domains on the monocyte cell surface, and be taken up into intracellular vesicles, thus following a similar

stimulation pathway as LPS. For LPS, previous studies have shown that unlike most transmembrane proteins that regulate their endocytosis by their cytosolic tail [51], a TLR4 mutant without cytosolic domain can still be internalized by LPS treatment [218]. Other studies showed that TLR4 is crucial for LPS signaling but not for LPS uptake [219, 220]. For example, in monocytes or endothelial cells, the same amount of LPS was taken up in WT and TLR4$^{-/-}$ cells [219]. This is similar to what we see with hS100A9 in TLR4$^{-/-}$ BM-DC. We did not quantify the uptake, but we did observe focal binding and internalization in these knock out cells. This indicates that although TLR4 is important for activating monocytes, it is not important for hS100A9 binding to plasma membrane and endocytosis.

CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein, which functions as a TLR4 co-receptor for LPS. Previous studies have shown that CD14 is important for LPS binding, signaling and endocytosis [17, 218, 221]. CD14 is also important for LPS induced inflammatory response. For example CD14$^{-/-}$ mice did not develop septic shock at a dose lethal to WT mice [222]. However, by increasing LPS concentration CD14$^{-/-}$ cells can be activated and TNFα release could be observed [223]. We obtained similar results in our study. Although higher concentration of LPS can compensate for the absence of CD14 during MyD88 dependent signal transduction, this is not the case when it comes to activation of the TRIF pathway. For example, there was minimal induction of expression of the TRIF dependent gene IP-10 in CD14$^{-/-}$ cells stimulated with high concentration of LPS, while TNFα and IL-1β were properly induced [224].

In the LPS binding and endocytosis mechanism, CD14 plays two different roles. The first role of CD14 is its function as a LPS transporter, delivering LPS to TLR4/MD2 and recruiting MyD88. The missing CD14 in this procedure can be overcome by increasing LPS concentration. The reason for this could because the existence of other receptors, for example CD36, which have been shown involved in LPS/*E.coli* induced pro-inflammatory responses [43, 225, 226]. The second role of CD14 is to assist in TLR4/MD2 endocytosis and to recruit TRIF and inducing IFN expression. The lack of CD14 in this mechanism can be overcome by forcing TLR4/MD2 to enter endosomes [223].

Our data indicate that CD14 may have a similar role in the hS100A9 induced stimulation of TLR4. First, we show in CD14$^{-/-}$ BM-DC, hS100A9 can bind to cell surface but cannot be endocytosed into intracellular vesicles. This means

that hS100A9 can bind to plasma membrane in a CD14-independent way but its endocytosis is CD14 dependent. To rule out the possibility that TLR4 would be responsible for the binding of hS100A9 detected on $CD14^{-/-}$ cells, we blocked surface CD14 on $TLR4^{-/-}$ BM-DCs, but could still detect S100A9-binding to the cell membrane.

S100A9 have been reported to bind to many cell surface molecule, which I summarized in the Introduction. It could be one/some of these receptor(s), which is responsible for the binding hS100A9 to cell surface of $CD14^{-/-}$ cells. But we didn't detect any TNFα secretion from $CD14^{-/-}$ BM-DC, maybe because this receptor(s) cannot deliver hS100A9 to TLR4/MD2. It could also be that the concentration we used in the experiment was not high enough to compensate the lack of CD14. The highest concentration we have tried for S100A9 is 40μg/ml and didn't get different result from what we show in the paper. One thing worth mentioning is that there was variability in the potency of various batches of the recombinant S100A9 proteins in inducing the inflammatory cytokine responses. It seems that these proteins may be sensitive, and their biological activity could be reduced during purification, which may also explain some of the conflicting data in this field (data not shown). As we know, during inflammation disease, the serum level of S100A8/S100A9 can be increased. For example in Juvenile rheumatoid arthritis patients, the serum level of S100A8/S100A9 could reach 2μg/ml and in the inflamed joints the local S100A8/S100A9 concentration could reach 40μg/ml [112]. Although some investigators have reported higher S100A8/S100A9 concentration, the optimal stimulatory concentration we choose probably could in some degree reflect the situation in inflammatory diseases. Additionally, we could also show that hS100A9 entered into early endosome and also localized to Golgi, similarly to what was seen upon LPS stimulation of TLR4 [227, 228].

As I mentioned above, except through caveloae, LPS can also be internalized by endocytosis through the clathrin-mediated pathway. This may be able to explain why in $Cav1^{-/-}$ we still can find hS100A9 binding and internalizing, however less than in WT cells.

In the paper, we have used hS100A9 for TEM experiments both in human THP1 cells and mouse BM-DCs. Since we know, although amino acid sequences between human and mouse S100A9 are quite different, they have similar in 3D structure, using human protein on mouse cells may not

influence the results. To confirm this, we repeated the TEM experiments by using mouse S100A9 with BM-DCs and obtained same results (data not shown).

Conclusion:

In this paper we show CD14 is an essential co-receptor for S100A9 activating TLR4 and also important for S100A9 internalization in monocytoid cells. However, CD14 is not essential for S100A9 binding to cell surface, which indicates existence of other receptor(s) for S100A9.

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Paper I

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Human S100A9 Protein Is Stabilized by Inflammatory Stimuli via the Formation of Proteolytically-Resistant Homodimers

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Abstract

S100A8 and S100A9 are Ca^{2+}-binding proteins that are associated with acute and chronic inflammation and cancer. They form predominantly heterodimers even if there are data supporting homodimer formation. We investigated the stability of the heterodimer in myeloid and S100A8/S100A9 over-expressing COS cells. In both cases, S100A8 and S100A9 proteins were not completely degraded even 48 hrs after blocking protein synthesis. In contrast, in single transfected cells, S100A8 protein was completely degraded after 24 h, while S100A9 was completely unstable. However, S100A9 protein expression was rescued upon S100A8 co-expression or inhibition of proteasomal activity. Furthermore, S100A9, but not S100A8, could be stabilized by LPS, IL-1β and TNFα treatment. Interestingly, stimulation of S100A9-transfected COS cells with proteasomal inhibitor or IL-1β lead to the formation of protease resistant S100A9 homodimers. In summary, our data indicated that S100A9 protein is extremely unstable but can be rescued upon co-expression with S100A8 protein or inflammatory stimuli, via proteolytically resistant homodimer formation. The formation of S100A9 homodimers by this mechanism may constitute an amplification step during an inflammatory reaction.

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Introduction

S100A8 and S100A9 proteins belong to the S100 protein family, which constitutes over 20 low molecular Ca^{2+}-binding proteins [1,2]. In particular, S100A8 and S100A9 are the focus of intense research, as they are associated with several inflammatory diseases and cancer [3,4]. In addition, they are well known protein markers for an increasing number of inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease and prostate cancer [5–7].

S100A8 (10 kDa) and S100A9 (14 kDa) proteins are expressed constitutively in circulating neutrophils and monocytes and their expression can be induced in resting tissue macrophages [8–12]. Although it has been documented that S100A8 and S100A9 can exist both as homo- and heterocomplexes, they preferentially form heterodimers or heterotetramers in a Ca^{2+} and Zn^{2+} dependent way [13–18]. Recent studies suggested that S100A8/S100A9 homo- and hetero-oligomers may have distinct roles in cell physiology. In particular, it has been reported that S100A8/S100A9 heterocomplexes could mediate apoptosis [19], induce neutrophil chemotaxis [20], activate the NFêB pathway [21], exhibit antimicrobial activity [22] and regulate NADPH oxidase activation upon arachidonic acid binding [23].

S100A8 knock-out (KO) mice show a lethal phenotype [24], while S100A9-KO mice are perfectly viable and no major differences in inflammatory response have been observed compared to wild type animals [25]. S100A9, but not S100A8 could bind heparansulphate [26], mediate neutrophil adesion to fibronectin [27] and increase Mac-1 affinity [28]. Furthermore, it has been shown that S100A9 protein is involved in tumour growth [29].

In the present work, we investigated the protein turnover and stability of S100A8/S100A9 homo- and heterodimers. In particular, we observed that human S100A9 (hS100A9) homodimers were unstable and readily degraded, while human S100A8 (hS100A8) homodimers were perfectly stable. Interestingly, S100A9 expression could be rescued upon challenge with inflammatory stimuli that stabilized the protein. Those findings showed that the cells could generate a qualitatively different S100A8/S100A9 oligomeric form in response to proper stimuli.

Materials and Methods

Cell Culture

The human monocytic leukemia cell line THP-1 was grown in RPMI 1640 culture medium (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS; Invitrogen, UK), 2 mM Glutamine (Sigma-Aldrich, USA), 1 mM sodium pyruvate, 10 mM Hepes, 100 U/ml of penicillin and 100 µg/ml of streptomycin (P/S; Invitrogen, UK), at 37°C in 5% CO_2. COS and LEP cells were

cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, UK), 2 mM Glutamine (Sigma-Aldrich, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, UK) at 37°C in 5% CO_2. LEP cells were cultured in the presence of Non-Essential-Aminoacids (NEA; Sigma-Aldrich, USA). LEP cell is an embryonic fibroblast cell line kindly provided by Prof. Vera Casslen [30].

COS and LEP Cell Transfection and Treatment

Three different transfections were performed:

1) hS100A8:1 µg pcDNA3.1-hS100A8+1 µg pcDNA3.1-EGFP;
2) hS100A9:1 µg pDream2.1-hS100A9+1 µg pcDNA3.1-EGFP;
3) hS100A8/hS100A9:1 µg pcDNA3.1-hS100A8+1 µg pDream2.1-hS100A9.

Briefly, COS or LEP cells were seeded in 24 well plate in serum-free medium the day before transfection. DNA-Lipofectamine 2000 mixture was prepared as follow: 2 µl Lipofectamine 2000/ reaction were incubated in 50 µl Optimem medium for 5 min at room temperature (RT). Plasmid DNA was added to the mixture and incubated for further 20 minutes at RT. Subsequently, the DNA-Lipofectamine 2000 mixture was added to the cells, which were incubated for 3 hrs at 37°C. Finally, 1 ml/well of medium was added to the transfected COS cells and a further 24 h incubation at 37°C was performed.

After overnight incubation, transfected COS cells were treated with different concentrations of proteasomal inhibitor MG132 (Millipore, Billerica, MA, USA), IL-1β (Invivogen, San Diego USA), LPS (Invivogen, San Diego, USA) and TNFα (Invivogen, San Diego, USA) as indicated in Figure Legends. In some experiments, to study protein dimerization, transfected COS cells were washed in PBS and incubated for 30 min on ice with 1 mM disuccinimidyl suberate (DSS, Sigma-Aldrich, USA) dissolved in PBS. To study protein stability, transfected COS cells were treated with 100 µg/ml of cycloheximide.

Western Blot

10 µg of total cell extract was loaded into 4–20% polyacrylamide gel (BioRad, Solna, Sweden). Proteins were subsequently transferred to PVDF membrane (Roche, Mannheim, Germany), which was saturated with 1% dry milk in PBS-Tween 0.05%. Membranes were incubated with the appropriate primary antibody diluted 1:5000 in PBS-Tween overnight at 4°C, washed 3 times in PBS-Tween, incubated for 1 h at RT with Goat anti-Rabbit or anti-Mouse secondary antibodies (Abcam, Cambridge, UK) diluted 1:5000, then washed 3 times in PBS-Tween and finally developed using ECL kit from Roche, (Mannheim, Germany).

The primary antibodies were the following: Mouse anti-human S100A9 (Novus Biologicals Inc., CO, USA), Rabbit anti-human S100A8 (kindly provided by Prof. Nancy Hogg, UK) and Rabbit anti-human β-tubulin (Novus Biologicals Inc., CO, USA).

Immunoprecipitation (IP)

Five million COS cells were co-transfected with pcDNA3.1-hS100A8 and pDream2.1-hS100A9, as described above. After washing, the cells were re-suspended in lysis buffer (75 mM Tris/ HCl pH 7.6, 1.25% NP-40, 100 mM NaCl and complete protease inhibitors) and cell debris removed by centrifugation. The supernatants were thereafter pre-cleared upon incubation with streptavidin (hS100A9) or protein G-conjugated beads (hS100A8) at 4°C for 1 hour with gentle shaking. Subsequently, the supernatants were incubated with biotinylated mouse anti-hS100A9 antibody (43/8-bio, produced in our lab) or rabbit anti-hS100A8 antibody (kind gift of Prof. Nancy Hogg, UK), at 4°C overnight. The following day, streptavidin- or protein G-conjugated beads (Invitrogen, UK) were added to the samples at 4°C for 1 hour. Subsequently, beads-protein complexes were harvested, washed 3 times in washing buffer (Invitrogen, UK), eluted in Laemmli Sample Buffer (BioRad, Solna, Sweden) and boiled at 70°C for 10 min. The samples were then analyzed by SDS-PAGE and Western blot. In some experiment, as a control, COS cells were transfected only with pDream2.1-S100A9, followed by the same procedure described above. TRIS buffer, NaCl and NP-40 were purchased from Sigma-Aldrich, (USA) while complete protease inhibitors were obtained from Roche (Mannheim, Germany).

Results

The hS100A8/hS100A9 Heterodimer is a More Stable form as Compared to hS100A8 or hS100A9 Expressed Alone

In order to investigate which S100A8/S100A9 oligomers were expressed in monocytes, we treated THP-1 cells with the cell-permeable cross-linker DSS. By Western blot, we could notice a band around 24 kDa, which was representative of the S100A8/S100A9 heterodimer complex, while we could not detect any homodimeric forms (Fig. 1a). Previously, it has been shown that in granulocytes the S100A8/S100A9 heterocomplex was extremely protease resistant [31]. We confirmed this finding by treating THP-1 cells, for different periods of time, with the protein translation inhibitor cycloheximide and, indeed, found that the S100A8/S100A9 heterodimer was not completely degraded even after 24 h (Fig. 1b).

Since we were able to detect only S100A8/S100A9 heterodimers in THP-1 cells, we decided to overexpress hS100A8 and hS100A9 in COS cells in order to investigate the stability of S100A8 and S100A9 individually. The results in Fig. 2a–b indicated that the hS100A8/hS100A9 heterodimer is the most stable form since hS100A8 expressed alone decayed within 24 hrs. Surprisingly, hS100A9 expressed alone was not detectable even at the earliest time point, suggesting that hS100A9 was unstable and immediately degraded.

In both experiments, we checked cell viability and even though both THP-1 and COS cells stopped proliferating upon cycloheximide treatment, they did not die (Fig. S1a–b).

The hS100A9 Protein is Proteolytically Degraded in COS Cells

To corroborate our hypothesis about the rapid turnover of hS100A9 protein, we treated the hS100A9-transfected cells with increasing concentrations of the proteasomal inhibitor MG132 and observed a marked increase of hS100A9 protein expression (Fig. 3a). Thus, hS100A9 expressed alone is unstable and subjected to rapid proteolytic degradation. In contrast, when COS cells were co-transfected with both hS100A8 and hS100A9 constructs, we observed a robust increase in hS100A9 protein expression (Fig. 3a), indicating that hS100A8 protein could rescue the hS100A9 protein from proteasomal degradation.

On the other hand, hS100A8 was readily detectable in the cells transfected with the hS100A8 construct alone. In this case, expression was not altered by MG132 addition, indicating that the hS100A8 protein was stable by itself (Fig. 3b). In addition, also the level of hS100A8 protein was increased, even though to a minor extent, when co-expressed with hS100A9 (Fig. 3b).

