Analysis of the functional roles of Mammary Serum Amyloid A3 protein

MEMÒRIA PRESENTADA PER ANNA DOMÈNECH GUITART

DIRIGIDA PER ANNA ARÍS GIRALT I ALÍCIA SERRANO GONZÀLEZ

TUTORITZADA PER NOEL XAMENA LÓPEZ

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT DE GENÈTICA
DEL DEPARTAMENT DE GENÈTICA I MICROBIOLOGIA

FACULTAT DE BIOCIÈNCIES

BELLATERRA, JUNY 2013

Programa de doctorat en Genètica

Analysis of the functional roles of Mammary Serum Amyloid A3 protein

Tesi doctoral

2013

Departament de Genètica i Microbiologia

Facultat de Biociències

Universitat Autònoma de Barcelona

Unitat de Remugants

Institut de Recerca i Tecnologia Agroalimentària

Memòria presentada per **Anna Domènech Guitart**, per optar al grau de Doctor per la Universitat Autònoma de Barcelona

Anna Domènech Guitart

Amb el vist-i-plau de la directora de la tesi i del tutor

Dra. Alícia Serrano Gonzàlez

Directora

Dr. Noel Xamena López

Tutor

La Serum Amiloide A3 (M-SAA3) mamària és una proteïna de fase aguda expressada principalment a la glàndula mamària. Els nivells d'expressió de la M-SAA3 varia en diferents situacions fisiològiques, el que suggereix un rol important a nivell funcional. Per tal d'analitzar les propietats de la proteïna, es van dur a terme quatre estudis. En el primer, la M-SAA3 va ser expressada de forma recombinant en un sistema bacterià. Aquest pas és important ja que ens proporciona una font de proteïna, en casos en que la purificació de fonts naturals representa clarament un coll d'ampolla. Es va obtenir la seqüència de dues isoformes, però només una va ser expressada recombinantment. La principal diferència entre les dues formes era una deleció en la regió del motiu SNARE, el que suggeria que aquest motiu pot estar implicat en una activitat antibacteriana directe. A més, en el primer estudi es va analitzar la primera funcionalitat. La M-SAA3 activava la fagocitosi mediada per macròfags, incrementant el nombre de macròfags actiu i la seva capacitat fagocítica. En el segon estudi es va analitzar l'efecte protector a nivell gastrointestinal. La M-SAA3 clarament reduïa la translocació de bacteris enteropatògens en cèl·lules CaCo-2, una línia d'epiteli intestinal comercial. La M-SAA3 també afectava la expressió de MUC3 i IL-8, incrementant els seus nivells, el que connecta de forma directa la proteïna amb la resposta immune innata. En el tercer estudi, es va desenvolupar un model intestinal en boví a partir de cultius *ex vivo* de Plaques de Peyer. Aquests cultius *ex vivo* ofereixen un ambient únic on coexisteixen diferents tipus cel·lulars i una aproximació més realista a la situació *in vivo*. En aquest context la infecció també va ser disminuïda i la M-SAA3 incrementava els nivells de IL-8 i INFγ. Per contra, les mucines no es van veure afectades, i la protecció va ser assolida per sobre-expressió de Occludina i Claudina-2, proteïnes que formen les tight junctions, encarregades de segellar la barrera epitelial. A més, es va demostrar que la M-SAA3 activa cèl·lules dendrítiques, incrementant l'expressió de citoquines i marcadors de maduració, migració i presentació d'antigen. En el quart i últim estudi, es va avaluar la possible aplicabilitat a nivell de la industria lletera. La M-SAA3 va ser infosa intra-mamàriament durant el secat, un període on es deixa de munyir les vaques per tal d'afavorir la regeneració cel·lular i augmentar la seva productivitat. La M-SAA3 va incrementar paràmetres relacionats amb una activació de l'involució tals com les metaloproteinases. Els nivells de proteïna i greix també eren augmentats i es va observar un augment numèric del recompte de cèl·lules somàtiques. També es va observar que la proteïna incrementava l'expressió de IL-8 i TNFα en cultius primaris de glàndula mamària, i també inhibia la infecció bacteriana. Finalment, les cèl·lules dendrítiques també eren activades en absència d'infecció.

Mammary Serum Amyloid A3 (M-SAA3) is an acute phase protein mainly expressed in the mammary gland. The levels of the protein vary in different physiological situations, indicating that may play an important functional role. In order to analyze the protein properties four studies were performed. In the first study, the protein was recombinantly produced in a bacterial expression system. This was important, as difficulty in protein purification from natural sources is a clear bottleneck for functional studies. Two M-SAA3 isoforms were obtained, but only one succeeded in the recombinant expression. Interestingly, the main difference was in a 3 amino acid deletion in the SNARE motif, which could be implicated in the direct bacterial killing. Moreover, the first functional role was evaluated. M-SAA3 clearly enhanced macrophages phagocytosis, increasing both the number of active macrophages and the phagocytic capacity. In the second study, the protective effect at a gastrointestinal level was assessed. M-SAA3 protein inhibited the translocation of enteropathogenic bacteria in CaCo-2 cells, a commercial intestinal epithelial cell line. In addition, M-SAA3 protein increased the expression of MUC3 and IL-8, which directly connected the protein with the innate immune response activation. In the third study, a bovine intestinal model was developed using *ex vivo* Peyer's Patches cultures. The *ex vivo* methodology offered a unique environment where different cell types coexist, and indeed, represent a more similar approach to an *in vivo* situation. In this context, the infection was also prevented, and a clear innate immune response was activated. M-SAA3 clearly activated the expression of IL-8, INFγ but in this case, mucins were not up-regulated. The bacterial translocation was achieved by an increase in the Occludin and Claudin-2 expression, tight junction genes that directly participate in the sealing of the epithelial barrier. Furthermore, the M-SAA3 directly activated dendritic cells functions, increasing their cytokine expression profile and cellular markers related to maturation, migration and antigen presentation. In the fourth and last study, the potential applicability in dairy industry was evaluated. M-SAA3 was infused in the mammary gland at dry off, a period of milking cessation which permits the mammary gland regeneration for an optimal production in following lactations. M-SAA3 increased parameters related to an increased involution of the mammary gland, such as metalloproteinases. Also protein and fat were increased and a numerical increase in the somatic cell count was observed. In addition, M-SAA3 raised the IL-8 and TNFα levels in primary mammary gland cultures, and inhibited bacterial infection. Finally, dendritic cells were also activated by M-SAA3 in absence of infection.

List of abbreviations

A/E	Attaching and effacing E. coli
ACTB	β-actin
AI	Association index
APC	Antigen presenting cell
APP	Acute phase protein
APR	Acute phase response
A-SAA	Acute phase SAA
At	Annealing temperature
BSA	Bovine serum albumin
CCR7	C-C chemokine receptor type 7
CD80	Cluster of differentiaton 80
CFU	Colony forming units
CRP	C-reactive protein
DC	Dendritic cells
EAEC	Enteroaggregative *E. coli*
ECM	Extracellular matrix
EHEC	Enterohemorragic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FCS	Fetal calf serum
FPLR1	Formyl peptide receptor like-1
GALT	Gut associated lymphoid tissue
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDL	High density lipoproteins
IEC	Intestinal epithelial cells
IL-1	Interleukin-1
IL-12	Interleukin-12
IL-23	Interleukin-23
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IS	Insoluble fraction
INFγ	Interferon gamma

iNOS	Nitric oxide synthase inducible
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani media
LCAT	Lecithin:cholesterol acyl transferase
LEE	Locus of nterocyte effacement
LPS	Lipopolysaccharide
LRR	Leucin repeats rich
LTA	Lipoteichoic acid
MHCI	Major histocompatibility complex I
MHCII	Major histocompatibility complex II
MMP	Matrix metalloproteinases
MR	Manose receptors
M-SAA3	Mammary SAA3
MUC2	Mucin 2
MUC3	Mucin 3
nCEH	Neutral cholesterol ester hydrolase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NI	Non-infected
NK cells	Natural Killer cells
O/N	Over-night
OmpA	Outer membrane protein A
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Saline Buffered
PFA	Paraformaldeihid
PI	Phagocytic index
PP	Peyer's Patches
PRR	Pattern recognition receptors
qPCR	Quantitative real time PCR
RPMI	Roswell park Memorial Institute medium
RT	Room temperature
SAA	Serum amyloid A
SCC	Somatic cell counts
SDS-PAGE	Sodium dodecyl sulfate polyacrilamide gel electrophoresis
SF	Soluble fraction
SN	Supernatant
SPI	Salmonella pathogenicity islands
sPLA2	Secretory phospholipase A2

SR	Scavenger receptors
SR-BI	Scavenger receptor class B Type I
STEC	Shiga-toxin producing E. coli
T3SS	Type III secretion system
TH-cells	T helper cells
TLR	Toll like receptors
TNFα	Tumor necrosis factor alpha

Table of contents

Chapter I

Literature review

Block I Innate immune response

1. Immune response: brief overview

The host defense is a precise system which integrates combined processes in order to fight against infection and other injuries. Two main phases are involved in the elimination of potential invading entities, the innate and the adaptive immune responses, which efficiently differentiate self from non-self. The **adaptive immune response** attacks specifically the microorganisms, by generating a repertoire of antigen receptors that finely discriminate between similar molecules and allows the precise destruction of the pathogens (Murphy, 2011). Nonetheless, in healthy conditions, only a few part of microbial challenges produce disease as they are destroyed before the activation of the adaptive immune response. In this context, **innate immunity,** also known as non-specific immune system, comprises the cells and mechanisms that defend the host from infection in a non-specific way, acting rapidly after a microbial challenge and also enhancing the adaptive immune response when necessary. Each pathogen has its own characteristic features such as transmission and replication modes, pathogenicity and response produced in the host. This wide variety challenges the immune system to develop a broad range of different responses. (Murphy, 2011; Mak and Saunders, 2004).

2. Innate immunity

The innate immune response represents the first line of defense during the initial hours post infection and it is composed by cellular and soluble components. The *immediate innate immunity is* responsible during the first four hours post microbial challenge and comprises different anatomical and physiological components that are present in the organism. If the infectious agent is not removed, the *early induced innate response* acts during the following four days. The induced response is composed by innate immune cells that recognize and discriminate host cells from invading agents. After the recognition, the cells become active and start a series of effectors mechanisms in order to eliminate the infection (Murphy, 2011; Mak and Saunders, 2004).

Innate immunity offers an important protection, controlling most of the potential pathogens in healthy individuals. However, innate immunity elicits a short-lasting protection and when overwhelmed it is necessary to activate the adaptive immune response, which encompass a long-lasting fight and also confers memory to efficiently counteract future infections. Even in this situation, innate immunity plays an essential role containing and delaying the infection in

the extent that the adaptive immunity becomes fully active (Murphy, 2011; Mak and Saunders, 2004).

2.1. Immediate innate immune response, anatomical and physiological barriers

Microorganisms are continually in contact with our bodies, through either external or internal surfaces. Skin and mucous membranes protect, respectively, the exterior and interior from environmental challenges. Pathogens must cross these anatomical barriers to gain access to the body. The skin is a large organ, composed of three layers: epidermis, dermis and hypodermis. Epidermis is a layer of **epithelial tissue** with a great resistance to infections, due to characteristic features such as a high resistance to water of the keratinized epidermic cells, a lack of blood vessels and a rapid cell turnover. Moreover, epithelial cells are efficiently sealed by tight junctions that separate and protect from the external media. The mucosal membranes overlay gastrointestinal, respiratory and urogenital tracts and permit the air and food passage (Murphy, 2011; Mak and Saunders, 2004).