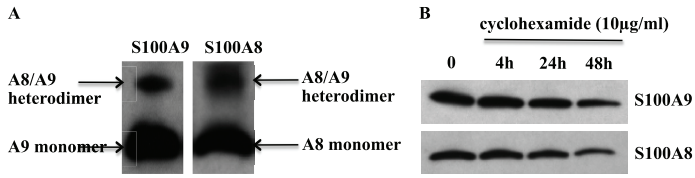

Figure 1. hS100A8 and hS100A9 form a stable heterodimer in THP-1. THP-1 were treated with DSS for 30 min in ice. Subsequently samples were used for Western blot and stained for hS100A9 and hS100A8 (**A**). THP-1 cells were treated with 10 μg/ml cycloheximide for 4 h, 24 h and 48 h. Samples were, then, collected and Western blot for hS100A9 and hS100A8 was performed (**B**).
doi:10.1371/journal.pone.0061832.g001

The hS100A9 Protein is Unstable Also in Fibroblasts

Next we wanted to determine whether the instability of hS100A9 protein was peculiar to the COS cells. For this purpose, we transfected a human lung embryonic fibroblast cell line (LEP) (Fig. 4a–b) with hS100A8 and hS100A9 constructs, alone or together. The results showed that hS100A9 was proteolytically degraded also in this cell line. The protein could again be rescued upon MG132 treatment or hS100A8 protein co-expression. Also in these cells, hS100A8 was stable by itself. Taken together, these data indicated that the instability of hS100A9 protein is not tissue-specific.

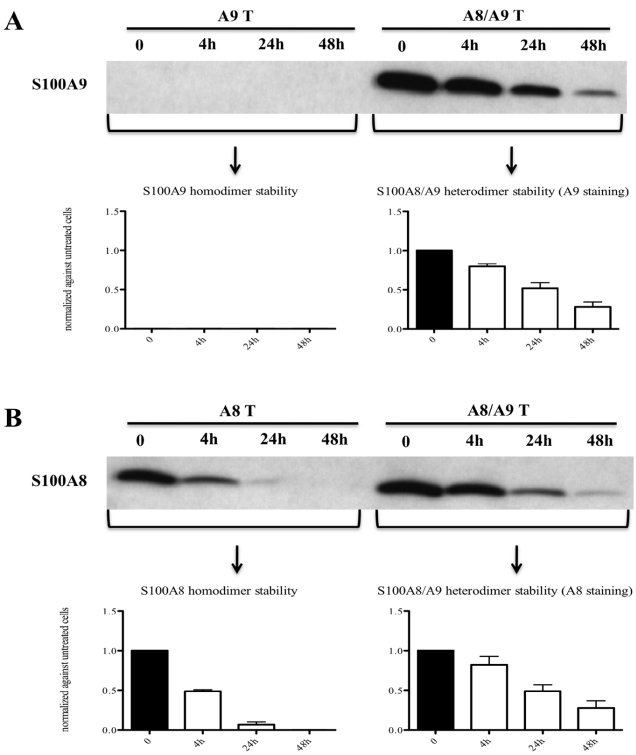

Figure 2. The hS100A8/hS100A9 heterodimers were more stable than hS100A8 and hS100A9 homodimers. COS cells were transfected with hS100A8 and hS100A9 separately or together. After 24 h, COS cells were treated with 100 μg/ml cycloheximide for 4 h, 24 h and 48 h. Samples were collected and analyzed by Western blot. Filters were stained with (**A**) anti-hS100A9 or (**B**) anti-hS100A8. In the lower charts, the relative band intensity compared to non-stimulated cells are indicated.
doi:10.1371/journal.pone.0061832.g002

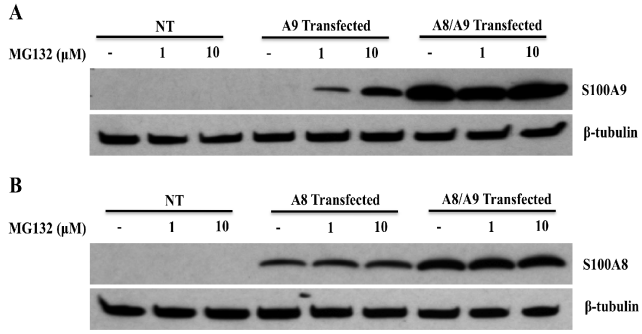

Figure 3. hS100A9 protein was unstable in COS cells but could be stabilized by MG132 or co-transfection with hS100A8. COS cells were transfected with hS100A8 and hS100A9 constructs either separately or together. 24 h after transfection, the cells were stimulated for 8 h with 1 or 10 μM MG132 and subsequently analyzed by Western blot using (**A**) anti-hS100A9 or (**B**) anti-hS100A8. The first three lanes (**NT**) of each panel represented non-transfected cells. From lane 4 to 6, COS cells were transfected with (**A; A9 Transfected**) hS100A9 or (**B; A8 Transfected**) hS100A8 separately, while from lane 7 to 9 (**A8/A9 Transfected**) cells were co-transfected.
doi:10.1371/journal.pone.0061832.g003

Formation of hS100A8/hS100A9 Heterodimers Stabilizes the hS100A9 Protein

From the data shown above, we hypothesized that the stabilization of hS100A9, observed upon co-expression with S100A8, might be due to formation of S100A8/A9 heterodimers. To confirm this, we co-transfected COS cells with both the hS100A8 and hS100A9 constructs and performed a co-immunoprecipitation (Co-IP) experiment, followed by Western blotting. We observed that, upon Co-IP of hS100A9, we could effectively detect the hS100A8 partner and vice versa (Fig. 5). Thus, hS100A9 was rescued from proteasomal degradation by forming complexes with hS100A8.

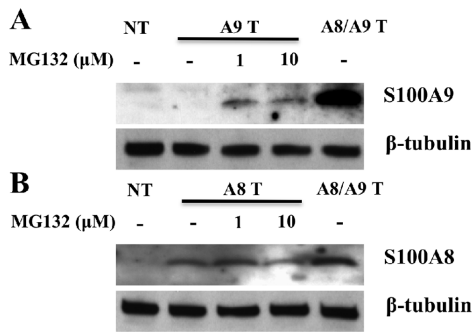

Figure 4. hS100A9 protein was unstable in LEP cells but could be rescued by MG132 and hS100A8. hS100A8 and hS100A9 expression vectors were transfected into human fibroblasts (LEP cells) as described before for COS cells. After SDS-PAGE and Western blots were performed either with (**A**) anti-hS100A9 or (**B**) anti-hS100A8. Lane 1 (**NT**) represented non-transfected cells; from lane 2 to lane 4 cells were transfected with hS100A9 (**panel A; A9 T**) or with hS100A8 (**panel B**; **A8 T**); in lane 5 (**A8/A9 T**) cells were co-transfected with both constructs.
doi:10.1371/journal.pone.0061832.g004

Inflammatory Stimuli Stabilize the hS100A9 Protein

Expression of S100A9 protein could, under inflammatory conditions, be detected in certain tissue cells such as keratinocytes or chondrocytes [32,33]. Therefore, we wanted to test whether pro-inflammatory stimuli could promote hS100A9 stability. For this purpose we treated hS100A9-transfected COS cells with IL-1β or LPS. As shown in Fig. 6a–b, both LPS and IL-1β were able to markedly increase hS100A9 but not hS100A8 expression. In addition, we confirmed our finding treating COS cells with TNFα, showing that hS100A9 stabilization was not peculiar to IL1β but it could occur also upon challenge with other pro-inflammatory stimuli (Fig. S2). The fact that also inflammatory stimuli promote hS100A9 expression, suggest that hS100A9 could escape proteasomal degradation in response to external signals.

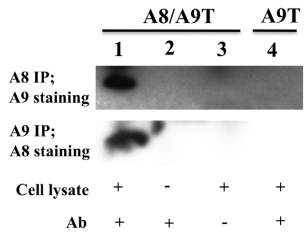

Figure 5. hS100A8 associates with hS100A9 in co-transfected COS cells. COS cells were co-transfected with expression vectors for both hS100A8 and hS100A9. 24 h later, hS100A9 was immunoprecipitated and analyzed for hS100A8 expression by Western blot (Panel 2, lane 1). The same experiment was repeated immunoprecipitating hS100A8 and staining for hS100A9 (Panel 1, lane 1). Lane 2 and 3 represented the controls. In brief, in control samples, COS cells were co-transfected and a full Co-IP experiment was performed but without the cell extract, or the antibody, respectively. In panel 1, lane 4, COS cells were transfected only with hS100A9-carrying vector. The hS100A9 protein was immunoprecipitated and Western blot performed with hS100A8 staining.
doi:10.1371/journal.pone.0061832.g005

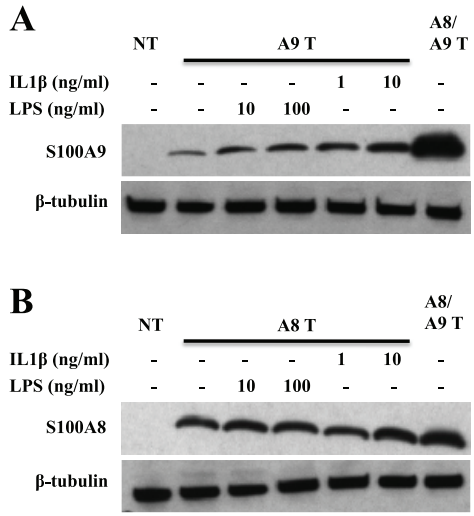

Figure 6. LPS and IL1β induced hS100A9 protein stabilization. COS cells were transfected either with hS100A8 or hS100A9 constructs, as described above. 24 h after transfection, COS cells were stimulated with either 1 or 10 ng/ml IL1β or alternatively with either 10 or 100 ng/ml LPS. Samples were collected and analyzed by Western blot. Filters were stained with (**A**) anti-hS100A9 and (**B**) anti-hS100A8. Lane 1 (**NT**) represented non-transfected cells; from lane 2 to 6, COS cells were transfected with (**A**; **A9 T**) hS100A9 or (**B**; **A8 T**) hS100A8 separately; in lane 7 (**A8/A9 T**) COS cells were co-transfected with both plasmids.
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IL1β Promotes the Formation of Protease-resistant hS100A9 Homodimers

To further analyze the composition of the different S100A8/S100A9 complexes, we transfected COS cells with hS100A8 and hS100A9 cDNA constructs alone or together. We thereafter treated COS cells with the membrane permeable cross-linker DSS and analyzed the S100 proteins by Western blotting staining for hS100A9 (Fig. 7a) or hS100A8 (Fig. 7b). When hS100A8 was expressed alone (Fig. 7b), we observed a band with an approximate Mw of 20 kDa representing the hS100A8 homodimer. When hS100A8 was expressed together with hS100A9, we observed an additional band at 24 kDa, representing the S100A8/S100A9 heterodimer. The 24 kDa heterodimer band was weaker than the 20 kDa hS100A8 homodimer band.

When hS100A9 was expressed alone and the cells were treated with MG132 to prevent its degradation (Fig. 7 a), we observed a band with an approximate Mw of 28 kDa, representing the hS100A9 homodimer. When hS100A8 and hS100A9 were co-transfected, we could only notice a S100A8/S100A9 heterodimer band at 24 kDa.

Lastly, we wanted to investigate if this event was peculiar for MG132 or if also inflammatory stimuli, such as IL1β, could promote protease-resistant hS100A9 homodimers. To this end, COS cells were transfected with hS100A9 alone, treated with IL1β and subsequently with DSS. After Western blot analysis, we detected a 28 kDa band in the sample treated with IL1β but not in the untreated control, confirming that hS100A9 was rescued from protein degradation by IL1β, via formation of protease resistant homodimers (Fig. 7a, lane 2).

Discussion

In the present work, we investigated the stability of the different oligomers formed by hS100A8 and hS100A9 in the myeloid cell line THP-1 and in transfected COS cells. We have shown that hS100A8 and hS100A9 could form homo- and heterodimers *in vivo*. While the hS100A8/hS100A9 heterodimer was the most stable oligomer, hS100A9 homodimers were unstable and barely detectable by Western blot. However, proteasome inhibition or inflammatory stimuli such as IL1β and TNFα could promote the formation of protease-resistant hS100A9 homodimers.

It is well accepted that the predominant form in which S100A8/S100A9 associates in physiological and pathological conditions is the heterodimer, even if it has been observed that S100A8 and S100A9 could also form homodimers [34,35]. An increasing amount of data indicate that S100A8 as well as S100A9 homodimers are important regulators of inflammation and cancer, exhibiting strong pro-inflammatory activity in various mouse models of diseases [36–38]. In particular it has been shown that, S100A8 homodimers could promote chondrocyte-mediated cartilage destruction, upon metalloproteinase activation, in experimental arthritis [38]. In addition, $CD8^+$ cells from subjects with lupus erythematosus stimulated with S100A8 or S100A9 showed an upregulation of IL-17 expression, leading to the development of auto-reactive lymphocytes [37]. Moreover, it has been shown that murine S100A8 homodimers were able to recruit leukocytes and had properties of an oxygen scavenger [39].

On the other hand, it has been observed that S100A9 homodimers interacted with TLR4 and RAGE [40], which are two receptors involved in the control of tumour growth in different systems [41,42]. In particular, it has been shown that inhibition of hS100A9/TLR4 interaction inhibited tumour growth [29]. In addition, hS100A9 is involved also in metastasis formation, most likely by interfering at an early stage of metastasis formation [43]. We have shown that treatment with an S100A9-binding molecule inhibited metastasis formation in a prostate cancer tumour model [44]. It has been observed that S100A9 was important for the development and function of myeloid-derived suppressor cells (MDSC) [45,46]. Also, our previous data has shown that human S100A9 was a TLR4-dependent pro-inflammatory molecule, activating NFκB in monocytes [47]. All together, these findings pointed out that S100A9, rather than S100A8/S100A9, could be the main mediator of inflammatory diseases and tumours and, more importantly, is emerging as a potential target for the treatment of malignant diseases.

Despite the emerging importance attributed to S100A8 and S100A9 homodimers, the most abundant form detected in serum is the heterodimer. Thus, in rheumatoid arthritis patients the amounts of heterodimer were 1000 fold greater as compared to S100A8 and S100A9 homodimers [21]. In addition, it has been shown with a two-hybrid system that hS100A8 and hS100A9 could not form homodimers in yeast [48]. Lastly, in granulocytes, the S100A8/S100A9 heterodimer was protease resistant while the S100A8 and S100A9 homodimers were not [31].

In our work, we showed by Western blot analyses that in THP-1 cells, no hS100A8 and hS100A9 homodimer could be found and we confirmed that the S100A8/S100A9 heterodimer was protease-resistant both in THP-1 and in COS cells over-expressing these proteins. However, we also showed that hS100A9 homodimers seemed to be rapidly degraded in COS cells, while hS100A8 homodimers were perfectly stable, which is in conflict with

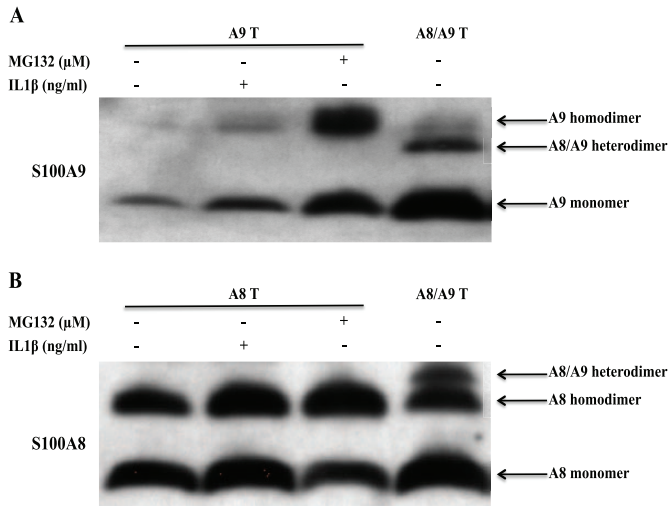

Figure 7. IL1β promotes the formation of protease-resistant hS100A9 homodimers. COS cells were transfected with hS100A8 and hS100A9 expression vectors, either separately or together. 24 h after transfection, transfected cells were treated either with MG132 or IL1β. Then, COS cells were incubated with 1 mM DSS on ice for 30 minutes and analyzed by Western blot (hS100A9 (**A**) and hS100A8 (**B**)).
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previously published data [31,48]. This apparent discrepancy could be due to the presence of different subsets of proteases in COS cells, yeasts and extracellular milieu, leading to a different hS100A8 protein turnover.

We speculate that hS100A9 degradation occurred via ubiquitination and proteasomal degradation, since hS100A9 protein expression could be rescued by the proteasomal inhibitor MG132. Using UbPred program (predictor of protein ubiquitination sites) we found that Lysine 93 (K93) of the hS100A9 protein could be a target for ubiquitination.

We also showed that hS100A9 homodimers could be stabilized by forming protease-resistant homodimers in cells exposed to inflammatory stimuli. We confirmed our data also in a fibroblast cell line. That inflammatory conditions could promote hS100A9 homodimer formation, and that hS100A9 is in itself a pro-inflammatory signal, suggests a mechanism for an inflammatory amplification step where an inflammatory cytokine would trigger the production of an additional pro-inflammatory signal. Further studies will be needed to dissect the detailed mechanism by which inflammatory stimuli stabilized hS100A9 homodimers. One possible explanation for the formation of protease-resistant hS100A9 homodimers could be due to the fact that, upon inflammatory stimuli, hS100A9 could potentially be post-translationally modified. Indeed, it is well established that S100 proteins can be subject to several post-translational modifications [49].

In summary, in this work we showed that hS100A9 homodimer, which was rapidly proteolytically degraded, could be rescued by inflammatory stimuli and co-expression with hS100A8. The stabilization of hS100A9 homodimers may allow hS100A9 to interact with its target receptors TLR4 and/or RAGE that, in turn, could start an intracellular signal cascade, mediating hS100A9 homodimer effects, which could be distinct compared to effects induced by S100A8/S100A9 heterodimers.

Supporting Information

Figure S1 THP-1 and COS cells viability. THP-1 (**A**) and COS (**B**) cells were treated with 10 and 100 ng/ml of the protein translation inhibitor cycloheximide respectively. Untreated and treated cells were collected at 4 h, 24 h and 48 h and subsequently counted using trypan blue dye.
(EPS)

Figure S2 TNFα induced hS100A9 protein stabilization. COS cells were transfected either with hS100A8 or hS100A9 constructs, as described above. 24 h after transfection, COS cells were stimulated with either 1 or 10 ng/ml TNFα. Then, samples were collected and analyzed by Western blot. Filters were stained with (**A**) anti-hS100A9 and (**B**) anti-hS100A8. Lane 1 (**NT**) represented non-transfected cells; from lane 2 to 4, COS cells were transfected with (**A**; **A9 T**) hS100A9 or (**B**; **A8 T**) hS100A8 separately; in lane 5 (**A8/A9 T**) COS cells were co-transfected with both plasmids.
(EPS)

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Author Contributions

Conceived and designed the experiments: MR TL FI. Performed the experiments: MR ZH EK. Analyzed the data: MR EK TL FI. Contributed reagents/materials/analysis tools: EK FI TL MR. Wrote the paper: MR ZH EK TL FI.

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Paper II

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Common Interactions between S100A4 and S100A9 Defined by a Novel Chemical Probe

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Abstract

S100A4 and S100A9 proteins have been described as playing roles in the control of tumor growth and metastasis. We show here that a chemical probe, oxyclozanide (OX), selected for inhibiting the interaction between S100A9 and the receptor for advanced glycation end-products (RAGE) interacts with both S100A9 and S100A4. Furthermore, we show that S100A9 and S100A4 interact with RAGE and TLR4; interactions that can be inhibited by OX. Hence, S100A4 and S100A9 display similar functional elements despite their primary sequence diversity. This was further confirmed by showing that S100A4 and S100A9 dimerize both *in vitro* and *in vivo*. All of these interactions required levels of Zn^{++} that are found in the extracellular space but not intracellularly. Interestingly, S100A4 and S100A9 are expressed by distinct $CD11b^{+}$ subpopulations both in healthy animals and in animals with either inflammatory disease or tumor burden. The functions of S100A9 and S100A4 described in this paper, including heterodimerization, may therefore reflect S100A9 and S100A4 that are released into the extra-cellular milieu.

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Introduction

S100 proteins constitute a protein family with more than 20 members, many of which have been associated with various diseases [1]. Interestingly, while seemingly divergent with regard to their primary sequence, the three-dimensional structure of most of the members is very similar [2], indicating that there might be shared functions between the protein family members.

S100A4, also called metastasin, has been shown to promote tumor growth and metastasis in several tumor models [3–5]. S100A4 has also been shown to be involved in the control of tumor growth in human cancer [6], and to be a potential prognostic marker [7–9]. S100 proteins can have both intracellular and extra-cellular functions and S100A4 has been shown to have a role in both compartments. The S100A4 protein is directly involved in the expression of the tissue degrading matrix metalloprotease MMP-13, both by interaction of extra-cellular S100A4 with RAGE [10], but also via a direct involvement in transcription of the MMP-13 gene [11]. S100A4 has also been shown to be involved in the regulation of cell motility [12] and angiogenesis [13].

S100A9 is a protein that is highly expressed in granulocytes but also expressed in some monocytic subpopulations [14]. S100A9 protein is present in plasma at microgram levels in the form of heterodimers together with S100A8 [15]. Furthermore, the S100A8/A9 protein level in plasma can be greatly increased in patients with inflammatory disease [16], or malignancies [17]. S100A9 can also be expressed as a homodimer in the absence of S100A8 [18].

With regard to biological function, S100A9 has been ascribed both intracellular and extracellular functions. S100A9 can be phosphorylated by p38 MAPK which in turn regulates its binding to the cytoskeleton [19], indicating that S100A9 may be involved in regulation of cell motility [20]. S100A9 has also been shown to be a ligand for two pro-inflammatory extra-cellular receptors, RAGE and TLR4 [21]. Interestingly, both RAGE and TLR4 have been shown to play a role in the control of tumor growth in different systems [22,23], and it has also been shown that inhibition of S100A9/TLR4 interactions can inhibit tumor growth [24]. S100A9 may also be involved in cancer progression by separate mechanisms. It has been demonstrated that S100A9 is important for metastasis, most likely by interfering at an early stage of metastasis formation [25]. Furthermore, treatment with an S100A9-binding small molecule will inhibit metastasis formation in a prostate cancer tumor model [26]. Lastly, S100A9 has also been shown to be important for the development and function of so called myeloid derived suppressor cells (MDSC) [27,28]. Thus, S100A9 is emerging as a potential pharmaceutical target for the treatment of malignant disease.

In this study we identify a chemical probe that inhibits the interaction between S100A9 and RAGE or TLR4. We could show that this molecule also inhibited tumor growth *in vivo*. Interestingly, the same chemical probe also bound and inhibited S100A4 interactions with the same receptors. Hence, our findings

demonstrate that it is feasible to develop small molecule inhibitors that can bind multiple S100 proteins and inhibit their interaction with biologically relevant receptors.

Results

Validating S100A9 as a Pharmaceutical Target Using a Chemical Probe

Given our previous interest in S100A9, and in particular its interactions with RAGE and TLR4 [21,24], we decided to screen an in house chemical library for new molecules that bind to S100A9 and inhibit its interaction with RAGE and TLR4. Early on in this screen we identified salicylic amides (Figure 1A) as a promising starting point for further development. Sub-libraries of compounds were designed, synthesized and tested as competitors of these interactions. The testing was performed using SPR technology to detect inhibition of S100A9 binding to RAGE and TLR4 (data not shown). By this method we defined a group of salicylic amides that showed specific binding to S100A9. The best compound in this series was oxyclozanide (OX) that was selected for further studies (Figure 1B).

OX inhibited in a dose-dependent fashion the interaction between S100A9 and both RAGE (Figure 1C) and TLR4 (Figure 1D). To verify that the interaction between OX and S100A9 resembled that which we have previously described for quinoline-3-carboxamides (Q compounds) [21], we also investigated the Ca^{++} and Zn^{++} dependency of the binding interaction of OX and S100A9. As shown in Figure 1E, the interaction between OX and S100A9 was dependent on the presence of both Ca^{++} and Zn^{++} for satiable binding to immobilized S100A9.

We have previously also shown that the inhibition of S100A9 and TLR4 expression influenced EL4 tumor growth, and that the inhibition of S100A9 interactions *in vivo* using Q compounds mimicked this effect [24]. Therefore, we performed an experiment where we investigated the effect of OX on EL4 tumor growth. As shown in Figure 2 A and B, OX inhibited EL4 tumor growth both as measured by tumor volume and tumor weight. We conclude from these data that a chemical probe selected only for its ability to bind S100A9 and inhibit its interaction with TLR4 and RAGE, show anti-tumor activity *in vivo*.

S100A4 and S100A9 Show Common Molecular Interactions

Other salicylic amides have previously been described as inhibitors of tumor growth, including niclosamide that has shown anti-tumor activity in models that are dependent on S100A4 [29]. Given the structural similarities between S100 proteins [2], as well as between niclosamide and OX, we proceeded to investigate whether OX could also interact with S100A4. We first investigated whether S100A4 could bind OX directly. As shown in Figure 3A, OX interacts with S100A4 in a similar Ca^{++} and Zn^{++} dependent manner as was described for the binding of OX to S100A9 above. Thus, a very similar conformational epitope, defined by OX binding, must be present in S100A4 and S100A9.

We then proceeded to investigate whether S100A4, in the presence of Ca^{++} and Zn^{++}, could also be a ligand of both RAGE and TLR4. Furthermore, we investigated the effect of OX on these interactions. Indeed, a specific interaction between S100A4 and RAGE could easily be demonstrated which could be inhibited by OX although with a ten-fold lower potency compared to the interaction between S100A9 and RAGE (Figure 3B). Very similar findings were observed when the interaction between S100A4 and TLR4 was investigated (Figure 3C). We conclude from these experiments that S100A4 in its Ca^{++}/Zn^{++} bound form is a ligand for both RAGE and TLR4.

S100A4 and S100A9 can form Heterodimers

S100A9 is commonly expressed as a heterodimer together with S100A8 [15], although it can also form homodimers [18]. Given the similarities of S100A9 and S100A4, we wanted to investigate whether S100A9 and S100A4 could interact directly and form heterodimers. To this end we immobilized either S100A4 or S100A9 on a biosensor chip and performed SPR analysis with various S100 proteins in the fluid phase. As shown in Figure 4A, homodimeric interactions were readily detected, independent of whether S100A4 or S100A9 was immobilized, but also interactions between S100A4 and S100A9. No interaction was detected of either protein with S100A1, S100A7 and S100A13 that were included as controls. All complex formation was dependent on Zn^{++} (Figure 4B). Thus, the conformational change induced by Zn^{++} is not only important for the ability of S100 proteins to interact with their ligands, but also seems to affect the formation of S100 protein heterodimers.

To verify that S100A4/S100A9 heterodimers could also be formed *in vivo*, we transfected 293T cells with expression plasmids carrying either human S100A4 or human S100A9 cDNAs. In cells transfected with the S100A4 or S100A9 constructs, S100A4 and S100A9 proteins could be detected. In cells transfected with theS100A9 alone there was very little S100A9 protein detected (Figure 4C). However, in cells transfected with both the S100A4 and the S100A9 constructs the amounts of both proteins were increased, indicating that the two proteins interact. Using a cell-permeable cross-linker, the presence of a protein with the predicted size of a S100A4/S100A9 heterodimer could be revealed, both using S100A4- and S100A9-specific antibodies. These data indicate that, given the appropriate conditions, S100A4/S100A9 heterodimers also form *in vivo*.

We next wanted to verify that S100A4/S100A9 heterodimers would be functional, i.e. interact with a relevant receptor. To address this we investigated the ability of S100A4 and S100A9 to interact with RAGE and TLR4 both as homo- and hetero-complexes. As is shown in Figure 5A, S100A4 interacts with RAGE in a similar way as S100A9 but at a lower response level and with a slightly slower off-rate. We could also demonstrate that the S100A4/S100A9 heterodimer binds to RAGE with kinetics resembling that of S100A9 and with a signal that is higher than the combined responses of the respective homodimers. This supports the notion that the heterodimer is biologically relevant and even more since similar results were obtained with TLR4 immobilized on the chip (Figure 5B). When the effect of OX on this interaction was investigated, OX inhibited S100A4/S100A9 binding to RAGE with somewhat lower potency than the S100A9 homodimer but more efficiently than the S100A4 homodimer (Figure 5C). This indicates that similar interfaces are involved when S100A4 and S100A9 interact with RAGE as homo- or hetero-complexes. Lastly, we investigated whether heparan sulfate (HS), a known inhibitor of the S100A9-RAGE interaction, was also able to displace the binding of S100A4 homo- and heterodimers to RAGE. As is shown in Figure 5D, HS inhibited binding of S100A4 homo- and heterocomplexes to RAGE almost as efficiently as S100A9. We conclude from these data that S100A4 and S100A9 show an overlapping reactivity with TLR4 and RAGE and that these proteins can form heterodimers with reactivity to these receptors.

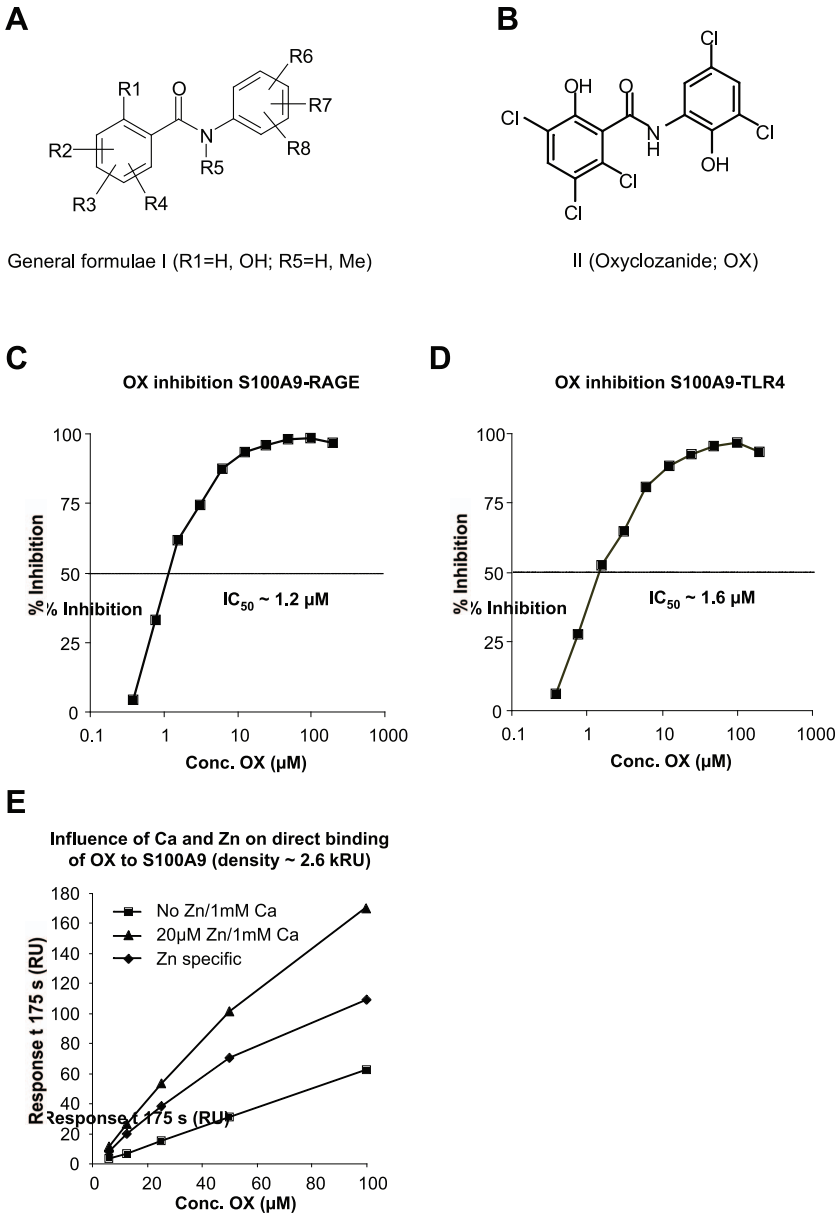

Figure 1. Oxyclozanide binds S100A9 protein. A: General formulae of synthesized salicylic and benzoic amides. B: Structure of Oxyclozanide (OX). C-D: Inhibition of S100A9 binding to immobilized RAGE (C) or TLR4 (D) by OX. 50 nM S100A9 was injected over RAGE or TLR4±0.4–200 µM OX in the presence of 1 mM $CaCl_2$, 20 µM $ZnCl_2$ and 1% DMSO. Binding was expressed as % inhibition of S100A9 response without OX and IC_{50} calculated after curve fit to a sigmoidal dose-response model. IC_{50} values of ~1.3 and 1.6 µM were obtained for OX inhibition of S100A9 binding to RAGE and TLR4. E: Effect of Zn^{++} on direct binding of OX to amine coupled S100A9. 3.125–100 µM OX was injected (2 min at 30 µL/min) over S100A9

(density ~2.6 kRU) in HBS-P with 1 mM Ca^{++} ±20 μM Zn^{++}. Responses at late association phase are plotted versus OX concentration and curves fit to a 1:1 model. Zn^{++} specific binding was obtained by subtraction of responses with Ca^{++} alone.
doi:10.1371/journal.pone.0063012.g001

S100A4 and S100A9 Expression in vivo

Having noted above that S100A4 and S100A9 can form heterodimers *in vitro* we wanted to investigate the expression pattern of these proteins *in vivo*. $CD11b^+$ cells in the mouse can be divided into various subpopulations dependent on their cell surface expression of the Ly6C and Ly6G markers (Figure 6A). We isolated by cell sorting $CD11b^+Ly6C^+Ly6G^+$ ($Ly6G^+$) and $CD11b^+Ly6C^{++}Ly6G^-$ ($Ly6C^{++}$) cells from bone marrow and spleen from normal C57BL/6 mice and analyzed these two populations for S100A9, S100A8 and S100A4 RNA expression using Q-PCR. The $Ly6G^+$ cell population expressed relatively high levels of S100A8 and S100A9 RNA, but was essentially negative for S100A4 expression (Figure 6B). On the contrary, $Ly6C^{++}$ cells expressed significant levels of S100A4 RNA, but relatively low levels of S100A8 and S100A9 compared to $Ly6G^+$ cells. In none of the experiments performed we observed a difference between bone marrow or spleen-derived $CD11b^+$ cells.