There are other physiological barriers that help to contain the infection. Mucosal membranes are surrounded by a layer of **mucus**, which is a viscous fluid with glycoproteins named mucins. Bacteria and other microorganisms are trapped into this fluid and the infection may be prevented or retarded. Also, peristalsis in the gastrointestinal tract helps the movement of the gut content and the release of bacteria. In addition, the gut is colonized by non-pathogenic bacteria known as **commensal bacteria**, which form the natural flora and help in the fight against infectious organisms competing for particular microorganisms binding sites or nutrient supplies. Furthermore, there are several kinds of direct **antimicrobial proteins and peptides** secreted by either the epithelial cells or other immune mediators. Lysozyme attacks peptidoglycan, the main component of the bacterial walls. Defensins, cathelicidins and histatins are short cationic peptides that disrupt bacterial membranes (Murphy, 2011; Mak and Saunders, 2004).

After breaking these initial components of the innate immunity, pathogens encounter the complement system, which are soluble proteins present in the blood that directly kill bacteria by cell membrane attack complexes and increase opsonization. **Opsonization** is the process by which bacterial cells are coated with antibodies or complement protein sin order to facilitate their destruction by phagocytosis (Murphy, 2011; Mak and Saunders, 2004).

2.2. Induced innate immune response

If the first defense is overwhelmed, the induced innate response acts during the first following days. Microorganisms are destroyed via **phagocytosis** mediated by specialized innate immune

response mediators, mainly referred as neutrophils and macrophages. The process implies bacterial recognition and invagination forming a large vesicle called phagosome where the microorganisms are digested.

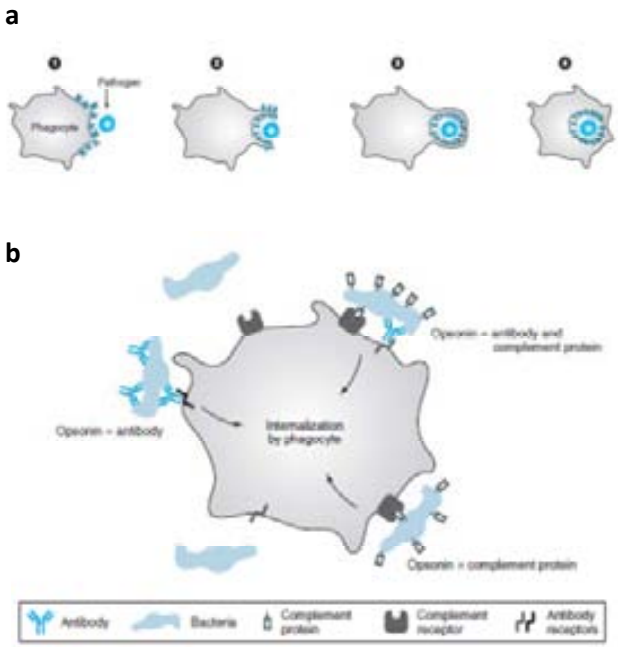


Figure 1. Phagocytosis (a) and opsonization (b) processes. a) phagocytes (mainly referred as macrophages and neutrophils) engulf bacteria for their elimination. b) antibodies, complement proteins and other molecules bind to the bacterial surface and favor bacterial phagocytosis (adapted from Mak and Saunders, 2004).

Inflammation is initiated in response to pathogen recognition or after detection of proteins released during cell damage of injured tissues. Locally, permeability of the blood vessels is increased by a series of factors such as kinins. Moreover, immune and non immune cells recognize bacteria and release **cytokines.** Cytokines are immunomodulators with a broad range of activities either autocrine, focused to the same secretor cell, or paracrine, aimed to affect adjacent cells. Cytokines are responsible for the inflammation process activating the immune response mediators and inducing the migration towards the infection site. Furthermore, they enter in the systemic circulation and promote the **acute phase response (APR)**, which involves the expression of **acute phase proteins (APP)**, mainly by the liver, increasing their concentration in systemic fluids.

3. Bacterial recognition

Innate immune cells discriminate self from non-self by **pattern recognition receptors (PRR)** which recognize conserved structures common to a broad variety of microorganisms. These structures are called **pathogen-associated molecular patterns (PAMPs)** and include cell wall molecules, DNA, RNA and microbial proteins (Mak and Saunders, 2004).

This system implies certain inflexibility in the pathogen recognition since the potential is fixed in the germ line, which produces a limited repertoire of receptor specificities. In contrast, the adaptive immune response offers a specific recognition of the pathogen that is obtained by somatic cell rearrangement, a system that is able to adapt to new bacterial challenges (Mak and Saunders, 2004).

Some of the most important PRR that participate in the pathogen recognition are Toll-like receptors (TLR), Scavenger receptors (SR), Manose receptors (MR) and NOD proteins.

3.1. Toll-like receptors

TLR are transmembrane proteins composed by an extracellular domain rich in leucine repeats (LRR) and a cytoplasmatic domain homologous to interleukin-1 (IL-1) receptor responsible for the signal transduction which ends up with the activation of the transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), one of the main signals that triggers inflammation and activation or migration of immune cells mediators (Takeda et al.., 2003). TLR are expressed in a broad range of leukocytes and other non-immune cells like intestinal epithelial cells (IEC) (Gribar et al.., 2008). TLR signaling is related to other functions apart from inflammation. In IEC they are involved in cell proliferation (Ruemmele et al., 2002) and tight junction remodeling which helps to repair the tissue after cell injury (Abreu, 2010).

So far, up to 13 members have been described. TLR2 and TLR4 are the most studied receptors of the family. TLR2 effectively recognizes lipoteichoic acid (LTA), present in Gram-positive bacteria. TLR4 binds lipopolysaccharide (LPS) from Gram-negative bacteria. Other members of the family bind other bacterial compounds, offering a wide spectrum of pathogen recognition (Takeda et al., 2003).

3.2. Scavenger receptors

Scavenger receptors encompass a broad range of molecules and mediate endocytosis of selected ligands. They are expressed mainly in macrophages and dendritic cells (DC). Three classes of SR have been identified: A, B and C. They were first described to bind polyanionic ligands, including modified low-density lipoproteins, activating the phagocytosis of apoptotic

cells. Later, they have been demonstrated to mediate bacterial recognition. Specifically, members of SR-A class bind Lipid A (a component of the LPS) and LTA (Peiser et al., 2002).

3.3. Mannose Receptors

The MR are members of the C-type lectins superfamily. They were first identified in macrophages, helping the clearance of endogenous glycoproteins. Later, they have been described to be expressed in endothelia and other cell types. The receptor is a transmembrane protein with a cytoplasmatic domain that mediates receptor internalization. The extracellular region contain three types of domains, one of them being mannose specific aimed to bind mannose ligands frequently found in the surface of many microorganisms (Gazi et al., 2008).

3.4. Nucleotide-binding oligomerization domain family (NOD proteins)

NOD-proteins are soluble sensors in the cytoplasm of the cell. NOD1 and NOD2 are members of the family and show analogous functions to the TLR. Indeed, they contain structurally related LRR domains and the transduction signal ends up with the NF-κB activation. Nevertheless, they are thought to bind structures non-recognized by TLR (Mak and Saunders, 2004).

4. Acute phase response (APR) and acute phase proteins (APP)

Bacterial infections usually involve a strong acute phase response reaction. Cytokines, produced by innate immune response phagocytes, change dramatically the systemic concentration of a broad range of proteins produced mainly in the liver. Specifically, cytokines such as tumor necrosis factor alpha (TNFα), IL-1 and interleukin-6 (IL-6) play a key role in the activation of the APR (Gruys et al., 2005). In addition, glucocorticoids also stimulate the APP expression. Two types of APP are known. A group where the proteins are diminished, known as **negative APP** and other group where the members are highly increased, called **positive APP**. Positive APP are subdivided depending on the level of their increase. Low APP rise up to 10-fold their basal levels (ceruloplasmin, fibrinogen, haptoglobin and α-globulins) meanwhile major APP are increased much more, reaching levels up to 1000-fold in determined instances (C-reactive protein (CRP) and Serum Amyloid A (SAA)). However, this is not a static classification, as the level of expression is species-specific. Table 1 shows different expression levels of the APP depending on the species (Cray et al., 2009).

Species	Major (>10-fold increase)	Moderate (<10-fold increase)
Human	CRP	α1-acid glycoprotein
	SAA	Fibrinogen
		Haptoglobin
Nonhuman primates	CRP	SAA
		Fibrinogen
		α2-macroglobulin
Pig	Haptoglobin	α1-acid glycoprotein
	SAA	
Cat	α1-acid glycoprotein	Haptoglobin
	SAA	
Dog	CRP	α1-acid glycoprotein
	SAA	Ceruloplasmin
		Haptoglobin
Mouse	Haptoglobin	CRP
	SAA	Fibrinogen
Rabbit	Haptoglobin	α1-acid glycoprotein
	SAA	CRP
		Fibrinogen
Cow	Haptoglobin	α1-acid glycoprotein
	SAA	CRP
		Fibrinogen
Goat	Haptoglobin	α1-acid glycoprotein
	SAA	CRP
		Fibrinogen

Table 1. Major and moderate acute phase proteins in different animal species. Adapted from Cray et al., 2009.

Negative APP include albumin and transferrin, and the decreased expression during the APR seem to be linked to a increased capacity of the liver to synthesize positive APP, which are the ones that directly participate in the immune response (Steel and Whitehead, 1994).

The biologic functions of the APP have not been totally elucidated but participate in a vast range of processes (Cray et al., 2009). Positive APP are mainly focused on general immune functions related to opsonization and trapping of microorganism or their products, activating complement, neutralizing enzymes and modulating host's immune response (Gruys et al., 2005).

The prolongation of the inflammatory response caused by the APPs may have detrimental effects, and therefore there is a need to accurately control the APR. Many different pathways are involved in its resolution. Glucocorticoids, which are important in the onset of the APR, offer a negative feedback which helps to the process finalization. Other inhibitory pathways involve transcriptional repressors and cytokines such as IL-1, TNFα and IL-6, which initiate a negative feedback to its own expression by inducing corticoesteroids (Jensen and Whitehead, 1998; Uhlar and Whitehead, 1999).

5. Dendritic cells: direct link among innate and adaptive immune responses

DC are **professional antigen presenting cells (APC)** with a key role in the onset of the adaptive immune response. Most of the cells of the organism are able to present antigens through the major histocompatibility complex I (MHCI), leading to the activation of the CD8+ T-cell which are cytotoxic and responsible for the killing of bacteria. On the other hand, professional APCs process and present foreign antigens through the major histocompatibility complex II (MHCII), which will be recognized by T helper cells (T_H-cells, CD4+) starting the cascade of adaptive immune response (Abbas et al., 2002). Among the different types of professional APC the DC gain interest, as they may be the only cell type able to activate naïve T cells (Bancherau and Steinman, 1998). In general, DC are divided in two families, DC resident in secondary lymphoid organs and DC in peripheral tissues that migrate to lymph nodes under inflammatory conditions. Immature DC present a high phagocytic activity and are responsible for antigen uptake. Maturation is reached, among other stimulus, by bacterial recognition, and produces a slight reduction in the phagocytic activity and enhances other features such interleukin-12 (IL-12) expression, antigen presentation and the capacity to migrate towards secondary lymphatic nodes (Macatonia et al., 1995; Bancherau and Steinman, 1998; Banchereau et al., 2000). In homeostasis, DC also play an important role related to immune tolerance, through the activation of immune regulatory mechanism which control antigens that do not cause pathology (Coombes and Powrie, 2008).