To further verify the differential expression of S100A9 and S100A4 we sorted the same cell populations as above from C57BL/6 spleen and prepared cell extracts for Western blot analysis. As shown in Figure 6C, the selective S100A4 expression in $Ly6C^{++}$ cells could be confirmed at the protein level. In addition, in splenic $CD11b^+$ cell from a mouse heterozygous for an allele where the S100A4 gene has been replaced with green fluorescent protein (GFP) the GFP protein appeared to be specifically expressed in the $Ly6C^{++}$ population (Figure 6D). In contrast, intra-cellular staining with anti-S100A9 antibodies revealed a more pronounced staining in the $Ly6G^+$ population (Figure 6D).

We subsequently investigated whether the same differential expression between S100A9 and S100A4 could be observed in a human monocytoid cell line. We therefore analyzed the S100A9 and S100A4 RNA expression in the human monocyte cell line THP-1 (Figure 6E). In these cells the S100A9 RNA expression was relatively low while S100A4 RNA expression was higher. Interestingly, after stimulation with LPS the S100A9 expression was significantly up-regulated while the S100A4 expression was down-regulated, again iterating the pattern of differential expression between the two genes. Also, stimulation of THP-1 cells with S100A9 itself, being a TLR4 ligand [30], also up-regulated the S100A9 expression while it down-regulated S100A4 expression.

The Selectivity of S100A4 and S100A9 Expression Remains during Disease

Lastly we wanted to investigate whether the differential expression pattern between S100A4 and S100A9 was modified under disease conditions. We focused on two different disease models; experimental autoimmune encephalitis (EAE) as a model for inflammatory disease and the EL-4 lymphoma model as a model for cancer. First we analyzed the S100A4 and S100A9 RNA expression in splenic $Ly6G^+$ and $Ly6C^{++}$ cells in control, EAE and EL-4 inoculated animals. As shown in Figure 7A, S100A4 expression remained largely restricted to the $Ly6C^{++}$ cell population also in disease. Interestingly, while a slight down-regulation of S100A4 expression was observed in EL4 tumor-carrying animals, induction of EAE rather increased the S100A4 RNA expression in splenic $Ly6C^{++}$ cells. A slight increase in S100A9 RNA expression was observed in splenic $Ly6C^{++}$ cells both in EAE and in EL-4 inoculated animals (Figure 7B). An increased S100A9 expression was observed in splenic $Ly6C^{++}$ cells in both EAE and EL-4 inoculated animals. Although these slight differences in expression levels could be seen, we conclude that the differential expression of S100A4 and S100A9 in splenic $CD11b^+$ cells remains also during inflammatory disease or tumor challenge.

To confirm this finding we performed immune-histology on brain sections from mice with EAE, as well as on EL4 tumors. As shown in Figure 7C, S100A4 and S100A9 expression could be detected in EAE brain. However, double positive cells were extremely rare. Essentially the same staining pattern was seen in sections from EL-4 tumors (Figure 7D). Most of these cells were

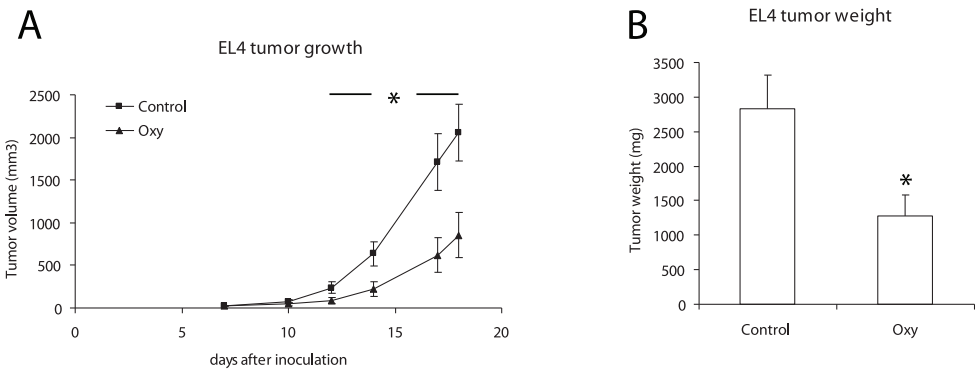

Figure 2. OX inhibits EL4 lymphoma growth *in vivo*. A: Anti-tumor effect of OX in EL4 tumors inoculated (s.c.) into wild type mice. OX was administrated p.o. at 30 mg/kg/day 7 days/week from day 1 throughout the experiment. Each data point represents mean ± SEM (n = 10; *$p<0.05$, Mann Whitney U). Control animals received only water. B: Tumor weights (*$p<0.05$; Mann Whitney U).
doi:10.1371/journal.pone.0063012.g002

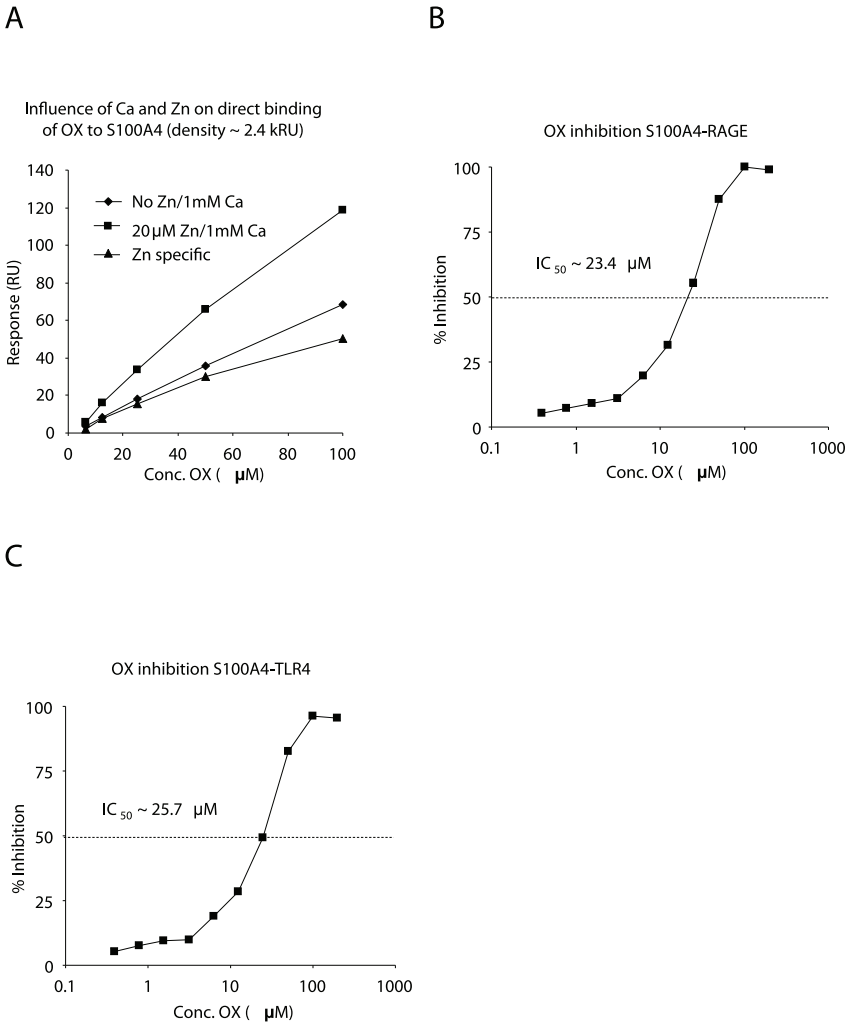

Figure 3. Oxyclozanide binds S100A4. A: Effect of Zn^{++} on direct binding of OX to amine coupled S100A4. 3.125–100 μM OX was injected (2 min at 30 μL/min) over S100A4 (density ~2.4 kRU) in HBS-P with 1 mM Ca^{++} ±20 μM Zn^{++}. Responses at late association phase are plotted versus OX concentration. Zn^{++} specific binding was obtained by subtraction of responses with Ca^{++} alone and curves fit to a 1:1 model. B-C: Inhibition of S100A4 binding to immobilized RAGE (B) and TLR4 (C) by OX. 100 nM S100A4 was injected over RAGE or TLR4±0.4–200 μM OX in the presence of 1 mM Ca, 20 μM Zn and 1% DMSO. Binding was expressed as % inhibition of response with S100A4 in the absence of OX. IC_{50} values (inserted) were calculated after curve fit to a sigmoidal dose-response model.
doi:10.1371/journal.pone.0063012.g003

also $CD11b^{+}$ (data not shown). We conclude from these data that S100A9 and S100A4 appears to be expressed in distinct $CD11b^{+}$ cell populations *in vivo*. Hence, if the proteins should be able to associate *in vivo* they would most likely have to interact in the extra-cellular space.

Discussion

In this paper we show that it is possible to define a novel small molecule compound (OX) that has anti-tumor effect *in vivo* by selecting for binding to S100A9 and inhibition of its interaction with RAGE. This finding also indicates that it is quite feasible to find small molecules that can inhibit specific protein-protein

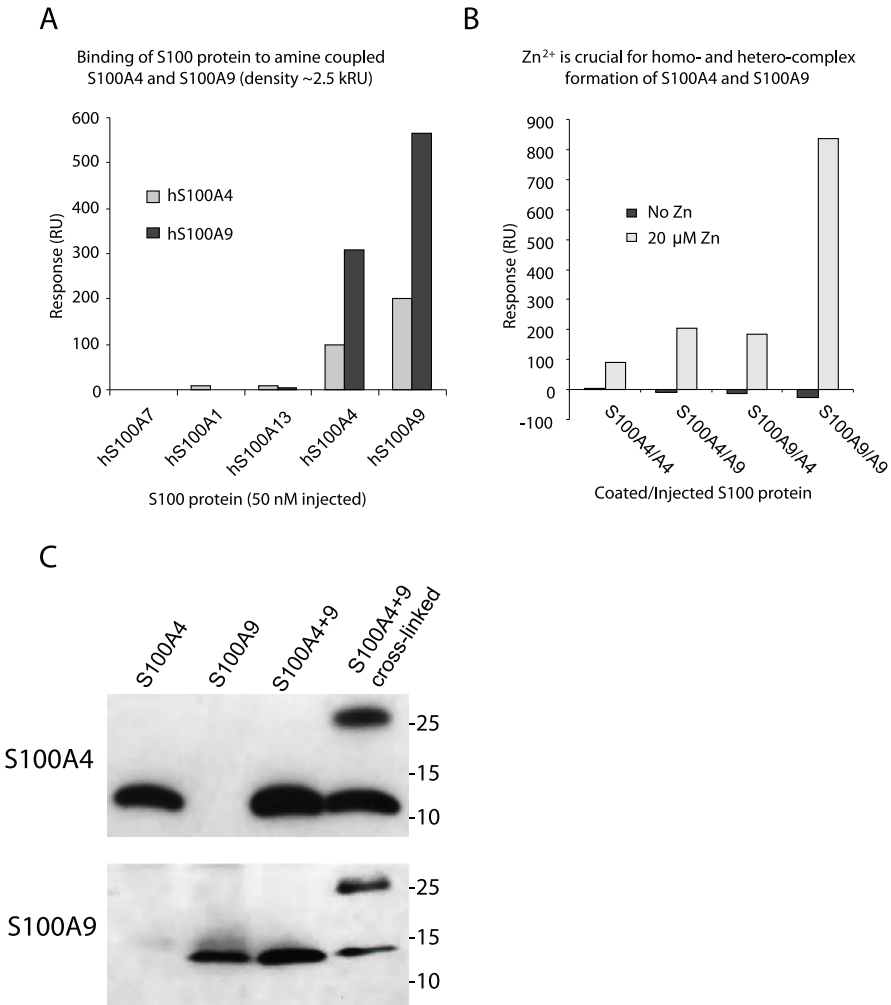

Figure 4. S100A4 and S100A9 can form heterodimers. A: Binding of S100 proteins to immobilized S100A4 and S100A9. S100A1, A4, A7, A9 and A13 (~1.3 µg/mL) were injected (2 min at 30 µL/min) over S100A4 and S100A9 (density ~2.5 kRU) in the presence of 1 mM Ca^{++} and 20 µM Zn^{++}. Responses were calculated at late association phase. B: Formation of homo- and hetero-complexes of S100A4 and S100A9 is Zn^{++} dependent. S100A4 and S100A9 were injected over immobilized S100A4 or S100A9 at ~1.3 µg/mL HBS-P containing 1 mM Ca^{++} ±20 µM Zn^{++}. Responses at late association phase were calculated. C: HEK293T cells were transfected either with S100A4 or S100A9 cDNA construct alone or the two together, as indicated. After 24 hrs of culture some of the transfected cells were exposed to the membrane permeable cross-linker DSS. Thereafter cell lysates were prepared, equal amounts (30 µg) of protein loaded on an SDS-PAGE gel and western blots were performed using either anti-S100A4 or anti-S100A9 antibodies as indicated. Representative results from one out of two experiments performed are shown.
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interactions. We have previously shown that Q compounds bind to S100A9 and inhibit its interaction with RAGE as well as TLR4 [21]. Also in the case of OX, selection for inhibition of the S100A9/RAGE interaction resulted in a compound that also inhibited the interaction between S100A9 and TLR4. Hence, a very similar molecular structure in S100A9 must be involved in both interactions. Interestingly, this molecular surface is most likely a conformational epitope since binding of S100A9 to OX, Q compounds, RAGE and TLR4 requires the presence of both Ca^{++} and Zn^{++} ([21] and herein). Especially the need for Zn^{++} is intriguing since the levels of free ions needed is unlikely to be available in the intra-cellular environment. Thus, it could be

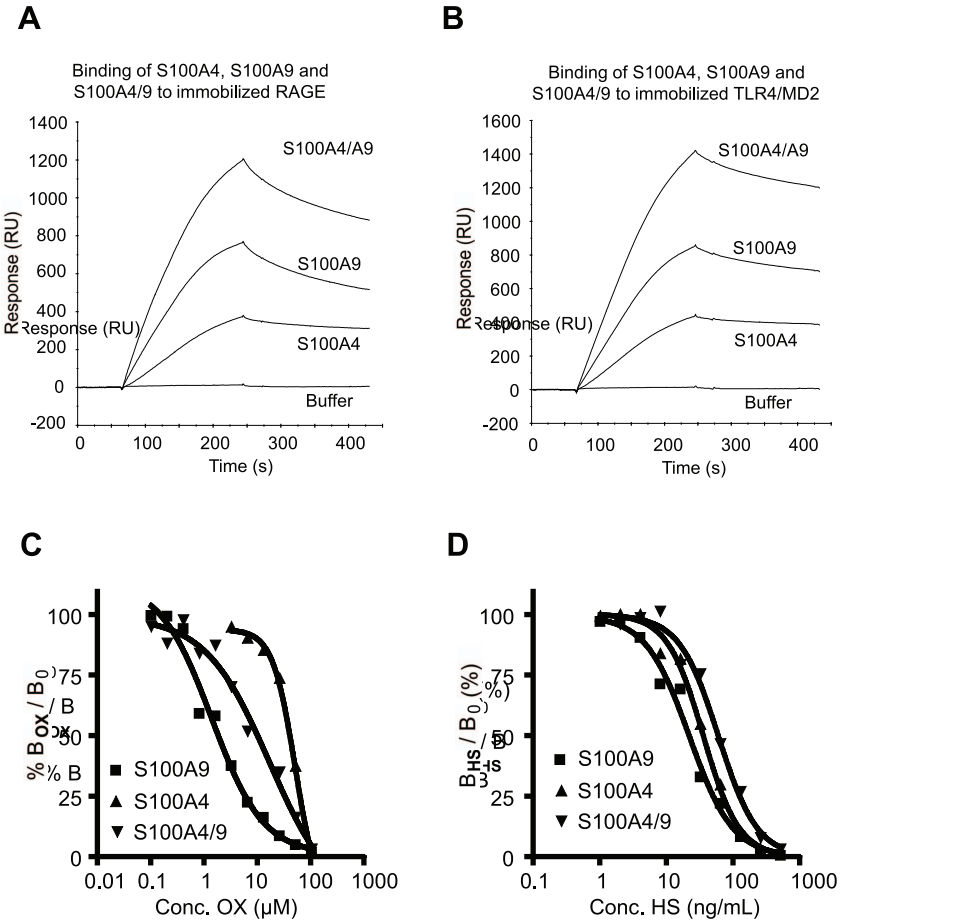


Figure 5. Binding of homo- and hetero-complexes of S100A4 and S100A9 to RAGE are displaced by OX and heparan sulfate (HS). A: Binding of homo- and hetero-complexes of S100A4 and S100A9 to immobilized RAGE and TLR4. S100A4, S100A9 and a 1:1 mixture of S100A4/9 were injected (2 min at 30 μL/min) over RAGE (left panel) or TLR4 (right panel). B: Inhibition of ~1.3 μg/mL S100A4, S100A9 and the 1:1 mixture with 0.195–100 μM OX in the presence of 1 mM Ca^{++}, 20 μM Zn^{++} and 1% DMSO. Samples were injected (2 min at 30 μL/min) over immobilized RAGE. C: Responses at late dissociation phase (expressed as % of signal in the absence of OX) were plotted against concentration of competitor and IC_{50} calculated by fit of curves to a sigmoidal dose-response or a one-site competition model. IC_{50} values of 1.4, 52 and 17 μM were calculated for S100A9, S100A4 and S100A4/S100A9. D: Corresponding experiment with 0.98–500 ng HS/mL as competitor. Conditions were identical except that DMSO was omitted from the sample buffer. HS inhibited binding of S100A9, S100A4 and S100A4/S100A9 to RAGE with 50% at 22, 36 and 60 ng/mL.
doi:10.1371/journal.pone.0063012.g005

argued that these functions are acquired when exported into the extra-cellular space and that these protein-protein interactions hence are mostly relevant in the extra-cellular compartment.

Q compounds have shown anti-tumor effects *in vivo* [26,31,32]. It has also been shown that treatment with Q compounds has a similar effect on EL4 lymphoma growth as seen in animals lacking either S100A9 or TLR4 expression [24]. We show here that OX, which has a very similar effect on S100A9-RAGE/TLR4 interactions as Q compounds, also shows anti-tumor effect *in vivo* in the same tumor model. It could be argued that since OX also interacts with S100A4 (see below), the anti-tumor effect could be due to S100A4 inhibition. However, OX displaces binding of S100A9 more efficiently than binding of S100A4 to both RAGE and TLR4. Moreover, Q compounds do not bind S100A4 (data not shown), which would argue for that, at least in the EL4 lymphoma model, the anti-tumor effect of OX is related to S100A9 binding. Thus, we would like to propose S100A9 as a pertinent target for the development of novel anti-cancer treatments.

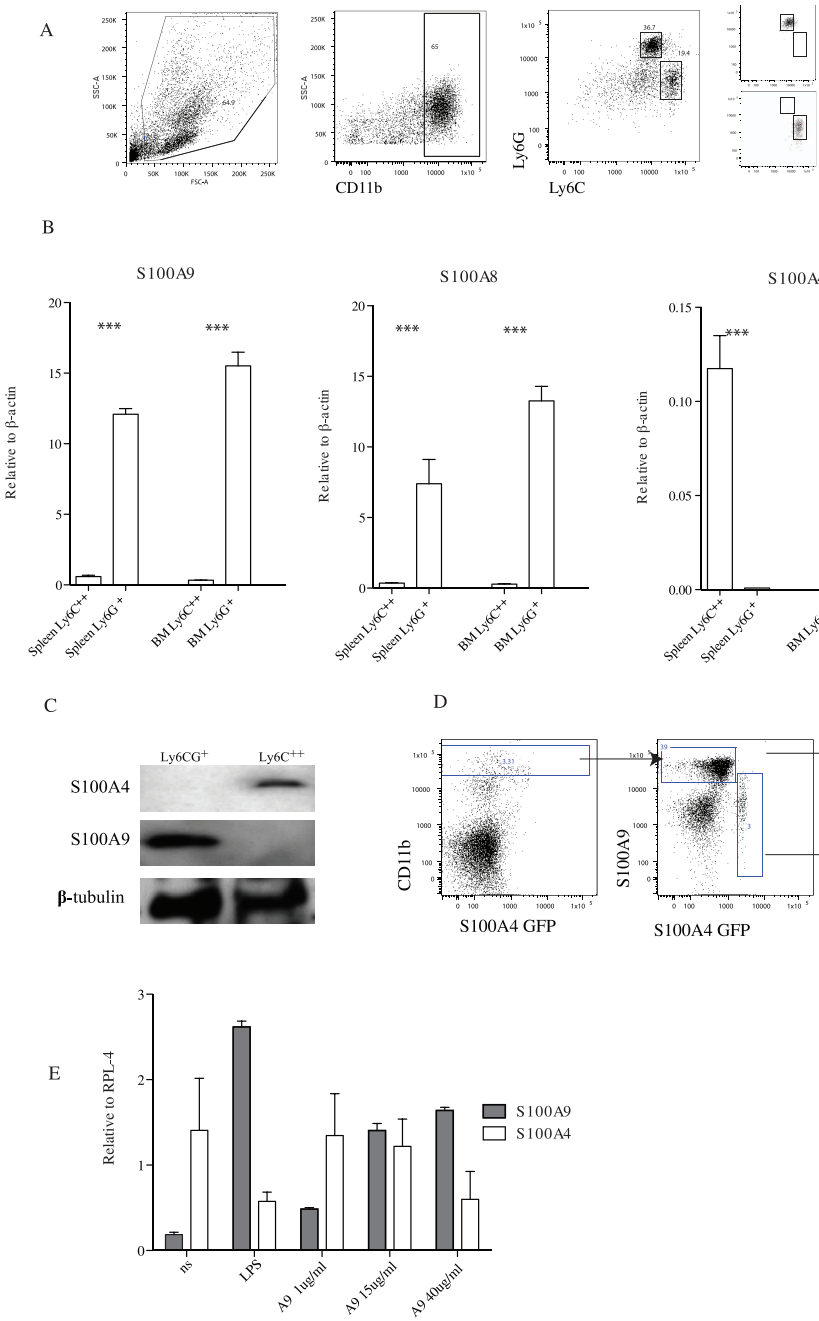

A
SSC-A
FSC-A
64.9
CD11b
65
Ly6G
Ly6C
36.7
19.4
B
S100A9
S100A8
S100A4
Relative to β-actin
***
Spleen Ly6C++
Spleen Ly6G+
BM Ly6C++
BM Ly6G+
C
Ly6CG+
Ly6C++
S100A4
S100A9
β-tubulin
D
CD11b
S100A4 GFP
S100A9
Ly6G
Ly6C
E
Relative to RPL-4
S100A9
S100A4
ns
LPS
A9 1ug/ml
A9 15ug/ml
A9 40ug/ml

Figure 6. Analysis of S100A9 and S100A4 expression *in vivo*. FACS sorting of spleen cells from C57BL/6 animals. Panel A: Left: The gate used for defining $CD11b^+$ cells is shown. Right: The $CD11b^+Ly6G^+C+/CD11b^+Ly6C^{++}$ populations used for comparison and defined as indicated. Purity is shown to the far right. Panel B: Quantitative real time RT-PCR analysis of S100A9, S100A8 and S100A4 RNA expression from indicated cell populations (>90% pure by FACS analysis) from the spleen and BM of C57BL/6, animals. The mean expression from 4 separate experiments is shown (*** = $p<0.001$; Students unpaired t test). C. FACS Aria sorted cells were used for Western blotting. Antibodies to S100A4, S100A9 and β-tubulin were used to reveal respective protein. Panel D. Indicates the gating strategy on S100A4 GFP mice on C57BL/6 background. E. Quantitative RT-PCR analysis of S100A4 and S100A9 expression from THP-1 cells cultivated alone or for 48 h with LPS or S100A9, as indicated. Two independent experiments with triplicate cultures were performed.
doi:10.1371/journal.pone.0063012.g006

The data showing that S100A4 is also able to bind to RAGE, TLR4 and OX was at first glance somewhat surprising. All these interactions were weaker than those between S100A9 and the same molecules but very specific and dependent on the presence of Ca^{++} and Zn^{++}, indicating a conformational epitope. It should be noted that the S100 proteins, although diverging at the level of primary sequence, show very similar three-dimensional structures [2]. Our current observation might also indicate that there will be some functional redundancy to be expected between proteins in the S100 protein family. Lastly, the fact that OX interacts with both S100A4 and S100A9 while Q compounds only interacts with S100A9 opens up the possibility for designing S100 protein binding molecules that will display variable patterns of S100 protein interactions.

That S100A4 and S100A9 can form heterodimers (Figure 4) is a novel observation. However, we also note that the formation of these heterodimers is dependent on Zn^{++}, which might indicate that such dimers are preferentially formed in the extra-cellular space. The fact that the two proteins seem to be produced by distinct subpopulations of $CD11b^+$ cells (Figure 6) would argue for this fact. However, we also show that the heterodimers can be formed intra-cellularly upon forced co-expression of the two proteins (Figure 4C) and it should be noted that S100A9 mRNA is present also in the $CD11b^+$ cells that express S100A4, although at low levels. These data indicate that intracellular conditions may still allow dimerisation to stabilize the proteins but that these dimers may not be functional until exported into the extracellular space. Lastly, the S100A4/S100A9 heterodimers are functional with regard to RAGE binding which in turn could be inhibited by OX. The behavior of S100A4/S100A9 heterodimers resembled S100A9 homodimers with regard to binding strength to RAGE, OX inhibition and also that the interaction could be inhibited by heparan sulphate (Figure 5C). However, the S100A4/S100A9 heterocomplex differed from the S100A4 homodimer with regard to the binding strength to both RAGE and TLR4; an effect that seems to be more than additive. This observation could indicate that the secretion of S100A4 in the presence of S100A9 may give rise to multimers with a more potent RAGE and TLR4 stimulatory activity than the secretion of S100A4 alone. In addition, qualitative changes cannot be excluded since both S100A4 and S100A9 can interact with other receptors than RAGE and TLR4 and heterodimerization may create a ligand that can bind additional receptors.

The differential expression of S100A4 and S100A9 in both healthy animals and in animals with ongoing inflammatory disease or tumor burden was striking. In sections from inflamed brain or tumors, double-expressing cells were very rare, if not completely absent. Whether this is of biological significance for the role of S100 proteins in the control of inflammation or tumor growth merits further investigation. An interesting observation was that while S100A4 expression was down-regulated upon inflammatory signals in THP1 cells S100A9 was up-regulated. We believe that this may reflect differentiation of S100A4 expressing THP-1 cells to S100A9 expressing cells. Alternatively, the cell line may contain two populations, one expressing S100A4 and the other expressing S100A9. Upon stimulation, the latter population might outgrow the former. In contrast, the S100A4 expression in $Ly6C^{++}$ monocytes was slightly increased during inflammation *in vivo* while S100A9 expression was substantially increased in the $Ly6G^+$ population (Figures 6 and 7). On the contrary, S100A4 was not up-regulated in $Ly6C^{++}$ cells from animals with tumors while S100A9 expression was up-regulated in $Ly6G^+$ cells in these animals, in line with what has previously been described for both proteins individually.

In conclusion, we have demonstrated novel interactions between S100 proteins, as well as their interactions with biologically relevant pro-inflammatory receptors. In addition, we believe that this work validates S100A9 as a potential molecular target for the treatment of malignant disease.

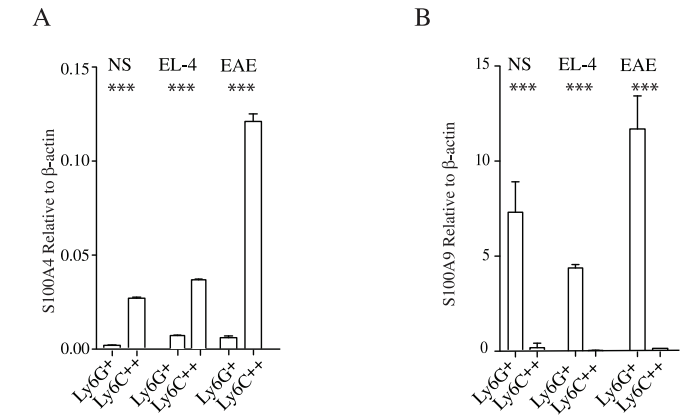
Materials and Methods

General Synthesis of Salicylic Amides

To a solution of the salicylic acid in dichloromethane were added 4 eq. of oxalylic chloride and one drop of DMF. The reaction mixture was stirred for three hours at room temperature and was then evaporated to dryness, co-evaporated twice with toluene and dried under high vacuum. To a solution of the aniline (2 eq.) and dimethyl aniline (2 eq.) in dioxane (20 ml/gram of acid chloride) at room temperature was added 1 eq. of solid salicylic acid chloride in one portion. The mixture was stirred at room temperature for 2 hours and then 2 M NaOH(aq) (2 eq.) was added, after which stirring was continued for two hours. 5 M sulfuric acid(aq) was added dropwise until pH 0–1 was reached and a precipitate was formed. Water was added to complete the precipitation and the crude product was isolated by filtration. After drying, the crude was purified by crystallization from HOAc/water or EtOH/water. Yields ranged from 20–50%. Oxyclozanide (OX) was purchased from Fluka (99.5%) and used without any further purification.