Block II Serum Amyloid A (SAA)

1. Serum amyloid A family

SAA proteins were identified in the early 1970s, when antibodies raised against the peptide responsible for inflammation associated to amyloidosis (amyloid A, or AA amyloid) reacted with a protein present in the acute-phase plasma (Kisilevsky and Manley, 2012). AA amyloidosis is characterized by deposition of fibrils formed by insoluble proteins in the extracellular space of organs and tissues and usually complicates chronic inflammatory disorders associated with a sustained acute phase response (Lachmann et al., 2007). In humans, more than 20 different proteins are able to deposit as fibrils, but SAA is the most abundant one (Upragarin et al., 2005).

SAA family comprises several apolipoproteins which are synthesized primarily in the liver and are divided in two classes depending on their responsiveness to inflammatory stimuli. **Acute-phase SAAs** (A-SAA) are highly increased during damage, infection or trauma. Their levels rise up to 1000-fold and plasma concentrations can increase from 1-5 µg/ml to 1 mg/ml (McAdam and Sipe, 1976; McAdam et al., 1978; Kindy et al., 1998; Eckersall et al., 2001). In contrast, **constitutive SAA** levels do not change during acute phase response (Uhlar and Whitehead, 1999). The constitutive form is the predominant member of the SAA family in human non-acute serum (Yamada et al., 1994; de Beer et al., 1995, 1996). During inflammation, A-SAA displace Apolipoprotein AI (Apo-AI) from the high density lipoproteins (HDL) becoming the most abundant apolipoprotein in acute serum (Coetzee et al., 1986).

The members of the SAA family differ in number depending on the species. Human and mouse species have been the most studied. Human genome is composed by four members, two A-SAAs (corresponding to SAA1 and SAA2), one pseudogene (SAA3) and one constitutive form (SAA4) (Jensen and Whitehead, 1998). In mouse, five members are observed and, in this case, the SAA3 is a normal and functional protein. Other species such as hamster, rabbit, dog, mink, cow, sheep, goat and horse have been described to contain more than one member in the family (Ulhar and Whitehead, 1999).

SAA protein sequence is highly conserved in all the family isoforms and among different species, observing high homology in a wide range of mammals, marsupials, fish, birds and invertebrates like the sea cucumber (Santiago-Cardona et al., 2003). This conservation among species along the evolution suggests an important functional role in the organism although the exact function has not been established yet (Kisilevsky and Manley, 2012). In humans, SAA1 and SAA2 proteins are the most similar forms with a 90% of homology. The constitutive human

SAA4 only shares 53-55% of amino acids compared with SAA1 and SAA2 and it contains an 8 amino acid insertion not present in the acute-phase isoforms (Uhlar and Whitehead, 1999). Human SAA3 is a pseudogene (Kluve-Beckerman et al., 1991), but mouse SAA3, shares 65% of the residues with murine SAA1 and SAA2. (Ulhar and Whitehead, 1999).

2. Gene and protein structure

The members of the family contain among 104 and 112 amino acid residues (length representing the sequences for both acute and constitutive SAA in human) with a 18 amino acid signal peptide (Uhlar and Whitehead, 1999; McDonald et al., 2001). All the genes form a cluster, localized in humans in the chromosome 11 and in mice in the chromosome 7 (Jensen and Whitehead, 1998) (Figure 1).

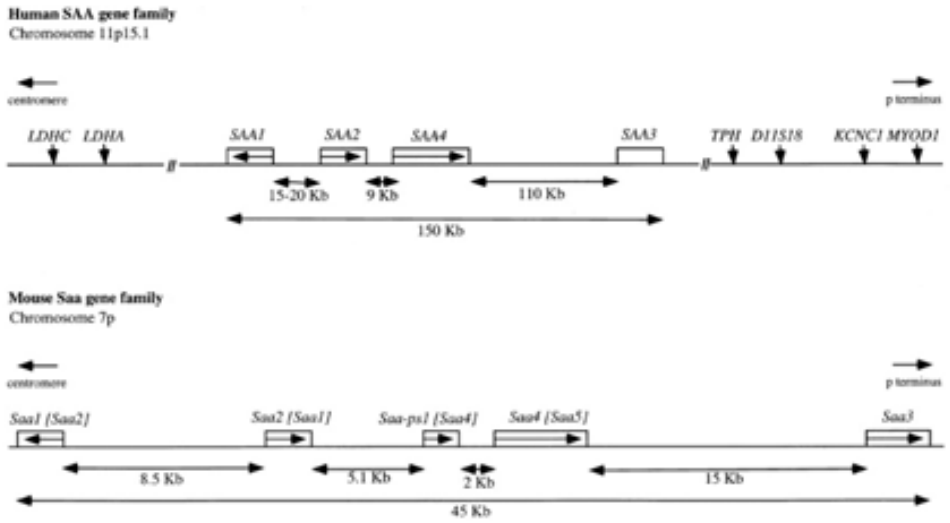


Figure 2. Human and mouse SAA gene families. All the members of the SAA family are clustered in the same region. Mouse genes are named in the new nomenclature (maintaining the old names in brackets). Human gene is finely localized between flanking genes corresponding to lactate dehydrogenase A and C (LDHA and LDHC), tryptophan hydroxylase (TPH), myogenic factor 3 (MYOD1), a member of the potassium channel family (KCNC1) and an unknown marker (D11S18). In mice, the exact position has not been described. (Adapted from Uhlar and Whitehead, 1999).

All of the genes studied so far share a structure of four exons/three introns which is characteristic of many other apolipoproteins (Uhlar and Whitehead, 1999), except the single duck SAA gene which has been described to contain three exons (Guo et al., 1996) (Figure 2).

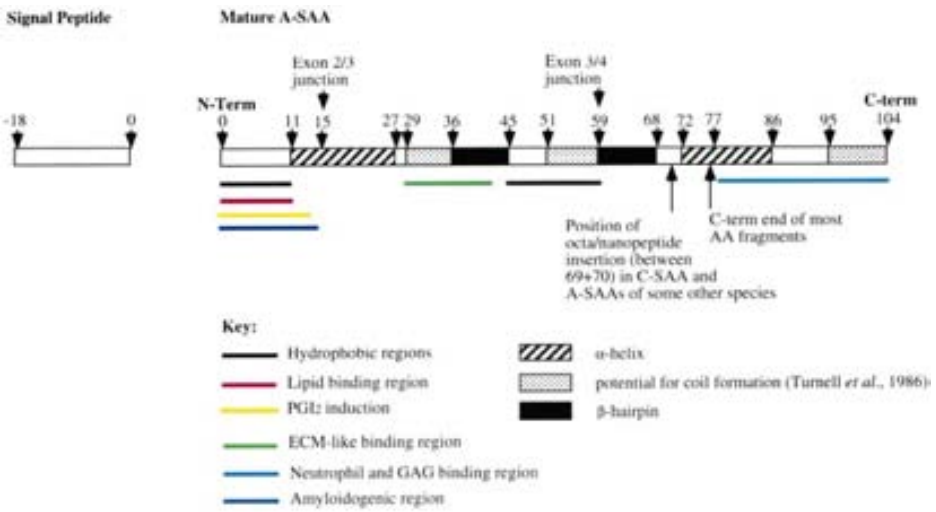


Figure 3. Structure of human A-SAA protein. The gene is organized in a 4 exon/3 introns structure. It contains a 18 amino acid signal peptide and a mature protein of 104 amino acids. Structural important regions of the protein are marked in colored lines. (Adapted from Uhlar and Whitehead, 1999).

Early work based on predictive methods suggested that A-SAA are likely to contain two regions of α-helix and other β-sheet regions (Ulhar and Whitehead, 1999). Further analysis suggested that SAA might consist between 33-44% helix (Meeker and Sack, 1998). Little is known about the structure of SAA beyond the primary and secondary structure. *Stevens* described homology between SAA and the N-terminal domain of hemocyanins, a copper-based oxygen transport protein in arthropods, suggesting that the helical content may compose around the 80% of the molecule. Further studies allowed the description of a putative model for the human SAA (Stevens, 2004) (Figure 3).

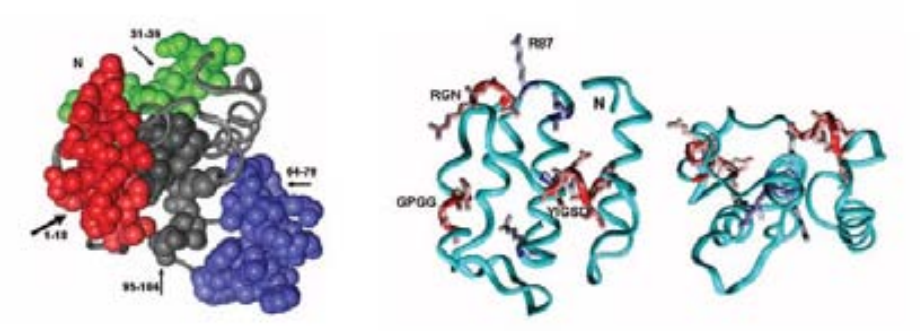


Figure 4. Putative model for the human A-SAA protein based on homology to the N-terminal domain of hemocyanin. Lateral view (left and center) and axial view (right). Important functional regions are highlighted: fibronectin (RGN), laminin (YIGSD), calcium (GPGG) and heparin/heparin sulfate (R87) binding sites. The cholesterol binding site at the N-terminus of the protein is indicated with a heavy arrow. The axial view indicates that the protein and calcium binding sites are located on the same face of the molecule (Adapted from Stevens, 2004).

Interesting functional regions are found in the SAA molecule (Turnell et al., 1986; Preciado-Patt et al., 1994; Liang et al., 1996; Sipe, 2000; Stevens, 2004).

- Cholesterol and HDL binding (residues 1-18 and 40-63)
- GPGG region for calcium binding (residues 48-51)
- YIGSR-like and RGD-like regions, cell binding domains of laminin and fibronectin, respectively (residues 29-42)
- Heparin/heparan sulfate (residues 74-104)

3. Serum amyloid A3 (SAA3)

The APP are mainly synthesized in the liver and during inflammation and acute response. It has been estimated that 2.5-3% of the protein synthesized in the liver corresponds to SAA members (Morrow et al., 1981). If the injure does not persist, levels fall to normal concentration after 7-10 days. Furthermore, it has been described in several species a widespread expression of SAA in many other tissues. This is an interesting feature, since extra-hepatic locally produced SAA may be involved in conditions that do not produce a systemic acute-phase response, playing a role focused to the site of expression (Urieli-Shoval et al., 1998).

It has been accepted that SAA1 and SAA2 are predominantly synthesized in the liver while **SAA3 is considered to be the main extrahepatic isoform** (Uhlar and Whitehead, 1999). In mice (see Table 2), SAA3 is produced in a wide range of tissues and cell types but SAA1 has only been documented in kidney and SAA2 in intestine (Meek and Benditt, 1986; Meek et al., 1989; Ramadori et al., 1985; Benditt and Meek, 1989).

In rats, SAA3 mRNA but not SAA1/2, has been found extrahepatically following LPS challenge in lung, ileum and large intestine (Meek and Benditt, 1989). However in another rodent such as hamster, the three A-SAAs have been found in a wide range of tissues (Webb et al., 1989; Hardardottir et al., 1997). In rabbits, only SAA3 mRNA has been found in a wide range of extrahepatic tissues and cell types (Rygg et al.; 1993; Marhaug et al., 1997).