Surface Plasmon Resonance (SPR)

SPR analysis was conducted using the Biacore 3000™ system from GE Healthcare, Uppsala, Sweden, mainly as described previously [21]. In the inhibition assay format, recombinant human S100A9 (produced in *E. coli* at Active Biotech; S100A9) or human S100A4 (R&D Systems; S100A4) was injected with OX over human RAGE or TLR4/MD2 immobilized on a CM5 chip at a density of ~3 kRU. In a first step OX was serially diluted in 100% DMSO and then diluted 50-fold in 10 mM HEPES, 0.15 M NaCl, pH 7.4, containing 0.005% Surfactant P20 (HBS-P buffer). Prior to injection, S100A4 or S100A9 was pre-incubated with OX for ≥1 h in assay buffer (HBS-P containing 1 mM Ca^{++}, 20 μM Zn^{++} and 1% v/v DMSO). In order to test the influence of Ca^{++} and Zn^{++} on direct binding of OX to immobilized S100A4 and S100A9, OX was injected in HBS-P without DMSO in the absence or presence of Ca^{++} and/or Zn^{++}. The ability of S100A4 and S100A9 to form homo- and hetero-complexes was also tested by injecting S100A4 and S100A9 over these proteins immobilized on the sensor chip and with recombinant human S100A1



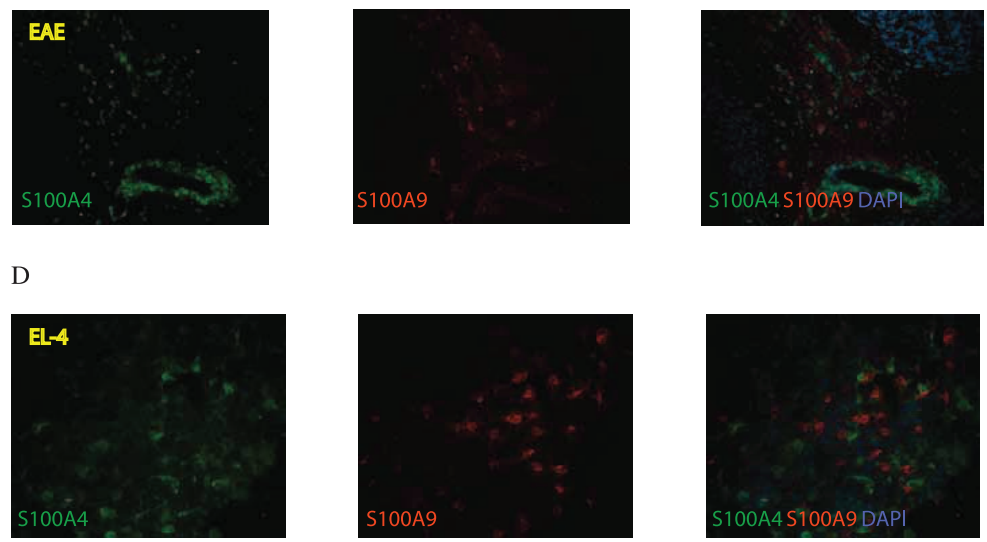


Figure 7. Analysis of S100A9 and S100A4 expression *in vivo* during disease. Panel A and B; Quantitative real time RT-PCR analysis (see Materials and Methods). Spleen cells, from control C57BL/6 animals, from mice inoculated with 50,000 EL4 lymphoma cells or spleen cells from EAE induced animals with MOG-peptide and with Pertussis toxin were FACS-sorted and used to analyze RNA expression of S100A4 (Panel A) and S100A9 (Panel B) (*** = $p<0.001$; Students unpaired t test). Panel C; Immunohistochemical analysis show cells expressing S100A4 (green), S100A9 (red) in brain sections from mice with MOG induced EAE. The labeled cells are localized close to and around a vessel. An overlay of the two first pictures including nuclear staining of DAPI (blue) is shown in the last picture. Scale bar represents 100 μm. Panel D; Expression of S100A4 (green) and S100A9 (red) respectively in sections from an EL-4 tumor. An overlay of the two first pictures including nuclear staining with DAPI (blue) is shown in the last picture. The overlay shows that S100A4 and S100A9 is expressed in different cells, no overlap can be detected. Scale bar represents 50 μm.
doi:10.1371/journal.pone.0063012.g007

(ProSpec, Rehovat, Israel), S100A7 (ProSpec) and S100A13 (R&D Systems) as negative controls. Formed homo- and hetero-complexes were also tested for binding to RAGE and TLR4 and whether binding could be displaced with OX and heparan sulfate (Sigma; HS). Evaluation of binding data was made using GraphPad Prism 4.0 and BIAevalution 3.0 software.

EAE and EL-4 lymphoma Model

All animal experiments have been approved by a local ethics committee ("Malmö/Lunds Djurförsöksetiska nämnd"). The issued ethical permits DNR M 275-08, M 60-10 and M 04-11 are specifically approved for the experiments performed in the current investigation. C57BL/6 mice, (Taconic M&B, Ry, Denmark) mice were kept in an SPF animal facility at BMC or Active Biotech AB, Lund. Nine weeks old animals were injected subcutaneously with 30,000 EL4 lymphoma cells in 100 µl PBS. After 7 days the animals were scored for tumor growth by palpation, day 10, 12, 14 and 18 the tumors were measured with a slide caliper and the volume calculated. OX was administered by oral gavage 30 mg/kg/daily from the day after cell inoculation to day 17. The mice were sacrificed by cervical dislocation and spleens were dissected. The cell suspension was thereafter passed through a 70 µm cell strainer and cells washed in Hank's BSS (Invitrogen Life Technologies, Paisley, UK).

Immunization EAE

Mice were immunized subcutaneously at the base of the tail with 50 µg MOG_{35-55} peptide (Schafer-N, Copenhagen, Denmark) in PBS emulsified in complete Freund's adjuvant (CFA). To induce the development of EAE, MOG-peptide immunized C57Bl/B6 mice were also injected twice (on the same day and two days later) with Pertussis toxin (200 ng; List Biological Laboratories, Inc. Campbell, Ca) in PBS.

Cell Culture Conditions

The human monocytic leukaemia cell line THP-1 (purchased from American Type Culture Collection, Manassas, VA) was grown in RPMI-1640 culture medium (Invitrogen, Stockholm, Sweden) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Sigma- Aldrich, St Louis, MO), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin and 100 lg/ml streptomycin (P/S; Invitrogen), at 37° in 5% CO2. The cells were cultured with or without 100 ng/ml ultra pure lipopolysaccharide and various concentrations of S100A9 as indicated in figure for 48 hr followed by harvesting the RNA for Q-PCR. 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 2mM Glutamine (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2, 1 mM sodium pyruvate, 10 mM HEPES.

293T Cell Transfection and Stimulation

Three different transfections were performed: 1) **hS100A4**:1 µg pcDNA3.1-hS100A4+1 µg pcDNA3.1-EGFP; 2) **hS100A9**:1 µg pDream2.1-hS100A9+ pcDNA3.1-EGFP; 3) **S100A4/S100A9**:1 µg pcDNA3.1-hS100A4+1 µg pDream2.1-hS100A9. Briefly, 293T cells were seeded in 24 well plate in serum-free medium. DNA-Lipofectamine 2000 mixture was prepared as follow: 2 µl Lipofectamine 2000/reaction were incubated in 50 µl Optimem medium for 5 min at room temperature (RT). Plasmid DNA was added to Lipofectamine 2000 solution and incubated for further 20 minutes at RT. Subsequently, the DNA-Lipofectamine 2000 mixture was added to the 293T cells, which were incubated 3h at 37°C. Finally, 1 ml/well of full medium was added to the transfected 293T cells and a further 24h incubation at 37°C was performed. In some experiments, to study protein oligomerization, 293T cells were washed in PBS and incubated for 1h in ice with 1 mM disuccinimidyl suberate (DSS) in PBS. Then, 293T cells were washed in PBS, lysed and SDS-PAGE followed by western blot was performed.

Q-PCR

Splenic $CD11b^+$ cells were purified using anti-CD11b magnetic beads and LS-columns (Miltenyi Biotech, Bergisch Gladbach, Germany),. Total RNA was extracted from $CD11b^+$ cell preparations by use of the Purelink RNA mini Kit (Invitrogen). RNA was reverse transcribed to cDNA by use of the SuperScript III Platinum synthesis system (Invitrogen). Real-time PCR (RT-PCR) was performed for the detection of S100A9, S100A8 and S100A4 RNA and quantified using a SYBR GreenER kit (Invitrogen) in a MYIQ (Bio-Rad) PCR machine. The threshold cycle number was determined and relative expression level of each mRNA was determined using the formula $2^{(Rt-Et)}$, where Rt and Et are the threshold cycles for the reference gene (β-actin or RPL-4) and the target gene, respectively.

Flow Cytometry

Flow cytometry analysis was performed on spleen cell suspensions, as indicated. Primary antibodies used were: anti-mouse CD11b-APC (eBioscience), Ly6G-FITC (BD Pharmingen) and Ly6C-biotin (BD Pharmingen). Biotinylated antibodies were detected with streptavidin-QD605 (Invitrogen). Data were acquired using a FACS LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Immunohistochemistry

Tissues analyzed with immunohistochemistry were embedded in OCT compound (Tissue-Tek®), and snap-frozen in liquid nitrogen. Cryosections (7–8 µm) were prepared on microscope slides, air dried and frozen at −20°C until staining procedures. Acetone fixed sections from EL-4 tumors or brains from animals with MOG induced EAE were blocked with 10% normal serum diluted in PBS with 1% BSA for 30 min. Thereafter the sections were incubated with primary antibodies, rabbit anti S100A4 (DAKO Denmark A/S, Denmark) and goat anti S100A9 (Santa Cruz Biotechnology Inc, CA, USA) or isotype controls for each antibody 1 hour in room temperature followed by incubation with secondary antibodies, donkey anti rabbit Alexa Fluor 488 (Invitrogen) and donkey anti goat Alexa Fluor 555 (Invitrogen, Oregon, USA) for 30 min. The slides were mounted using ProLongGold antifade kit containing DAPI (Invitrogen, Oregon, USA). The stainings were analysed and photographs taken using a Leica DMX microscope and Leica application suite 3.7 software.

Western Blot

Spleen cells were stained as described above and $Ly6G^+$ and $Ly6C^{++}$ subpopulations were sorted using a FACSAria flow cytometer (BD Biosciences). For Western blot, 10 µg of proteins was loaded onto 12% polyacrylamide gels (C.B.S. Scientific, San Diego, CA, USA). Proteins were subsequently transferred to PVDF membrane (Roche), which was saturated with 1% dry milk in PBS-Tween. Thereafter, the membranes were incubated with Rat anti-Mouse S100A9, Rabbit anti-mouse S100A4 and Rabbit anti-β Tubulin (R&D Systems) as primary antibody and Rabbit anti-Rat-HRP or Goat anti-Rabbit-HRP (SouthernBio-

tech Birmingham, Alabama, USA) as secondary antibodies and filters developed using ECL kit (GE Healthcare, UK).

Author Contributions

Conceived and designed the experiments: TL FI DL. Performed the experiments: UW PB EK AO ZH MT MR. Analyzed the data: PB EK AO MT DL FI TL. Wrote the paper: DL FI TL.

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Paper III

PLOS ONE

RESEARCH ARTICLE

CD14 Is a Co-Receptor for TLR4 in the S100A9-Induced Pro-Inflammatory Response in Monocytes

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Abstract

The cytosolic Ca^{2+}-binding S100A9 and S100A8 proteins form heterodimers that are primarily expressed in human neutrophils and monocytes. We have recently shown that S100A9 binds to TLR4 in vitro and induces TLR4-dependent NF-κB activation and a pro-inflammatory cytokine response in monocytes. In the present report we have further investigated the S100A9-mediated stimulation of TLR4 in monocytes. Using transmission immunoelectron microscopy, we detected focal binding of S100A9 to monocyte membrane subdomains containing the caveolin-1 protein and TLR4. Furthermore, the S100A9 protein was detected in early endosomes of the stimulated cells, indicating that the protein could be internalized by endocytosis. Although stimulation of monocytes with S100A9 was strictly TLR4-dependent, binding of S100A9 to the plasma membrane and endocytosis of S100A9 was still detectable and coincided with CD14 expression in TLR4-deficient cells. We therefore investigated whether CD14 would be involved in the TLR4-dependent stimulation and could show that the S100A9-induced cytokine response was inhibited both in CD14-deficient cells and in cells exposed to CD14 blocking antibodies. Further, S100A9 was not internalized into CD14-deficient cells suggesting a direct role of CD14 in endocytosis of S100A9. Finally, we could detect satiable binding of S100A9 to CD14 in surface plasmon resonance experiments. Taken together, these results indicate that CD14 is a co-receptor of TLR4 in the S100A9-induced cytokine response.

Introduction

It is well established that both intracellular proteins, as well as fragments of extracellular proteins released upon tissue injury, can become ligands mediating sterile inflammation (reviewed in [1–4]). Such proteins are denoted damage associated molecular patterns (DAMPs). Binding of DAMPs to receptors such as TLR4 or RAGE, has been shown to induce the production of pro-inflammatory cytokines both in immune cells such as dendritic cells and macrophages as well as in other tissue resident cells.

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Competing Interests: The authors have the following interests: TL is a part time employee and PB and MR are employees of Active Biotech AB that develops small molecule compounds for treatment of inflammatory disease and Cancer. FI holds a grant from Active Biotech AB. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

S100 proteins are low molecular weight Ca^{2+} binding proteins, which are expressed in a tissue-specific manner in various cells of the human body (reviewed in [5–7]). Most of these proteins reside in the cytosol of the cells, while some are secreted. The S100A9 protein is normally expressed as a heterodimer together with the S100A8 protein in myeloid cells. In human cells, these proteins are co-expressed both in neutrophils and monocytes/macrophages [8–11], while in the mouse they are mainly expressed in neutrophils. The S100A8/A9 heterodimer is highly abundant in human neutrophils and constitutes a large part of the total protein content of these cells [9, 10].

The S100A8/A9 heterodimer can be secreted by activated monocytes [12], but the molecular mechanism of secretion is still largely unknown. Further, these proteins are released in high amounts by neutrophils during various inflammatory conditions and can be used as markers of inflammation (reviewed in [13–15]). In the extracellular milieu, both the S100A8 [16–19] and S100A9 proteins [17, 18, 20–22] have been reported to possess pro-inflammatory function and are therefore considered to be damage associated molecular patterns (DAMPs). Thus, both huS100A8 [19, 23] and huS100A9 [21, 24] interact with TLR4 and stimulate production of pro-inflammatory cytokines in monocytes. Interestingly, we also found that moS100A9 could induce activation of inducible nitric oxide synthase (iNOS) expression in bone marrow-derived dendritic cells (BM-DCs). That finding suggested to us that S100A9 could also stimulate the endosomal pathway of TLR4 stimulation involving activation of β-interferon (IFNβ) expression [25]. The activation of iNOS expression by S100A9 would, in analogy with the response to LPS [25], be expected to involve endocytosis of the S100A9/TLR4 complex in the responding monocytes.

The mechanism of activation of TLR4 by bacterial lipopolysaccharide (LPS) and the downstream intracellular signaling pathways has been extensively investigated [26, 27]. Seminal studies by Beutler and coworkers demonstrated that TLR4 was the LPS-receptor [28]. Subsequently reports from several laboratories showed that binding of LPS also involved the LPS binding protein MD2 and CD14 [29–31]. CD14 is a glycosylphosphatidylinositol-anchored membrane protein [32], which has several functional roles in the LPS-induced stimulation of TLR4. First, and most importantly, CD14 is essential for the internalization of the LPS/TLR4 complex by endocytosis [33] and hence for the IFNβ response induced by the cytosolic TRIF/TRAM-dependent pathway of TLR4 activation [34]. Second, CD14 binds LPS and enhances the LPS-responsiveness by TLR4/MD2 [35]. Interestingly, CD14 is also involved in the internalization of TLR3 and enhances signaling from that receptor [36].

The role of CD14 in DAMP-induced TLR4 stimulation has also been investigated previously (reviewed in [4]), but the functional role of the CD14 protein is not fully understood. In this report we have further investigated the mechanism of the S100A9-induced stimulation of the TLR4-dependent cytokine response in monocytes. We show that TLR4 is neither essential for the binding for S100A9 to the plasma membrane nor for the internalization of S100A9 into these cells. Further, we present findings indicating that S100A9 can bind CD14 and that CD14 is an essential co-receptor for S100A9-mediated TLR4-stimulation.

Materials and Methods

Mice

C57BL/6 mice were bought from Taconic Europe (Ry, Denmark). TLR4-KO and CD14-KO mice were originally bought from Jackson Laboratories (Bar Harbor, MN USA). Caveolin-1 deficient (cav-1-KO) mice [37] were bred onto C57BL/6 genetic background [38]. TLR4-KO and cav-1-KO mice were bred in the animal facilities of the Biomedical Center at Lund

University. All experiments in this study involving the use of cells from animals were approved of by the local ethics committee of animal experiments of Malmö and Lund (permit M12/13).

Cell culture

The human monocytic leukemia cell line THP-1 (from ATCC, Mannassas, VA, USA) was cultured in RPMI-1640 medium (Invitrogen, Stockholm, Sweden) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100U/ml penicillin and 100μg/ml streptomycin (all supplements from Invitrogen). Bone marrow derived dendritic cells (BM-DCs) were prepared by culturing cells from femurs and tibias of mice in the same medium as above. The medium was supplemented with 10% tissue culture supernatant of J558L cells transfected with a GM-CSF cDNA construct and cells were harvested on day 7 of culture. Peritoneal wash cells from C57BL/6 mice were used in some experiments. For in vitro stimulation experiments cells were cultured in triplicate using the above medium and stimulated with huS100A9, Ultra-pure E.coli K12 LPS (InvivoGen, San Diego CA) or the synthetic lipopeptide Pam_3Cys (EMC Microcollections GmbH, Tuebingen, Germany) as indicated in Figure legends.

Cytokine assays

Supernatants were harvested from in vitro cultures and stored at -80°C until use. TNFα concentration was determined using the Cytokine Bead Array (CBA; BD Biosciences, San Jose California) and the human and mouse TNF Flex sets (BD Biosciences), according to the manufacturer's protocols. Analysis was performed using an LSR II flow cytometer (BD Biosciences).

Antibodies

For CD14 blocking experiments, mouse anti-human CD14 antibody (clone M5E2; Novus Biologicals, Littleton CO, USA) and rat anti-mouse (clone 4C1/CD14; BD Biosciences) were used. The following antibodies were used for staining specimens for electron microscopy: rabbit anti-caveolin 1 antibodies (BD Biosciences), goat anti-mouse TLR4 (M16) (Santa Cruz Biotechnology, CA USA), rabbit anti-Rab5 (ab18211, Abcam, Cambridge, UK) and rat anti-mouse CD14 (clone Sa14-2; Biolegend, Nordic Biosite, Täby, Sweden).

Recombinant S100A9 proteins

Preparation of the human S100A9 (huS100A9) protein was described in detail in our previous paper [21] and purification of mouse S100A9 (moS100A9) was performed using the same protocol. Endotoxins were removed using Detoxi-Gel columns (Thermo Scientific). The endotoxin content was tested using LAL Chromogenic Endpoint assay (Hycult Biotechnology, Uden, The Nederlands). The huS100A9 and moS100A9 protein batches used in here contained 0.12 EU/ml and 0.036 EU/ml endotoxin, respectively. In experiments analyzing the biological activity of the proteins, polymyxin B was included in control cultures.

Transmission electron microscopy

THP-1 cells or mouse BM-DCs were incubated with recombinant human S100A9 protein conjugated with colloidal gold (10nm particles) for 15 min at 37°C. The cells were harvested and washed once with PBS. Thereafter the cell pellet was re-suspended in 150mM sodium cacodylate/2.5% glutaraldehyde, pH 7.4 (EM-fix solution) and incubated at room temperature over night. Cells were then prepared for immunostaining and transmission electron microscopy as

recently described [39]. Briefly, ultrathin sections of the cells were subjected to antigen retrieval with metaperiodate and then incubated over night at 4°C with primary antibodies in PBS (≈10 μg/ml). Binding of the primary antibodies was detected with secondary antibodies of appropriate specificity, conjugated with 25nm colloidal gold (Electron Microscopy Sciences, Fort Washington, PA, USA; titer 1:1 to 1:20). Specimens were examined in a Philips/FEI CM100 transmission electron microscope operated at 60 kV accelerating voltage. Images were recorded with a side-mounted Olympus Veleta camera with a resolution of 2048x2048 pixels (2Kx2K).

Surface plasmon resonance (SPR) analysis

Binding of CD14 to recombinant human S100A8 and S100A9 (produced at Active Biotech AB, Lund, Sweden) was analyzed using the surface plasmon resonance (SPR) technology on a Biacore 3000^{TM} system (GE Healthcare, Uppsala, Sweden). Briefly, the S100 proteins were immobilized in separate flow cells on a CM5 chip through standard amine coupling. Then recombinant human CD14, derived from CHO cells (R&D Systems cat no 383-CD-050/CF), was injected (for 2 min at a flow rate of 30 μl/min) in 10 mM HEPES, 0.15 M NaCl, pH 7.4, containing 0.005% v/v surfactant P20 (HBS-P buffer) and 1 mM Ca^{2+} and 20 μM Zn^{2+}. Regeneration was performed by a 30 μl pulse of 3 mM EDTA in HBS-P buffer. Resulting sensorgrams were fit to a 1:1 model using the BIAevaluation software 4.1.

Results

S100A9 binds to monocyte membranes and can be internalized by these cells

We have previously shown, and confirm in here (Fig 1A) that the huS100A9 protein can induce a cytokine response in the human THP-1 monocyte cell line. Further, huS100A9 can stimulate a cytokine response also in mouse macrophages (Fig 1B). As expected, in both cases addition of polymyxin B strongly reduced the LPS-induced response. Importantly, this compound had little impact on the S100A9-induced response, indicating that putative LPS contamination of the recombinant protein preparation contributed minimally to the response.

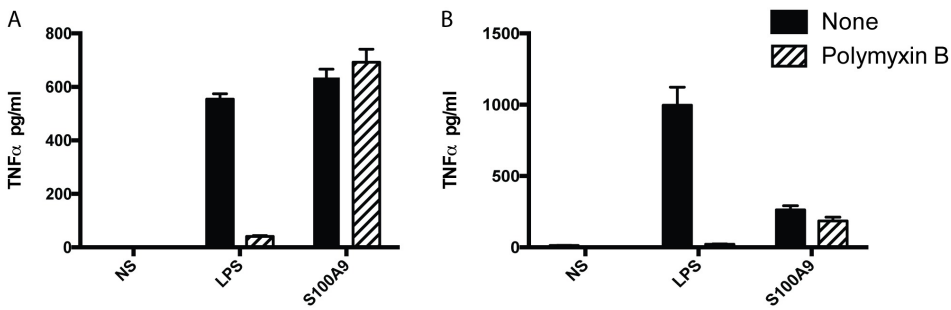

Fig 1. S100A9 induces a TNFα-response in monocytoid cells. (A) THP-1 cells (1x10^6/ml) or (B) peritoneal wash cells (1x10^6/ml) were stimulated either with huS100A9 (40μg/ml) or LPS (A, 100ng/ml; B, 10ng/ml), with or without pre-incubation with polymyxin B (50μg/ml) for 30min. Supernatants were harvested after (A) 4hrs or (B) 24 hrs of culture and TNFα concentration determined using CBA assay. Results are representative of 3 (A) and 2 (B) independent experiments.

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Our previous data suggested that the S100A9-protein, similarly to LPS, might also be internalized into the responding cell upon TLR4 stimulation [21]. To address this possibility, we labeled huS100A9 with colloidal gold and incubated THP-1 cells with the gold-conjugated huS100A9. Transmission electron microscopy analysis revealed that huS100A9 displays focal binding in pit-like structures on the THP-1 cell surface (Fig 2A). Further, the protein was also detected in vesicles in the cytosol of these cells, suggesting that the protein had been internalized by endocytosis. Staining of specimens from these cells with TLR4 antibodies revealed similar focal co-localization of huS100A9 with TLR4 in pit-like structures (Fig 2B). Cytosolic vesicles in which huS100A9 co-localized with TLR4 (S1A Fig) as well as with the early endosomal marker Rab5 (Fig 2C) could also be detected in these cells. These data further support that stimulation with S100A9, similarly to stimulation with LPS [40, 41] may involve internalization of TLR4.

S100A9 binds to caveolin-1 containing membrane subdomains

We next wanted to investigate the nature of the membrane subdomains with focal binding of S100A9 and accumulation of TLR4. Previously published findings indicate that upon

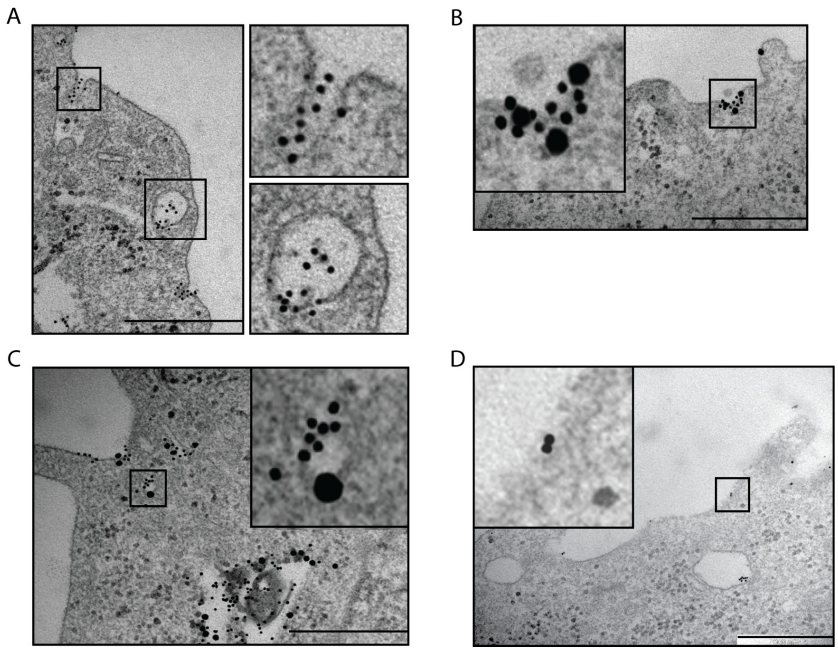

Fig 2. Binding of S100A9 and TLR4 expression coincide on monocyte cell surface. (A,B) THP-1 cells were incubated with 1μM colloidal gold-labeled S100A9 (10nm grains) protein for 15 min at 37°C and specimens prepared for TEM analysis. (B) Specimens from the same preparation were immuno-stained with TLR4 antibody, followed by secondary gold-labeled (25nm grains) anti-goat Ig antibody. The images show representative sites of surface and vesicular binding of the S100A9-protein and TLR4-expression. (C) Specimens from the preparation of THP-1 cells used in (A) were immuno-stained with Rab5 antibody conjugated with colloidal gold (25nm); bar 500nm. (D) THP-1 cells exposed to MβCD do not display focal S100A9 binding. THP-1 cells were cultured in presence of 15mM MβCD for 30 min, thereafter washed and incubated with colloidal gold-labeled S100A9 as in (A). Bar: 500nm.

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stimulation of monocytes with LPS, both TLR4 and CD14 accumulate in membrane subdomains containing lipid rafts [42, 43]. Treatment of cells with methyl-β-cyclodextrin (MβCD) sequesters cholesterol and is known to interfere with lipid raft function [44, 45]. Interestingly, in MβCD-treated THP-1 cells there was only occasional binding of the protein to the plasma membrane (**Fig 2D**) and there was no focal binding similar to what was seen in untreated THP-1 cells (compare to **Fig 2A**).

Both monocytes and DCs express the protein caveolin-1 (cav-1), which is known to associate with caveolae-like membrane subdomains in such cells (reviewed in [46]). We therefore stained specimens of S100A9 exposed mouse BM-DCs with caveolin-1 specific antibodies. As can be seen (**Fig 3A**), S100A9 bound to similar membrane subdomains in the BM-DCs and the sites of focal S100A9 binding co-localized with cav-1 staining. Interestingly, we could also detect occasional binding of S100A9 to the membranes of caveolin-1 deficient (cav-$1^{-/-}$) BM-DCs and the protein could also be internalized by these cells (**Fig 3B**). These data taken together suggest that S100A9 binds to membrane subdomains containing lipid rafts and cav-1, but that presence of cav-1 is not essential for the binding and internalization.

TLR4-independent internalization of S100A9 in BM-DCs

The above data indicated that S100A9 could be internalized into BM-DCs by endocytosis. While, as shown in our previous report [21], TLR4 expression was essential for the S100A9-induced cytokine response in BM-DCs, we also wanted to know whether TLR4 would be required for the internalization of S100A9. TEM analysis of TLR4-KO BM-DCs incubated with gold-labeled S100A9 revealed focal binding of S100A9 to the plasma membrane (**Fig 4A**). The finding that S100A9 was also detected in cytosolic vesicles of these cells, suggested that internalization of this protein is TLR4-independent. Also in the TLR4-KO cells, the focal binding to the plasma membrane (**Fig 4B**) as well as S100A9-binding in cytosolic vesicles (**S1D Fig**), co-localized with cav-1 expression. These data indicate that S100A9 can be internalized at cav-1 containing membrane subdomains through a TLR4-independent mechanism.

CD14 is a co-receptor of TLR4 in the S100A9-induced cytokine response

The above data indicated that there is at least one cell membrane associated receptor molecule that can bind and internalize S100A9 even in the absence of TLR4. Previous reports have

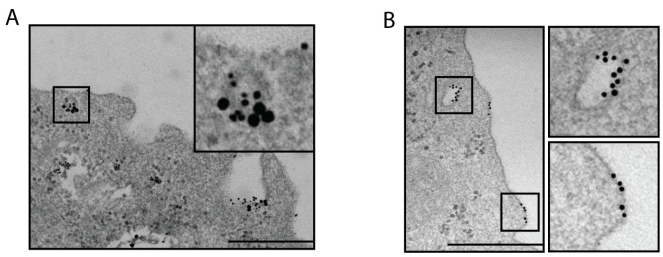

Fig 3. S100A9 binds focally both at membrane sites expressing and lacking cav-1. BM-DCs from (A) C57BL/6 or B) cav-1-KO mice were incubated with gold-labeled S100A9 (10nm grains) as in Fig 2. (A) The specimen was thereafter immuno-stained with rabbit anti-cav-1 antibody, followed by gold-labeled (25nm grains) secondary antibody. The images show representative sites of surface and vesicular binding of the S100A9-protein and TLR4-expression. Bars: 500nm.

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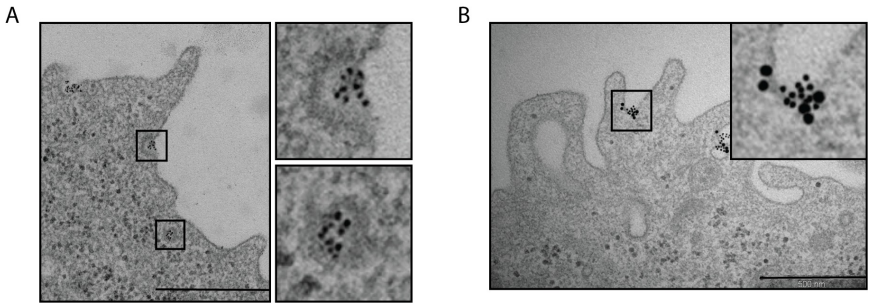

Fig 4. Focal S100A9 membrane binding and internalization is TLR4-independent and coincides with Cav-1 expression. (A,B) BM-DCs from TLR4-KO mice were incubated with gold-labeled S100A9 (10nm grains) as in Fig 2. (B) The specimen was thereafter immuno-stained with rabbit anti-cav-1 antibody, followed by gold-labeled (25nm grains) secondary antibody. The images show representative sites of surface and vesicular binding of the S100A9-protein and cav-1-expression. Bars: 500nm.

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described the involvement of CD14 in the internalization of LPS and an essential role of this protein in the LPS-induced IFNβ-response [33, 47]. We therefore next wanted to address whether CD14 might also be involved as a co-receptor in the S100A9-induced response. The observation that the focal binding of S100A9 to TLR4-KO BM-DCs co-localized with focal CD14 staining provided support for this possibility (**S2A Fig**).