Tissues	SAA isoform	Reference
Adipocytes	SAA3	Benditt and Meek (1989)
Adrenal gland	SAA1, SAA2, SAA3	Meek and Benditt (1986)
Brain	SAA3	Meek and Benditt (1986)
Pituitary gland	SAA3	Meek and Benditt (1986)
Heart	SAA3	Ramadori et al. (1985); Meek and Benditt (1986)
Stomach	SAA1, SAA3	Meek and Benditt (1986)
Small intestine	SAA2 (weak), SAA3	Ramadori et al. (1985); Meek and Benditt (1986)
Duodenum	SAA3	Meek and Benditt (1986)
Jejunum	SAA2 (weak), SAA3	Meek and Benditt (1986)
Ileum	SAA2, SAA3	Meek and Benditt (1986)
Large intestine	SAA1, SAA3	Meek and Benditt (1986)
Epithelial lining the mucosa of the ileum and large intestine	SAA2	Meek et al. (1989)
Kidney (ephitelial lining of the tubules)	SAA1, SAA2, SAA3	Ramadori et al. (1985); Meek and Benditt (1986), Meek et al. (1989); Marhaug et al. (1997)
Lung	SAA1, SAA2, SAA3	Ramadori et al. (1985); Meek and Benditt (1986)
Kupfer cells	SAA3	Benditt and Meek (1989)
Macrophages	SAA3	Ramadori et al. (1985); Meek and Benditt (1986); Rokita et al. (1987)
Pancreas	SAA3	Meek and Benditt (1986)
Skeletal muscule	SAA3	Meek and Benditt (1986)
Spleen	SAA3	Ramadori et al. (1985); Meek and Benditt (1986), Meek et al. (1989)
Testes	SAA3	Meek and Benditt (1986)

Table 2. Extrahepatic sites of SAA mRNA expression in mice after an LPS stimulus (Adapted from Upragarin et al., 2005).

In humans, extrahepatic expression of A-SAA has been documented in a wide variety of tissues such as breast, intestine, stomach esophagus, pancreas, prostate, lung, skin, brain, kidney spleen and liver principally localized into the epithelial components of these tissues (Urieli-Shoval et al., 1998). In this case, though, the A-SAA expression is restricted to SAA1 and SAA2. The SAA3 isoform was considered to be non-expressed since no mRNA was found for long time (Kluve-Beckerman et al., 1991; Urieli-Shoval et al., 1998). More recently, mRNA expression has been described in mammary epithelial cell lines in response to prolactin and LPS (Larson et al., 2003). *Larson et al.*, cloned the mammary SAA3 cDNA, which contained an open reading frame that would hypothetically translate into a 60 amino acid precursor protein containing an 18 residue signal peptide typically found in other SAA proteins. The final mature protein would be 42 amino acids of length and would be considered a pseudogene because of its abnormal

functionality. The sequence alignment of the predicted human SAA3 protein with other known SAA3 (rabbit, hamster, bovine, and mouse), showed a great amino acid identity (94%) for first 48 residues (Larson et al., 2003).

In cow, horse and sheep, high concentrations of SAA3 were found in colostrum decreasing at 4 days post-parturition (McDonald et al., 2001). In this context, the protein is widely known as **mammary SAA3 (M-SAA3)**. The fact that high levels of SAA3 in milk have been detected in healthy animals indicates that SAA3 also participates in non-pathological states and is probably related to the well-being of the mammary gland.

M-SAA3 expression in mammary gland depends on the physiological state. Mammary gland in non-pregnant animals produces low levels of protein meanwhile in pregnancy the levels of M-SAA3 increase, being dramatically high after calving (McDonald et al., 2001; Molenaar et al., 2009). In commercial farms, milk production is maximized when dairy cows are pregnant during approximately 70% of the lactation period. Between lactations and during late gestation it is necessary a **dry period** (no-milking period), taking place a **mammary gland involution and regeneration** processes where the senescent epithelial cells are renewed to ensure an optimum milk production in subsequent lactations (Capuco et al., 1997; De Vries, 2010). Because of the hormonal levels resulting from pregnancy, involution of the mammary gland is slower than in other species where involution does not coexists with pregnancy (Noble et al.., 1999; Pezeshki et al., 2009). During this dry period, there is an increase of the SAA expression observing a dramatic rise at 76 hours post last milking, which suggest that M-SAA3 is also induced in non-lactation phases of pregnancy (Molenaar et al., 2009). This result indicates that M-SAA3 is expressed in mammary gland in a temporally restricted manner, similar to other proteins implicated in mammary defense such as lactoferrin, and supports the idea that M-SAA3 has important roles in the defense and well-being of the mammary gland.

In pathological events such as mastitis on dairy cows, mammary gland increases the M-SAA3 expression and their levels are highly increased in milk. In non-mastitic milk the levels of M-SAA3 are low (depending on the lactating moment), around 0.2-0.54 μg/ml. Naturally occurring infections rise the M-SAA3 levels either in mild (presence of clots in the milk) or moderate (clots and observable inflammation such as heat, pain, redness or swelling) mastitis. Levels of M-SAA3 in mild mastitis are 2.6 μg/ml (range 0.2-32 μg/ml) and in moderate mastitis, 20.6 μg/ml (range 1.2-95 μg/ml) (Eckersall et al., 2001). Other studies described levels above 100 μg/ml during subclinical mastitis by *S. aureus* (Kovacevic-Filipovic et al., 2012; Molenaar et al., 2009). The evident increase during pathological conditions also suggests that M-SAA3 participate actively in the mammary gland defense against pathogens and it can be used as

indicator of infection in mammary gland, gaining interest the potential for evaluating subclinical infections which are difficult to diagnose in normal circumstances (Eckersall et al., 2010; Kovacevic-Filipovic et al., 2012).

4. A-SAAs functionality

The SAA family has been related to a wide variety of different functions. After their discovery, the lipid metabolism and transport were the main area studied. Thereafter, a great interest in the immunologic related properties has arisen.

4.1. SAA, lipid related functions and cholesterol metabolism

In homeostasis, phagocytes eliminate senescent and dying cells. During the acute inflammatory response there is an increase of cells related to inflammation and tissue repair. At the site of injury, macrophages ingest and degrade dead or dying cells and other debris. In this process there is a recycling of their constituents, importantly membrane cholesterol and phospholipids (Manley et al., 2005). Cholesterol is essential for growth and cell viability, as it acts as an essential membrane component participating in its permeability and fluidity (Yawate, 2003). Moreover, cholesterol is a precursor for the biosynthesis of steroid hormones and bile acids (Pollard et al., 2007). However, too much cholesterol in cells can be detrimental to membranes and cause cytotoxicity (Oram, 1996). A major function of HDL is the cholesterol transport from peripheral cells to the liver for its catabolism and excretion from the body, a process known as reverse cholesterol transport (Oram, 1996).

In acute inflammation, SAA binds more than 80% of the HDL molecules (Coetzee et al., 1986). Due to this association, specific roles for the SAA-HDL complex have been postulated during inflammation. It has been described that SAA targets HDL to macrophages, preferentially than hepatocytes, affecting cholesterol reverse transport (Banka et al., 1995, Kisilevsky and Subrahamanayan, 1992). Two opposite theories have been launched. First, reverse transport is modified in order to handle the large amounts of cholesterol liberated at sites of injure, enhancing the clearance of excess cholesterol from macrophages. And secondly, the SAA-HDL would be redirected to macrophages to deliver phospholipids and cholesterol helping the tissue repair. Although some studies suggest that SAA-HDL complexes are 30 % lower than the non acute HDL complexes (Banka et al., 1995) and the free cholesterol uptake is increased (Liang and Sipe, 1995), the first theory seems to be more consistent. The levels of free cholesterol in plasma increase in parallel to SAA levels during inflammation, indicating that it facilitates cholesterol efflux (Lindhorst et al., 1996). Also, in favor of this theory, *Steinmetz et al.* describes that SAA decreases lecithin:cholesterol acyl transferase (LCAT) activity, an

enzyme responsible for free cholesterol esterification and cytoplasmatic storage (Steinmetz et al., 1989), and *Lindhorst et al.* described an increase in neutral cholesterol ester hydrolase (nCEH), which deesterificates stored cholesterol esters to be transported across the cell membrane through the ABCA transporters, where are accepted by the ApoA1-HDL complex (Lindhorst et al., 1996; Manley et al., 2005). Moreover, SAA has been described to enhance secretory phospholipase A_2 (sPLA2) activity, an enzyme that is also highly increased in inflammatory conditions being considered also an APP (Tietge et al., 2002). In homeostasis, pPLA2 has been related to hepatic lipolysis and the reverse cholesterol transport, helping the release of cholesterol from the HDL-ApoA1 complex and the transport to hepatocytes. In inflammatory conditions, high sPLA2 concentrations are found in the site of injury, and as SAA increases their activity, more cholesterol would be released there (Pruzanski et al., 1995). Finally, *Oram* extended the support to the first theory pointing out the possibility that the main function of SAA is the displacement of ApoA1 itself, as free-lipid ApoA1 has higher affinity for free cholesterol and this would help to the clearance of cholesterol from macrophages (Oram, 1996).

4.2. Immunological related functions

Several immune-related functions have been described for the SAA family, most of them related to processes occurring during inflammation and injury:

- Induction of extracellular matrix degrading enzymes
- Chemotaxis of immune cell mediators
- Cytokine release
- Bacterial opsonization and phagocytosis
- Inhibition of bacterial infections in gastrointestinal tract
- Direct antimicrobial activity

4.2.1. Induction of extracellular matrix degrading enzymes

Matrix metalloproteinases (MMP) play a key role in the degradation of extracellular matrix and their fine regulation is essential in physiologic processes, being implicated in pathological responses when deregulated (including tumor invasion, arthritis and other inflammatory diseases). They degrade matrix substrates including gelatin and collagen types IV, V and VII (Birkedal-Hansen et al., 1993). The SAA proteins have been described to increase the expression of collagenase in monocytes, synovial and corneal fibroblast in a concentration dependent manner (Mitchell et al., 1991; Strissel et al., 1997; Lee et al., 2005).

4.2.2. Chemotaxis of immune cell mediators

The movement of a cell directed by a gradient of a chemical compound is known as chemotaxis. Leukocytes migrate towards the site of injury mainly through **chemokines**, a type of cytokines involved in immune cell recruitment. One of the most important chemokines increased in the innate immune response is the interleukin-8 (IL-8) (Abbas et al., 2002).

SAA proteins have been widely related to the chemotactic activity. Immune cells such B and T lymphocytes, monocytes and neutrophils have been described to migrate in SAA gradients (Olsson et al., 1999; Xu et al., 1995; Badolato et al., 1994; de Jesus et al., 2009). Moreover, not only immune related cells are affected since smooth muscle cells have also shown the same behavior (Kumon et al., 2002). Furthermore, the concentration of SAA necessary to observe this chemoattractant activity would be around 0.8-4 μM. This concentration is higher than normal SAA serum levels which move around 0.08 μM, but are in agreement with acute phase serum levels, which arise up to 80 μM – corresponding to 1 mg/ml (Olsson et al., 1999; Xu et al., 1995).

Moreover, SAA have been shown to increase expression of surface adhesion proteins such as CD18/CD11b and CD18/CDS11c in neutrophils and monocytes, increasing the adhesion to endothelial cells (Badolato et al., 1994). T lymphocytes have been also described to increase the endothelial adherence after SAA treatment, but in this case, they failed to find increased expression of adhesion proteins (Xu et al., 1999). This greater adherence would help to transmigration and infiltration into tissues.

Also, a greater infiltration of immune response mediators has been described after subcutaneous injection of SAA *in vivo* (Patel et al., 1998; Xu et al., 1999; Badolato et al., 1994).

4.2.3. Cytokine release

Cytokines are responsible for the onset of inflammatory phenotype and their modulation may influence the course of the inflammation process. SAA stimulates cytokine production in a variety of cell types and tissues. *He et al.*, described increased production of IL-8 and TNFα in a range of 2-5 fold in SAA treated neutrophils. This response to SAA required *de novo* protein synthesis and transcription of the genes. In contrast, no increased expression of IL-6 and IL-1β was observed (He et al., 2003). Macrophages also showed significant upregulation of several proinflammatory cytokines such as C-C motif chemokine 4, 1 and 3 (CCL4, CCL1 and CCL3), Inteleukin 23 (IL-23) and IL-8 (Leow et al., 2012) and IL-1β (Patel et al., 1998). The increased proinflammatory response after SAA treatment was not only reduced to immune related cells

since intestinal epithelial cells also increased the production of IL-8 mRNA and protein and enhanced the effect of other cytokines such as IL-1 and TNFα (Jijon et al., 2005).

4.2.4. Bacterial opsonization and phagocytosis

Phagocytosis of the infective bacteria is one of the main goals of innate immune response. It has been described that SAA binds to a large number of Gram-negative bacteria, including *Escherichia coli, Salmonella typhimurium, Shigella flexneri, Klebsiella pneumonia, Vibrio cholera* and *Pseudomonas aeruginosa* but not other Gram-positive organism such *Streptococcus pneumoniae* and *Staphylococcus aureus*. Maximal binding was achieved around 1 µg/ml, which falls within the normal serum levels and do not require acute phase levels. The ligand was described to be the outer membrane protein A (OmpA), a protein conserved among the *Enterobacteriaceae*, and related members are found in almost all Gram-negative bacteria (Hari-Dass et al., 2005). Further investigation showed that this binding would function as an opsonization mechanism and that bacterial phagocytosis of neutrophils and macrophages was increased in SAA treated cells (Shah et al., 2006).

4.2.5. Inhibition of bacterial infections in gastrointestinal tract

Synthetic peptides derived from the M-SAA3 isoform have been described to efficiently inhibit the binding of enteropathogenic *E. coli* to epithelial intestinal cells *in vitro* (Mack et al., 2003). Other studies have demonstrated an upregulation of Mucin 3 (MUC3) along with the decreased infectivity after treatment of intestinal cells with SAA peptides (Larson et al., 2003; Manuell et al., 2011). A 4 amino acid motif, TFLK, located in the N-terminal region of the protein has been directly related with this protective action (McDonald et al., 2001). According to McDonald, the TFLK motif is only present in the mammary isoform of bovine SAA3 but not in the hepatic isoforms. Further studies using the truncated human SAA3 (42 amino acid peptide), also showed an *in vitro* protective effect but failed to prevent enteric infection in *in vivo* murine model (Gardiner et al., 2009).

4.2.6. Direct antimicrobial activity

A direct killing activity against a broad range of bacteria has been reported for M-SAA3. *Molenaar et al.*, showed decreased cell viability in Gram-negative and positive bacteria (*E. coli, Streptococcus uberis* and *P. aeruginosa*) after being incubated with M-SAA3. The antibacterial activity was dose-dependent and almost complete killing occurred at M-SAA3 concentrations above 63 µg/ml, levels found in milk during naturally occurring infections. This property has only been described for the mammary isoform.

5. Cell receptors for SAA

SAA is a small protein exerting a wide range of functions. However, many of the described functions are not a direct effect of the protein itself but a signal transduction cascade activated by the binding of different cellular receptors.

SAA activates a seven-transmembrane G protein-coupled receptor called formyl peptide receptor like-1, **FPRL1**, mediating the chemotactic activity, the IL-8 secretion and the upregulation of MMP-9 via activation of MAP kinases ERK1/2 and p38 (Lee et al., 2005; He et al., 2003; Su et al., 1999).

Toll like receptors are also a target for SAA protein. Both the **TLR2** and **TLR4**, respectively detect Gram-negative and positive bacteria and have been described to be functional receptors for SAA. The recognition of these receptors would lead to the activation of the proinflammatory response via the NF-κB transcription factor for the TLR2 and the activation of the MAPK for the TLR4 (Cheng et al., 2008; Sandri et al., 2008; Baranova et al., 2010).

The scavenger receptor class B Type I, **SR-BI,** has been demonstrated to mediate the cholesterol efflux when associated to HDL-SAA (van der Westhuyzen et al., 2005). The human orthologue of the SB-BI is the CD36 and LIMPII Analogous-1 receptor (**CLA-1).** It mediates the SAA uptake via endocytic vesicles and activates downstream SAA signaling, increasing the phosphorilation of MAPKs, ERK1/2 and p38 along with the IL-8 expression (Baranova et al., 2005). Another scavenger receptor of the class B family, **CD36**, performs as novel receptor for SAA, inducing also the IL-8 expression by activating the MAP kinase cascade of ER1/2 and JNK1/2/3 (Baranova et al., 2010).

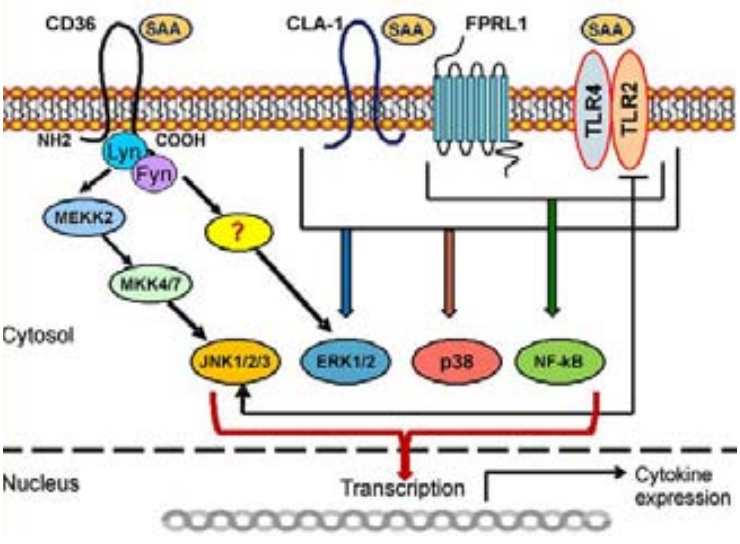


Figure 5. Schematic diagram illustrating SAA-induced signaling mediated by SAA receptors. FPRL1, TLR2, TLR4, CLA-1 and CD36 have been described to mediate signaling via MAPK or NF-κB pathways. (Adapted from Baranova et al., 2010)

Block III Mastitis and gastrointestinal infections in cattle

1. Bovine mastitis

Mastitis is classically defined as the inflammation of the mammary gland produced by injury or microbial infection. It is, indeed, a complex disease with a diverse etiological causes, pathogenesis, intensity, duration and therapy. Mammary glands offer a sterile environment but internalization of pathogenic bacteria through the teat canal may initiate the infectious process as long as they survive and divide inside the gland. The severity of the disease depends on the pathogen and the natural mechanism of defense of each cow. Mastitis can course as **sub-clinical** infection, with elevated somatic cell count in milk and decreased milk production, or manifest **clinical** symptoms, characterized by visual inflammation such as swelling, heat, redness, pain and arriving to systemic signs of fever, anorexia, shivering and weight loss (Jain, 1979).

Bovine mastitis is considered to be one of the most economically important diseases for the diary industry in develoed countries (Petrovski et al., 2006) accounting for 38% of the total expenses related to common diseases in animal production (Bradley, 2002). Losses are difficult to estimate and are associated to loss of milk production (discarded milk plus non-productive periods), premature culling and costs of specialized treatment and management (De Vliegher et al., 2012). There are several reports that estimate the average financial impact of clinical mastitis. *Heikkila et al..* calculated the costs in a model for Holstein-Friesians and Ayrshires cows (high and moderate milk producers, respectively, as higher milk yields increase the risk of mastitis). When premature culling was included in the model, the cost per clinical case was 623 and 596 €, converted to a cost per cow-year they were 147 and 121 €, respectively (Heikkila et al., 2012). In addition sub-clinical mastits are difficult to detect due to the absence of any visible indications and it also has major costs implications (Viguier et al., 2009).

Mastitis pathogens have been classified as either **contagious** or **environmental**. Contagious pathogens are considered to be adapted to survive in the host and particularly into the mammary gland. They typically produce subclinical mastitis and spread from direct contact. On the other hand, environmental pathogens are opportunistic, and usually generate a rapid infection (Bradley, 2002).

1.1. Common etiological causes

The prevalence (number of affected cows in a given population in a given time) of clinical mastitis is around 30% (Pitkala et al., 2004; Nam et al., 2010). Several different factors may affect the distribution of bacterial isolates from clinical mastitis cases such as age,

management and kind of study. Different reports give diverse results in the incidence of each bacteria, but they agree in the range of pathogenic bacteria that affects the different herds around the world. Some of them described the coagulase negative *Staphylococcus* species (CNS) as the most frequent pathogens (Pitkala et al.,2004; Nam et al., 2010) meanwhile others describe *Streptococcus uberis* to be the main cause (De Vliegher et al., 2012; Veterinary Investigation Diagnosis Analysis database – VIDA 2011 report –United Kingdom).

In summary, the main contagious pathogens are considered *Staphylococcus aureus, Streptococcus dysgalactiae* and *Streptococcus agalactiae*, meanwhile the major environmental pathogens comprise *Enterobacteriaceae* particularly *E. coli* and *Streptococcus uberis*.

1.1.1. Streptococcal mastitis

They comprise mainly three species: *S. dysgalactiae* and *S. agalactiae* which are considered as contagious pathogens and *S. uberis* as environmental. *S. agalactiae* had high prevalence decades ago, but as they are obligate udder parasites that do not survive in the environment, they become easy to eradicate with a proper antibiotic therapy and management. *S. uberis* and *S. dysgalactiae* can survive for long periods in the environment and in cow skin and therefore are more difficult to eradicate (Jain, 1979). Specifically *S. uberis* are among the most common causes of clinical and subclinical mastitis (Hogeveen et al., 2005).

1.1.2. Staphylococcal mastitis

S. aureus and coagulase negative staphylococcus species (CNS) are common causes of mastitis. The group of CNS comprise multiple staphylococcal species (45 different species and subspecies of which a dozen are commonly found in milk) that are mainly environmental (Nam et al., 2010; De Vliegher et al., 2012), and have become one of the most important etiological causes in well-managed herds (Pitkala et al., 2004; Nam et al., 2010). *S. aureus* are a contagious pathogen that spreads easily cow to cow and provokes chronic subclinical mastitis and occasionally acute episodes (Hogeveen et al., 2005).The principal reservoirs are the udder, teat skin and the milk of infected glands. *S. aureus* have the capacity to penetrate tissues producing deep reservoirs complicating the antibiotic therapy and their eradication. *S. aureus* damages neutrophils and inhibit phagocytosis difficulting its eradication (Jain, 1979).

1.1.3. Coliform mastitis

E. coli is the main coliform producing mastitis and it is considered to be an environmental pathogen. Coliforms induce a typical acute disease, short limited and mild, as they do not cause extensive damage to mammary tissue. They are also effectively controlled by natural antibiotics and phagocytes present in milk (Jain, 1979; Hovegeen et al., 2005).

1.2. Mastitis and dry period

The dry period is a necessary non-milking period during lactations in order to maximize milk production. The preferred method is an abrupt cessation of milking. After the last milking, the mammary tissue still secretes milk which is accumulated in the mammary gland, promoting involution. After two or three days, when the milk accumulation is maximal, the fluid volume decreases and concentration of milk components such as immune cells and proteins increase (Nickerson, 1989). During the first 2-3 weeks of dry off, there is an increased risk of mastitis due to the udder pressure exerted by the milk stasis which allows bacterial penetration as the teat canal is shortened and dilated (Bradley, 2002; Green et al., 2002). Moreover, the milk leakage during the beginning of the dry period also promotes bacterial growth and increases the opportunity of infections into the mammary gland. As involution progresses the udder become more resistant to infection, due in part to the formation of the keratin plug in the teat canal which prevent bacterial multiplication and penetration (Nickerson, 1989).