To determine whether CD14 would be involved in the S100A9-induced cytokine response we used an antibody known to block the interaction between LPS and CD14. This antibody readily reduced the S100A9-induced TNFα response in THP-1 cells (**Fig 5A**). We also confirmed that the antibody could block the TNFα response induced by a low concentration of LPS. This blockade, consistently with previously published data [33, 48], could be overcome by increasing concentrations of LPS. As expected, the antibody did not interfere with the TLR2-mediated Pam_3Cys-induced response. We obtained similar results when mouse peritoneal macrophages were stimulated with S100A9 in the presence of an antibody that blocks binding of LPS to mouse CD14 (**Fig 5B**). To confirm these data we performed similar stimulation experiments using mouse BM-DCs. As can be seen, while the S100A9 protein induced a robust TNFα-response in wt BM-DCs, the response was strongly reduced in CD14-KO BM-DCs (**Fig 5C**). The response was also strongly reduced in TLR4-KO BM-DCs, thereby confirming our previous data [21]. The addition of polymyxin B to the cultures (**Fig 5A and 5B and S2B Fig**) had only limited effect on the TNFα-response. Taken together, these data indicate that the S100A9-induced TNFα-response is both TLR4- and CD14-dependent.

While the TNFα-response was CD14-dependent, we could still detect S100A9 binding to the plasma membrane of both CD14-KO BM-DCs (**Fig 5D**) and TLR4-KO BM-DCs that had been pre-incubated with the CD14 blocking antibody (**S2C Fig**). These data suggest that there are also other S100A9-binding membrane molecule(s) except CD14 and TLR4 on these cells. However, there was no internalization of S100A9 neither in the CD14-KO BM-DCs nor in cells BM-DCs exposed to the blocking anti-CD14 antibody. Taken together, these data indicate that CD14 is an essential co-receptor in the S100A9-induced cytokine response and suggest that CD14 may also be essential for S100A9-internalization.

SPR analysis was used to investigate whether human CD14 could directly interact with human S100A9. For this purpose, human S100A8 and S100A9 were immobilized to the same level on a chip and CD14 was passed over these surfaces. **Fig 6** shows sensorgrams obtained after

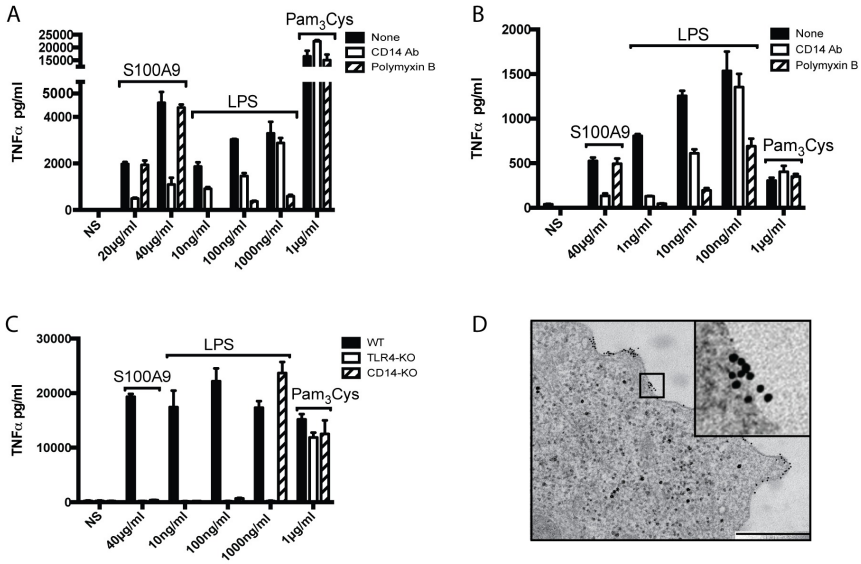

Fig 5. CD14 is essential for the S100A9-induced cytokine response. (A,B) Blocking of CD14 inhibits S100A9-induced cytokine response. (A) THP-1 cells ($1x10^6$/ml) or (B) peritoneal wash cells ($1x10^6$/ml) were pre-incubated with (A) mouse-anti-human CD14 blocking antibody (5μg/ml), (B) rat-anti-mouse CD14 blocking antibody (10μg/ml), (A,B) polymyxin B (50μg/ml) or medium (NS) for 30min, as indicated. The cells were subsequently cultured in medium (NS) or stimulated with indicated concentration of S100A9 protein, LPS or TLR2-stimulator Pam_3Cys (1μg/ml); cells were simulated with 40μg/ml S100A9 in (B). Supernatants were harvested after (A) 4hrs or (B) 24 hrs of culture and TNFα concentration determined using CBA assay. Results from one representative experiment out of 3 (A) and 2 (B) independent experiments are shown. (C) BM-DCs from indicated mouse strains were stimulated with 40μg/ml moS100A9, or with LPS or Pam_3Cys for 24 hrs and culture supernatants analyzed as in (A,B). Results are representative of two experiments. (D) CD14-KO DCs were incubated with gold-labeled S100A9 (10nm grains) and analyzed as in Fig 2. Bar: 500nm.

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injection of 50 to 800 nM CD14 over S100A9 (Fig 6A) or S1008 (Fig 6B). As is shown in Fig 6C, CD14 demonstrated satiable binding only to S100A9 with an affinity of 0.1 to 0.2 μM calculated after kinetic analysis of sensorgrams using a 1:1 model, whereas binding of CD14 to S100A8 was low and non-satiable in the concentration range used. Taken together, these data support the hypothesis that CD14 is an essential co-receptor for S100A9-induced TLR4-stimulation.

Discussion

In this report we have investigated the mechanism of S100A9-induced TLR4 stimulation. We show by TEM analysis that the S100A9 protein, which is capable of inducing a TLR4-dependent TNFα-response in monocytes, displays focal binding to the plasma membrane of such cells. Previous studies from other laboratories had shown that the cav-1 protein is expressed in THP-1 cells [49, 50]. Analysis of plasma membrane from THP-1 cells induced to differentiate to macrophages, revealed that cav-1 associated with detergent resistant membrane domains i.e. lipid rafts [51]. Lipid rafts are membrane subdomains involved in signaling (reviewed in [52, 53]) that can be found in caveolae and are known to be involved in TLR4-stimulation (reviewed in [54]). Such cav-1-associated membrane subdomains in monocytoid cells thus most probably represent caveolae (reviewed in [46, 55]).

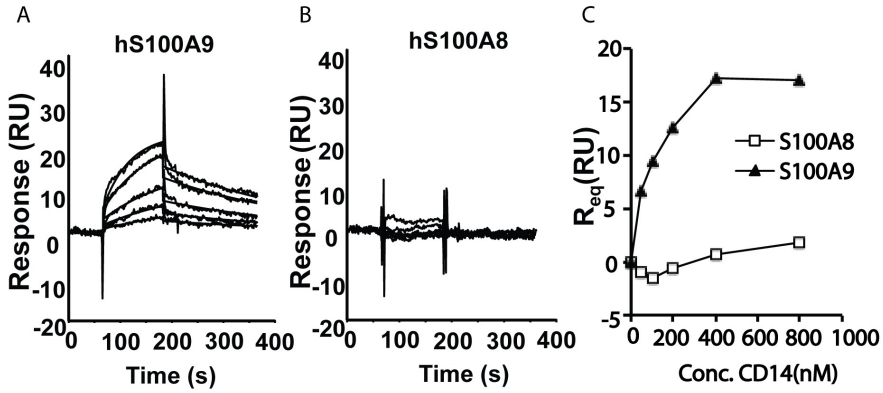

Fig 6. Interaction of CD14 with immobilized S100A8 and S100A9 using SPR analysis. Sensorgrams from bottom to top were obtained after injection of 50 to 800 nM CD14 over S100A9 (A) or S100A8 (B). Responses at steady-state (R_{eq}) were calculated either by fit of sensorgrams to a 1:1 model (S100A9) or at late association phase (t 175 s) due to too fast kinetics (S100A8) and plotted vs CD14 concentration in C. A K_D of 0.1 to 0.2 μM was calculated for CD14 binding to S100A9 whereas non-satiable binding was obtained to S100A8. The data shows representative results from one out of two experiments performed.

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We speculated that the focal binding of the S100A9 protein might represent binding to membrane subdomains. It is known that TLR4 is recruited into lipid rafts upon stimulation of monocytoid cells with LPS [43] and that recruitment is reactive oxygen species dependent [56]. We could show that the sites of focal S100A9-binding on THP-1 cells coincided with focal expression of both TLR4 and the cav-1 protein. These results are consistent with a model according to which the stimulation of cells with the S100A9 protein via TLR4 would induce the recruitment of both TLR4 and S100A9 into lipid raft/caveolar membrane subdomains. The lack of focal S100A9-binding in MβCD-treated THP-1 cells was consistent with this model. In contrast, other investigators have reported that THP-1 cells lack caveolae-like membrane structures and have proposed that the TLR4-ligand LPS would be internalized by macropinocytosis [57, 58]. In these reports however, cav-1 expression was not investigated and these structures may therefore not have been detectable. We show in here that the S100A9 protein also binds to the plasma membrane of cav-1-KO BM-DCs and the protein could be detected in cytosolic vesicles of such cells. These data suggest that caveolae are neither essential for the binding nor for the internalization of the S100A9 protein. We speculate that the binding seen in these cells might reflect binding to S100A9 receptors located in lipid rafts. That hypothesis would be consistent with the only occasional, non-focal membrane binding detected in MβCD-treated cells.

Further, TLR4 is important for the LPS-induced inflammatory response but not for the internalization of LPS [59, 60]. In consistency with those findings, we previously showed [21] and confirmed in here that TLR4 is essential for the S100A9-induced TNFα-response in BM-DCs. In addition, we could also detect focal S100A9-binding to the plasma membrane of TLR4-KO BM-DCs and internalization of the protein in these cells. Previous studies in this field have shown that CD14 acts as a co-receptor during LPS-induced TLR4 stimulation [34]. Upon stimulation, CD14 plays important roles both for the recruitment of the TLR4/MD2/LPS complex into lipid raft membrane subdomains [43, 56] and for the internalization of that complex through endocytosis [33, 61, 62]. However, neither TLR4-signaling [33] nor the carboxy-terminal tail of the protein [62] is needed for the internalization. After internalization, the

TLR4/MD2/CD14 complex can subsequently be detected in the early endosomal compartment defined by the EEA1 and Rab5 markers [25, 41]. The Rab7b [63] and Rab11a proteins [64] regulate the further cytosolic sorting of TLR4/CD14.

In analogy with these previous findings we show herein that the focal S100A9-binding detected both on the plasma membrane and in cytosolic vesicles of TLR4-KO cells, co-localized with CD14 expression. Further, we show that the S100A9 co-localized with Rab5 in such vesicles and there was no detectable internalization of S100A9 into CD14-KO cells. Thus, similarly to LPS, the internalization of S100A9 is CD14-dependent. The TLR4/MD2/CD14 complex is known to recycle from the plasma membrane to Golgi apparatus [35, 65]. While this mechanism is not essential for TLR4 signaling, LPS was shown to follow that route of recirculation upon stimulation of TLR4 [35, 66]. In our experiments we could also detect gold-labeled huS100A9 in association with the Golgi apparatus and also with rough endoplasmic reticulum in the cytosol of THP-1 cells (**S2D Fig**). The finding of S100A9 association with the Golgi apparatus suggests that also in this respect S100A9-mediated TLR4-stimulation may follow the same general pathway as stimulation by LPS.

Most importantly, we could show that the co-localization of CD14 and S100A9 proteins is functionally relevant. Thus, the S100A9-induced cytokine response was clearly CD14-dependent as it was eliminated in CD14-KO BM-DCs. That finding was further supported by the experiments showing that CD14 antibodies blocking LPS-induced TLR4 stimulation, could also block S100A9-induced TLR4-stimulation in human THP-1 cells. These data provided functional data strongly supporting the hypothesis that CD14 also acts as a co-receptor in the S100A9-induced response. Further, we could detect specific binding of S100A9 to CD14 in SPR analyses, but failed to detect specific binding of huS100A8 to CD14. Our previous paper showed that huS100A8 binds less well than huS100A9 to TLR4 [24]. Taken together, these results indicate that CD14 is a co-receptor for the S100A9-induced stimulation of TLR4.

Unexpectedly, we could detect binding of S100A9 to the surface of CD14-KO BM-DCs, indicating that these cells express other S100A9-binding receptors as well. The BM-DCs were generated by culturing BM cells in the presence of GM-CSF. BM-DCs most probably originate from monocytes and these cells express lower level of cell surface CD14 than macrophages [33, 67]. The integrin CD11b, also known as complement receptor 3 (CR3), has been shown to facilitate the uptake of LPS in BM-DCs and myeloid DCs [67]. Further, several other membrane proteins CD85j [68], CD147 [69] and CD33 [70] have been shown to be receptors for S100A9. At present we do not know the nature of the S100A9-binding detected on the CD14-KO BM-DCs, but these previously described receptors are potential candidates since they are all expressed in monocytoid cells. However, deletion of either TLR4 or CD14 was sufficient to completely inhibit the S100A9-induced cytokine response, defining these as essential receptors of that response.

While several previous studies [33, 61, 62] have shown that the CD14 protein is essential for LPS-induced internalization of TLR4, a recent study provided evidence indicating that both an agonistic TLR4/MD2 specific antibody and a small synthetic TLR4 ligand could induce CD14-independent internalization and endosomal TLR4 signaling [71]. Thus, at least some ligands can cause internalization of TLR4/MD2 through a CD14-independent pathway. Upon ligand-binding MD2 was shown to promote the dimerization and internalization TLR4/MD2 [62, 72]. Both the TLR4/MD2-specific antibody and the synthetic TLR4-ligand used by Rajaiah et al [71] could potentially cause dimerization of TLR4/MD2 and thereby induce the CD14-independent internalization. As shown here, however, the stimulation of BM-DCs with S100A9 is both TLR4- and CD14-dependent. In addition, we did not detect internalization of the S100A9 protein in CD14-KO cells, suggesting that S100A9, similarly to LPS, may induce CD14-dependent internalization of TLR4/MD2.

We used recombinant huS100A9 produced in E. coli bacteria in our experiments and it was important to take precautions to avoid the involvement of LPS [73] in the cytokine response induced upon stimulating monocytes with the protein. Thus, LPS contaminants were removed by affinity chromatography during S100A9 protein preparation and addition of polymyxin B to stimulation cultures could confirm that induced cytokine responses were largely insensitive to this compound. Additionally, the CD14 blockade with specific antibodies strongly reduced the S100A9-induced cytokine response, while it did not affect the response induced by the TLR2-agonist Pam_3Cys, indicating that bacterial TLR2 stimulators do not contaminate the protein either.

Taken together, we show herein that CD14 is an essential co-receptor of the S100A9-induced cytokine response in monocytoid cells. Our findings further indicate that while CD14 can bind S100A9 and may be essential for the endocytosis of the S100A9 protein, there are other putative S100A9 receptors present on the surface of such cells. The identity of these putative receptors is currently unknown.

Supporting Information

S1 Fig. (A) S100A9 co-localizes with TLR4 in cytosolic vesicles. THP-1 cells were prepared as in Fig 2B. (B) Vesicular co-localization of S100A9 and cav-1 in TLR4-KO BM-DCs. Same specimen as in Fig 4B.
(TIF)

S2 Fig. (A) BM-DCs from TLR4-KO mice were incubated with gold-labeled S100A9 (10nm grains) as in Fig 2. The specimen was thereafter immuno-stained with rat anti-mouse CD14 antibody, followed by gold-labeled (25nm grains) secondary antibody. The image shows representative sites of surface and vesicular binding of the S100A9-protein and CD14-expression. Bar: 500nm. (B) Parallel cultures stimulated as those in Fig 5C were exposed to polymyxin B and the TNFα-response analyzed. (C) BM-DCs from TLR4-KO mice pre-incubated with anti-CD14 antibodies as in Fig 5B and subsequently incubated with gold-labeled S100A9 (10nm grains) as in Fig 2. The image shows representative sites of surface binding of the S100A9-protein. Bar: 500nm. (D) Co-localization of S100A9 with Golgi apparatus (lower right quadrant) and rough ER (upper right quadrant) in THP-1 cells. Specimen was prepared as in Fig 2A. Bar: 500nm
(TIF)

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Author Contributions

Conceived and designed the experiments: FI TL MR PB MM. Performed the experiments: ZH MR PB MM. Analyzed the data: FI TL MR PB ZH MM KS. Contributed reagents/materials/analysis tools: KS MM. Wrote the paper: FI ZH MR TL KS PB MM.

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