2. Bovine gastrointestinal infections

Enteric disease is an important health problem in calves and it normally curses with diarrhea. The incidence (number of new cases per population in a determined period) varies depending on the study, and the range is around 5 to 28% (Virtala et al., 1996; Wittum et al., 1996; Gullisken et al., 2009). Calves during the first month of life are the most affected group, and the disease is associated with reduced weight gain with delayed growth and increased mortality with the consequent economic impact (Virtala et al., 1996; de Graaf, 1999; García et al., 2000).

Diarrhea has multifactorial etiology, being caused and affected by viruses, bacteria, protozoa and human related factors such as management (housing, feeding and hygienic conditions) (Bartels et al., 2010; Lanz Udhe et al., 2008).

The most common pathogens found in diarrheic calves are *Cryptosporidium*, rotavirus, coronavirus, *Salmonella*, attaching and effacing *E. coli* and other *E. coli* such as F5(K99) (Blanchard, 2012).

Pathogen	Positive samples (%)
Cryptosporidium sp	37.2
Coronavirus	30.5
Rotavirus	26.6
Salmonella spp	15.7
Salmonella group D1 (*Salmonella* Dublin)	5.8
Salmonella group C2 (*S. newport*)	4.2
Salmonella group B *(S. typhimurium*)	2.7
Salmonella group E	2.3
Salmonella group C1 and others	0.7
Attaching and effacing *E. coli*	10.5
K99 *E. coli*	4.5
Bovine viral diarrhea virus	1.3

Table 3. Summary of enteric pathogens from necropsied calves less than 35-days old with diarrhea. California Animal Health and Food Safety Laboratory (CAHFS) data, n=2311, from 2008-2011 (Adapted from Blanchard, 2012).

2.1. *Escherichia coli*

Diarrheic *E. coli* are assigned to groups based on their virulence properties and clinical manifestations.

- **Enterotoxigenic *E. coli* (ETEC):** It is the most important cause of diarrhea in beef and dairy cows in the first 4 days of life (Moxley and Smith, 2010). The main feature is the production of fimbria and enterotoxins. The fimbria allows the attachment to the intestinal villi and is known as F5 or K99. After attaching, the heat stable toxin (STa) produces fluid efflux into the intestine lumen. Attachment factors are mostly produced in immature villi so natural intestinal maturation ends up protecting from the infection (Blanchard, 2012; Foster et al., 2009).

- **Attaching and effacing *E. coli*: Enterohemorragic (EHEC), Enteropathogenic (EPEC):** The attaching and effacing (A/E) lesion results in the microvilli effacement forming pedestals beneath the site of attachment and leading to a deregulation of ion exchangers and water malabsortion. The process is regulated by the genes of the locus of enterocyte effacement (LEE), which encode a Type III secretion system (T3SS) and the *eae* gene (which encodes for intimin). The T3SS translocates bacterial effector proteins into the host cell cytoplasm, such as Tir (the intimin receptor). Intimin is an outer membrane protein and mediates the attachment by binding to the Tir receptor (Croxen and Finlay, 2009). When the A/E bacteria do not secrete toxins are classified as enteropathogenic (EPEC) and if they secrete Shiga toxin are known as enterohemorragic (EHEC) (Foster et al., 2009). Cattle are a key reservoir for EHEC (Croxen and Finlay, 2009).

- **Shiga toxin-producing *E. coli* (STEC):** Typically described as Shiga Toxin producing *E. coli* with no *eae* genes (Blanchard, 2012).
- **Enteroinvasive *E. coli* (EIEC):** They invade the host intestinal epithelial cells and multiply, causing destruction and acute inflammatory response (Desmarchelier and Fegan, 2011), in a similar manner than other enteric pathogens such as *Shigella* (Croxen and Finlay, 2009).
- **Enteroaggregative *E. coli* (EAEC):** They adhere in aggregative pattern resembling micrololonies (Desmarchelier and Fegan, 2011).

2.2. *Salmonella* ssp

The most common causes of enteric *Salmonella* in calves less than 21-days old are *S. typhimurium* and *S. newport. Salmonella enterica* subsp. *enterica* serovar Dublin (*Salmonella* Dublin)is the most common afer 30-days of age, and is considered to be host-adapted to cattle but infrequently infect other mammals including humans (Uzzau et al., 2000). Affects both young and adults but mainly infects calves between 2 weeks and 3 months of age, producing acute enteritis and systemic disease leading to important economical and welfare losses (Uzzau et al., 2000; Blanchard, 2012). The colonization of the intestinal epithelium is mainly at the Peyer's Patches (PP) level.

PP are part of the gut-associated lymphoid tissue (GALT) and are distributed throughout the mucosa and submucosa of the gastrointestinal tract. They form a dome with lymphoid follicles of B and T lymphocytes. Overlying the follicles a specialized ephithelium which contain M-cells is observed. The M-cells are responsible for the antigen uptake from the intestinal lumen and the transport to the antigen presenting cells scattered around the follicles (dendritic cells and macrophages) (Hathaway and Kraehenbul, 2000). *Salmonella* preferentially infects M cells but other routes can be used (enterocytes and dendritic cells), finally reaching systemic circulation (Jones, 1997; Tam et al., 2008).

Multiple virulence factors have been identified, located in *Salmonella* pathogenicity islands (SPI), which encode T3SS able to inject bacterial proteins into the host epithelia, helping to the invasion replication and survival of the bacterial cells (Coburn et al., 2007).

Chapter II

Objectives

Mammary SAA3 levels are high in colostrum and greatly variable in milk under different infection conditions and along lactation. However, biological functions of M-SAA3 are practically unexplored.

The main objective of the thesis was to evaluate the specific roles of the mammary expressed SAA3 protein (M-SAA3), at immunological level. The specific objectives were:

1. To clone M-SAA3 directly from mammary tissue and recombinantly produce it in order to conduct functional experiments.
2. To evaluate the effect of M-SAA3 protein in innate immune related cells, such as macrophages and dendritic cells.
3. To investigate the potential protective role of M-SAA3 at gastrointestinal and mammary level against pathogenic bacteria.
4. To analyze the potential capacity of M-SAA3 to activate the pro-inflammatory response in epithelial cells and other cell types.
5. To explore a possible industrial application of M-SAA3 in ruminant production.

To achieve these objectives, four studies were conducted:

Study 1 *"Recombinant expression of goat milk serum amyloid A: Preliminary Studies of the protein and derived peptides on macrophage phagocytosis"*

Molecular cloning and recombinant expression of the M-SAA3 and evaluation of the first functional role of the protein: the macrophage phagocytosis activation endured by both the whole M-SAA3 protein and derived peptides.

Study 2 *"Mammary Serum Amyloid A3 reduces bacterial translocation and enhances the immune response in epithelial intestinal cells"*

Evaluation of the protective role of M-SAA3 at the gastrointestinal tract using an intestinal epithelial cell line, related to a direct inhibition of the bacterial translocation and the activation of the pro-inflammatory response.

Study 3 *"Mammary Serum Amyloid A3 protection against Salmonella Dublin in bovine ex vivo intestinal model and dendritic cells"*

Production of an *ex vivo* intestinal infection model in order to study the protective effect of M-SAA3 in a more physiological condition and evaluate the function of the protein on dendritic cells, an important mediator between the innate and adaptive immune response.

Study 4 *"Enhancement of early-cow dry period performance by Mammary Serum Amyloid A3"*

Evaluations of *in vivo* applicability of the protein in the optimization of cow dry period, a bottleneck in the dairy industry.

Chapter III – Study 1

Recombinant expression of goat milk serum amyloid A: preliminary studies of the protein and derived peptides on macrophage phagocytosis

Anna Domènech[1], John G. Raynes[2], Anna Arís.[1], Alex Bach[13] and Alícia Serrano

1. Ruminant Department of IRTA, Institute of Research in Agriculture and Technology. Torre Marimon, km 12,1 C-59, Caldes de Montbuí, 08140, Barcelona, Spain.

2. Immunology and Infection Department, London School of Hygiene and Tropical Medicine, LSHTM. 481, Keppel St, London WC1E 7HT, UK

3. ICREA, Institució Catalana de Recerca i Estudia Avançats, Barcelona (Spain)

This research has been published in:

Protein and Peptide Letters, 2012; Vol 19 Nº3

1. INTRODUCTION

Serum Amyloid A3 protein is a member of the Serum Amyloid A family, which comprises both acute phase and constitutive proteins. They are mainly produced in the liver although extrahepatic synthesis has been described in several tissues, where the major form is frequently the SAA3 isoform which is an acute phase form (Bendit, 1986; McDonald et al., 2001; Molenaar et al., 2009). The SAA members have been related to several immune functions, like chemotaxis (Badolato et al., 1994; de Jesus Rodriguez et al., 1998), cytokine modulation (Patel et al., 1998; He and Ye, 2003) and an antibacterial role (Larson et al., 2003; Mack et al., 2003; Gardiner et al., 2009; Molenaar et al., 2009), although the precise mechanism and receptors involved in these functions are not completely defined.

When considering functions of SAA3, 10-mer peptides derived from the N-terminal milk derived SAA3 (M-SAA3) have been described to prevent the binding of enteropathogenic *Escherichia coli* (EPEC) to both *in vitro* human intestinal epithelial (Larson et al., 2003; Mack et al., 2003; Gardiner et al., 2009). Those studies led to the conclusion that the 10-mer peptides are the active portion of the protein and that within this the TFLK motif appears to be essential for activity (Larson et al., 2003; Mack et al., 2003). According to *Larson et al*., the conservative 4 amino acid N-terminal TFLK motif is only present in the mammary isoform of bovine SAA3, and it is associated with a gastrointestinal protection role via intestinal mucin overexpression. In contrast, more recent studies indicate that the TFLK motif is also present in non-mammary SAA3 forms (GenBank sequence MGC:133567) and macrophages (GenBank sequence AF540564.1).

On the other hand, serum SAA1 has been described to have an indirect role in the clearance of Gram-negative bacteria via bacterial opsonization, binding to the outer membrane protein A of *E. coli* and facilitating the destruction of the bacteria by macrophages and neutrophils (Hari-Dass et al., 2005; Shah et al., 2006). More recently, *Eckhardt et al*. reported a reduction of *E. coli* viability when co-cultured with intestinal epithelial cells (IEC) *in vitro* in the presence of SAA1 and SAA2. They hypothesized a possible mechanism in which SAA would reduce bacterial load in the intestine lumen via IEC phagocytosis of SAA opsonized *E. coli* (Eckhardt et al., 2010).

However, no reports about a possible opsonic mechanism of the milk derived SAA3 form (M-SAA3) have been described yet, despite its potential in having an effect on both the maternal side contributing to the mammary gland health itself and also in the intestinal-epithelial immune homeostasis of the new-born via maternal milk intake.

In this work, we describe the cloning and sequencing of goat M-SAA3 (g-MSAA3) from milk, mammary gland tissue and liver. We describe the optimization of expression of the recombinant protein through culture strategies and molecular approaches in order to obtain a soluble active and purified protein. To our knowledge no recombinant gM-SAA3 expression studies have been carried out. Purification of SAA from *ex vivo* sources and particularly SAA3 is problematic. The optimal expression and purification of gM-SAA3 would facilitate further functional investigations of the protein both *in vitro* and *in vivo* studies, usually the bottleneck for final utilization of recombinant proteins and/or its derivates. We also explored the possible antimicrobial effect of the gM-SAA3 and its derived peptides via the recognition of Gram-negative bacteria and macrophage activation as previously described for the plasma SAA forms. These observations provide new tools and suggest wider importance for SAA family members in host protection.

2. MATERIALS AND METHODS

2.1. Cloning of C-ter 6 histidine tagged gM-SAA3

The SAA3 was cloned from three different origins: milk, mammary gland and liver. In milk, the somatic cells were processed as in *Boutinaud et al*. Briefly, 300 ml of fresh goat milk was centrifuged at 2000 g during 15 min with EDTA added to 0.5 mM final concentration. The fat layer and the supernatant were removed and the pellet was washed twice with PBS containing 0.5 mM EDTA. In liver and mammary gland, a biopsy of approximately 0.5 cm^2 was homogenized manually with a pestle (Boutinaud and Jammes, 2002). The RNA of all the samples was extracted with TRIzol® (Invitrogen, California, USA). RNA quality and integrity was evaluated by visualization in 0.8 % (w/v) agarose gel with 500 ng/ml of ethidium bromide (Sigma, Missouri, USA) and by electropherogram analysis using the Experion™ Automated Electrophoresis system (Bio-Rad, California, USA) and the Experion RNA StdSens chips (5-500 ng/µl total RNA). Total mRNA was retrotranscribed to cDNA using the IScript cDNA synthesis kit (Bio-Rad, California, USA). SAA3 gene amplification was carried out using the specific primers, Fw 5'-CAACCATGAACCTTTCCACGGGCATC-3' and Rv 5'-GTACTTGTCAGGCAGGRCAGCAG-3' designed based on the GenBank database sequences EF564257, EF564270, DQ839400. The PCR conditions were a denaturation step (95°C, 5 min), 24 cycles of (94°C, 1 min, 68°C, 1 min, 72°C, 1 min) plus a final elongation step (72°C, 10 min). The PCR was performed with High Fidelity PCR system (Roche, Manheim, Germany), 2 µl of cDNA (50 ng/µl) and primers at 2.5 µM. The PCR product was visualized in 1 % (w/v) agarose gel with 500 ng/ml of ethidium bromide (Sigma) and it was recovered using the Minielute kit (Qiagen, Maryland, USA). The SAA3 gene was cloned into the PET101/D-TOPO vector

(Invitrogen, California, USA) which introduces a 6 histidine C-terminal tail. *E. coli* TOP 10 were used for plasmid maintaining and sequencing purposes and *E. coli* BL21 Star (DE3) was the final recombinant expression strain. Cells were chemically transformed with the plasmids and transformants were selected in LB-ampicillin (100 µg/ml) plates. Screening PCR was performed as previously described to check which clones contained the SAA3 insert. The plasmids were isolated from the PCR positives clones using the Miniprep Extraction kit (Qiagen, Maryland, USA) and they were sequenced using the T7 priming sites of the original vector. Vector NT 11 Advanced was used for sequence alignment.

2.2. Obtaining a N-ter 6 histidine tagged gM-SAA3

PCR was performed using as a template the plasmid containing the C-terminal histidine tagged gM-SAA3 and the following specific primers at 2.5 µM: Fw 5'-CACCATGCATCACCATCACCATCACGACGATGACGATAAGAACCTTCCACGGGCATG-3' and Rv 5'-TCAGTACTTGTCAGGCAGGRCAGC-3'. The Fw primer introduces a enterokinase target and a 6 histidine tail, and the Rv a stop codon before the C-terminal histidine sequence of the original template plasmid. The PCR cycle conditions were a denaturation step (95°C, 5 min), 24 cycles of (94°C, 1 min; 55°C, 15 sec; 72°C, 40 sec) plus a final elongation step (72°C, 10 min). PCR product was visualized, purified, cloned, sequenced and transformed as described above.

2.3. Recombinant gM-SAA3 expression cultures and cell viability

Growth was monitored by optical density at 600 nm (OD_{600}) with the Ultrospec 2100 pro spectrophotometer using the respective culture media as blank. Growing conditions were 200 rpm and 37°C unless otherwise mentioned. Pre-inoculums were 5 ml overnight (O/N) cultures inoculated with 1 bacterial colony in Luria-Bertani media (LB) with ampicillin (100 µg/ml). Expression cultures were 200ml of LB with ampicillin (100 µg/ml) at an initial DO_{600} of 0.05. Growth was monitored every 15 min the first hour after induction and each hour until reaching 4 hours of expression. Non-transformed *E. coli* BL21 (DE3) was used as a negative expression control strain. Recombinant expression was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at different conditions: IPTG 0.1, 0.25, 0.5 and 1 mM, time of expression of 1, 2, 3 or 4 hours, DO_{600} of induction at 0.6 and 1, use of LB media or rich 2xYT, ampicillin concentration at 100, 250 and 500 µg/ml and temperature of growth (O/N at 16°C). Cell viability was calculated by culture dilution in NaCl 0.9%. 100 µl of serial dilutions were plated in LB and LB-ampicillin (100 µg/ml) plates by duplicate. Plates were grown O/N at 37°C and cell viability was expressed as colony forming units (CFU/ml) discarding those plates with a number of colonies below 15 or above 300.

2.4. Purification and quantification of gM-SAA3

400 ml cultures were centrifuged at 6000g during 10 min at 4°C, and cell pellet was frozen at 80°C until use. Cell pellets were enzymatic and mechanically-treated. Pellets were resuspended in 6 ml of 20mM Na_2HPO_4 /NaH_2PO_4, 0.5M NaCl, pH 7.4 buffer, with lysozyme (0.2 mg/ml), DNase I and RNase A (20 µg/ml), cocktail inhibitor of proteases (1 mM), $MgCl_2$ (1mM) during 30 min at room temperature. The suspension was mixed with pre-weighted 0.1 mm glass beads (range 26-36 mg per ml of sample) (Biospec Product, Inc, Bartlesville, USA). Three cycles of beating of 45 sec each, with 1 min on ice within cycles, were carried out in the MiniBead Beater (Biospec Product, Inc, Bartlesville, USA). The disrupted solution was centrifuged for 15 min at 20000 g at 4°C. The supernatant and pellet were considered as soluble (SF) and insoluble (IF) fraction respectively. The IF was resuspended with the same volume of 20 mM Na_2HPO_4 /NaH_2PO_4, 0.5M NaCl, 4M Urea pH 7.4 buffer. The gM-SAA3 protein was purified from the SF using a His Spin Trap column (GE Healthcare, Uppsala, Sweden) following the manufacturer's instructions. The sample application and the washing buffer contained 80 mM imidazole. The recombinant gMSAA3 protein was eluted using 500 mM of imidazol and dialyzed O/N against phosphate buffered solution (PBS) at 4°C and gentle agitation. The protein was aliquoted and stored at -80°C until use. DC_Assay (Bio-Rad, California, USA) was used for total protein quantification (assay limits from 0.2 mg/ml to 1.6 mg/ml) using chicken ovoalbumin as standard and following manufacturer's instructions.

2.5. gM-SAA3 derived peptides

Synthetic peptides from mammary goat SAA3 were produced by Biomedal (Parque científico y tecnológico Cartuja, Sevilla, Spain) and analysed by HPLC and Mass Spectophotometry. The region of the sequence was chosen based in the studies of *Larson et al.*, using the goat isolated sequence (N – GWGTFLREAG – C) and its respective TFLK-like region scrambled peptide (N – GWGFRTLEAG – C) (Larson et al., 2003)

2.6. Antibodies against qM-SAA3

Two rabbits were immunized with a 12-amino acid peptide designed from the gM-SAA3 sequence obtained from milk. An antigenicity study of the protein was performed to select the most antigenic peptide, based on hydrophilicity, exposed surface and accessibility criteria. The final chosen peptide comprised the 109-120 amino acid sequence of the putative protein. Rabbits were bled at day 90 post immunization and the antibodies were purified by protein G affinity chromatography.

2.7. SDS-PAGE, Coomassie and Western-blot

1 ml culture samples were collected and centrifuged 5 min at 6000g, 4°C. Cell pellets were stored at -20°C until analyses. Proteins were visualized by SDS-PAGE using 12% polyacrylamide gels. Before loading, cell pellets were resuspended with water to a DO_{600} of 0.03 and samples were boiled 10 min with Laemmli 2x (Sigma, Missouri, USA). The gels were run for 60 min at 200V. Proteins were either stained with Coomassie brilliant blue (1 hour) or transferred to Immuno-Blot PDVF membranes (at 100V during 60 min). In Western-blot, the membranes were blocked with Tris-buffer solution with 0.05% (v/v) Tween-20 (TBST) and 5% dry skimmed milk. First and second antibodies were a monoclonal anti-polyhistidine (Ref. H1029-Sigma, Missouri, USA) diluted 1:3000 in TBST, and an anti-mouse IgG (whole molecule) alkaline phosphatase conjugated produced in rabbit (Ref. A4312-Sigma, Missouri, USA) diluted 1:20000 in TBST. BCIP/NTB solution premixed (Sigma, Missouri, USA) was used as a substrate. The purified caprine SAA3 antibody was used as a primary antibody in Western-blot at a concentration of 1:3000 (initial concentration 0.3 mg/ml) and a anti-rabbit IgG (whole molecule)-alkaline phosphatase conjugated produced in goat (Ref. D0487-Dako, Glostrup, Denmark) diluted 1:1000 as a secondary antibody.

2.8. Macrophages isolation from goat milk

Macrophages from milk were extracted as described previously by *Denis et al*. Briefly, 100 ml of fresh goat milk kindly provided from a British farm were centrifuged at 400 g during 10 min at 4°C (Denis et al., 2006). After washing the cell pellet three times with 15 ml of Roswell park Memorial Institute medium 1640 (RPMI, Gibco, California, USA) it was finally resuspended in 4 ml of RPMI, quantified in a haemocytometer and seeded in 24-well plates ($2 \cdot 10^6$ cells/well). Macrophages were allowed to stick on the coverslips during 2 hours, washed 3 times with RPMI and cultured with 1 ml of supplemented RPMI plus 10% fetal calf serum (FCS) during 24 hours.

2.9. PBMC isolation from human blood and macrophages differentiation

Anticoagulated-blood (heparin 100 UI/ml) was obtained from volunteers at London School of Hygiene and Tropical Medicine (LSHTM) under informed consent, and was diluted 1:2 with warm RPMI 1640 medium (Gibco, California, USA) supplemented with 2 mM L-glutamine (Gibco, California, USA), 1 mM sodium pyruvate (Sigma, Missouri, USA), 100 µg/ml streptomycin/penicillin (Gibco, California, USA). The diluted blood was layered onto Histopaque (1077 g/ml, Sigma, Missouri, USA) in a 1:2 ratio (histopaque/diluted blood) and centrifuged 30 min at room temperature (RT) at 400g (brakes off). PBMC (Peripheral blood

mononuclear cells) were isolated from the interphase, washed with media and centrifuged 10 min at 250g. The cell pellet was resuspended and incubated in 15 ml of Red Blood Lysis buffer (Sigma, Missouri, USA) for 20 min, in order to eliminate the contaminating erythrocytes. Cells were washed twice with RPMI media, the cell pellet was resuspended in 2 ml of RPMI and quantified by haemocytometer counting. The PBMCs were seeded into 24-well plates with glass coverslips inside, at $2 \cdot 10^6$ cells/well and let stuck for 2 hours at 37°C, 5% CO2. Adherent cells were washed twice with 1 ml of RPMI and cultured during 6 days in supplemented RPMI plus 10% FCS for macrophage differentiation.

2.10. Bacterial culture for phagocytosis assay

Escherichia coli BL21 – GFP+ (green fluorescent protein) were incubated over-night in 10 ml LB-amp (100µg/ml) media at 37°C at 250 rpm. Bacteria were centrifuged 10 min at 2000 g and the cell pellet was resuspended in 10 ml of PBS. Concentration of bacteria was calculated using spectophotometric quantification (OD_{600}=1 corresponds to 10^9 CFU/ml) using PBS as a blank.

2.11. Phagocytosis assay

Differentiated macrophages were washed once with supplemented RPMI media and were incubated either with gM-SAA3 (3 and 30 µg/ml) or derivative peptides (at 1 and 100 µg/ml), using a human serum sample as a positive control 4% (v/v)) and PBS as a negative control, in a final volume of 500 µl. For bacterial incubation RPMI supplemented with 1 mM pyruvate and 2 mM glutamine and 0.2% (w/v) BSA was used. No antibiotics were added. Immediately after, bacteria were added at MOI 1:50 and 1:25, and the plate was incubated 1 hour at 37°C, 5% CO_2. After the incubation, the cells were washed 3 times with PBS and fixed by adding 300µl of warm paraformaldeihid (PFA) 4% in each well during 30 min After a wash with PBS, 300 µl/well of NH_4Cl 50 mM was added and incubated for 30 min in order to avoid fluorescence fading. Finally wells were washed with PBS and dried coverslips were mounted into glass slides using Vectashield-DAPI (Vector Laboratories, California, USA). The coverslip edges were sealed with nail polish and kept at 4°C until analysed using fluorescence and confocal microscopy (LSM 510, Zeiss, Germany). Association of bacteria (AI) was determined by fluorescence microscopy of full depth images and phagocytosis (PI) determined by analysis of z stack 3 d images. The number of bacteria for each cell was recorded and expressed as AI or PI (number of bacteria per 100 cells) and percent of cells with associated bacteria or phagocytosed bacteria GFP+.

The two different donors reported different basal levels of phagocytosis, corresponding to the incubation with PBS values, and therefore were treated separately. The set of data was no normally distributed and it was analyzed by Kruskal-Wallis and Wilcoxon non-parametric tests.

2.12. Determination of macrophage viability after the treatment with the recombinant protein and peptides

96-well plate was incubated with 5 x10^4 macrophages/well in a final volume of 100 µl and cultured during 6 days. Cells were incubated during 1 hour with 4% (v/v) serum, PBS, Recombinant gM-SAA3 protein (3 and 30 µg/ml) or derived peptides (1, 50 and 100 µg/ml). For the viability assay, the volume on each well was removed and it was added 100 µl of media + 10 µl of WST-1 reagent (Cell proliferation Reagent WST-1, Roche, Manheim, Germany). Absorbance at 450 nm was recorded after 3 hours.

3. RESULTS AND DISCUSSION

3.1. Cloning and sequencing of gMSAA3

Goat SAA3 from different origins was successfully cloned. Total goat milk RNA was highly degraded meanwhile mammary and liver RNA were obtained in a high concentration and well conserved. Despite the quality differences, all RNA samples reported similar results in SAA3 amplification and final cloning. Analyses of the cloned sequences revealed two different SAA3 nucleotide sequences in all cases, one of 483 bp and another of 492 bp, mainly differing in a 9 nucleotide deletion/insertion within them. The two different goat SAA3 mRNAs seemed likely to be equally present in milk, mammary gland and liver, as suggested by the ratio of positives clones obtained of each isoform. Thus, potentially both goat SAA3 proteins could be produced in blood and milk, although further research is required to confirm this. The presence of these two isoforms in goat SAA3 had been previously reported in the GenBank nucleotide database (EF564257, F564270, DQ839400). The 486 bp nucleotide SAA3 sequence obtained from milk, mammary and hepatic origin in goat were 99% homologous to EF564257 and F564270, and the 492 bp SAA3 sequence was 98.2% homologous to DQ839400. To our knowledge, the existence of two mRNA sequences for SAA3 (basically differing from the 9 nucleotide deletion) has been only described in *Capra hircus* meanwhile in other mammals only isoforms of the long 492 bp form have been reported.

Two theoretical protein sequences of 128 and 131 amino acids length were obtained from the 483 and 492 bp nucleotide sequences respectively (Figure 1). The theoretical amino acid sequence derived from the short and long SAA3 isoforms were 98.4% and 96.9% homologues to the goat GenBank sequences. Analyses of the gM-SAA3 obtained sequences revealed common interesting regions within them. The N-terminal region contained a 4-amino acid TFLR motif similar to the previously described bovine TFLK one. In bovine, the TFLK motif had been described to be only present in the milk and colostrum derived SAA3 and it was attributed to

have a functional protective role in the organism (Larson et al., 2003; Mack et al., 2003). However, the TFLR motif of the gM-SAA3 was present in both liver and mammary cloned sequences. Other features could be also described from gM-SAA3 sequence analyses. Human SAA1 and SAA2 have been reported to contain between the 47-59 amino acids two extracellular matrix binding domains, the YIGSR-like and RGD-like domains which bind to laminin and fibronectin receptors (Preciado-Patt et al., 1994). The presence of those motifs had been related to the chemoattractant capacity of the protein activating cell migration. In the gM-SAA3, as well in the bovine SAA3, the fibronectin-like binding domain was maintained whereas the laminin-like binding was not.

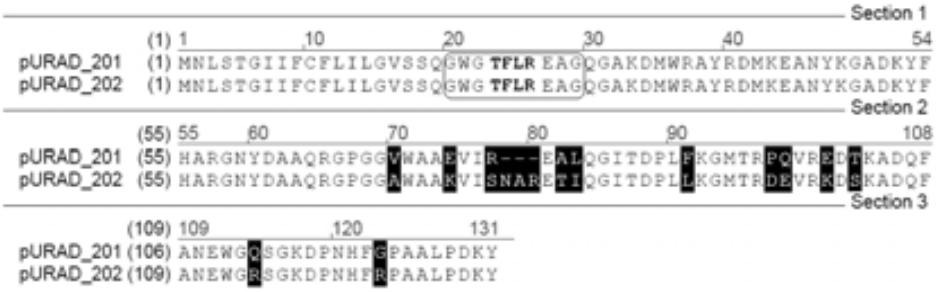


Figure 1. Alignment of the two predicted aminoacid gM-SAA3 sequences, short (pURAD_201) and long (pURAD_202) isoforms. Non-similar amino-acids are shaded. Square indicates the region for peptide design.

3.2. Expression of gMSAA3

One plasmid containing each of the SAA3 sequence (483 and 492bp) was chosen to conduct the recombinant protein expression studies. These plasmids were named pURAD201 and pURAD202, respectively, and EcUR206 and EcUR207 were the final recombinant expression strains containing them. EcUR206 grew in a typical logarithmic way whereas EcUR207 stopped its growth 20 min after IPTG induction and visual cell lyses was observed. Later, EcuR207 recommenced growing until it reached the final OD_{600} of non-transformed *E.coli* BL21 control strain (Figure 2A). The same behavior was described using 4 different clones and visual lysis of the cells was observed each time shortly after induction. In addition, Western-blot analyses revealed no gM-SAA3 protein expression from EcUR207. So, recombinant protein induction of EcUR207 not only caused host cell lysis but also failed in the expression of the recombinant protein (Figure 2B). Considering that EcUR207 subsequently recovered growth, plate counts of the culture were performed and showed that pURAD202 plasmid decreased in 3 orders of magnitude (data not shown). The plasmid loss and the following overgrowth of cells without plasmid had been previously described in pET vectors carrying SAA transcripts (Yamada et al., 1994) and it was always attributed to a toxic effect of the SAA protein expressed at basal levels. In the present study BL21 (DE3) strain was used for expression purposes in order to limit

basal expression and the sudden drop described in the culture absorbance was a better indicator of cell lysis rather than plasmid loss. In this context, cell lysis of human SAA1.1 but not SAA4, as well as mouse SAA2 and hamster SAA1, have been previously reported and related to the ability of SAA to form channels into the bacterial outer membrane (Hirakura et al., 2002). Protein Sequence analysis of the corresponding sequences (Human SAA1: GenBank CAG47037, human SAA4: GenBank CAG46558, Mouse SAA2: GenBank AAH24606 and Hamster SAA1: GenBank AAB27187) indicated that the isoforms that produced cell lysis contained an amino acid SNARE-like sequence (SDARE) whereas the human SAA4 (which did not produced cell lysis) did not (SRSRV). These data could indicate a possible functional bactericidal role of that SNARE region, since this is found in the long form of the gMSAA3. Further investigation of this is needed.

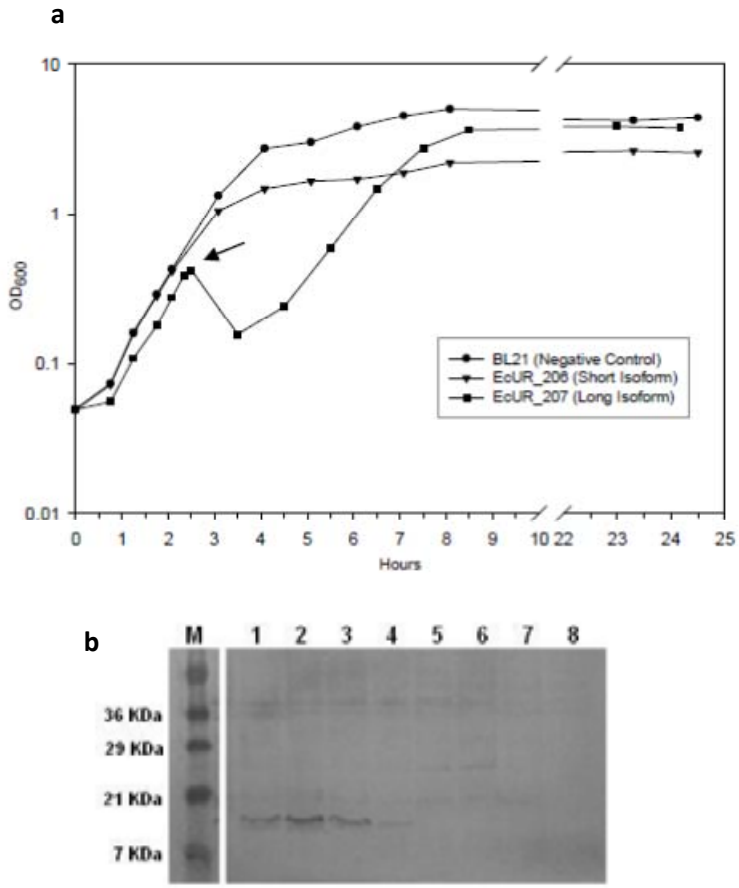


Figure 2. a) Typical growth pattern of EcUR_206 (containing short C-ter gMSAA3) and EcUR_207 (containing long C-ter gMSAA3) of one of 4 clones). Time of induction is marked with a black arrow. b) 12 % SDS PAGE and Western-blot using anti-his-tag of total cell extract from EcuR_206 (lanes 1 to 4) and EcuR_207 (lanes 5 to 8) at induction times of 1h (lanes 1 and 5), 2h (lanes 2 and 6), 3h (lanes 3 and 7) and 4h (lanes 4 and 8) of induction.

Different culture parameters were studied in order to improve C-terminal histidine tagged gM-SAA3 (C-ter gM-SAA3) production from the EcUR206 strain. The yields of recombinant C-ter

gM-SAA3 protein were higher at 1 and 2 hours after induction, with the use of a low concentration (0.1mM) of IPTG, and at an OD_{600} of induction closer to 1. Higher concentrations of ampicillin (250 and 500 µg/ml), the use of rich medium 2YTX and the growth temperature (O/N at 16°C) did not improve recombinant protein production (data not shown). The final optimal recombinant expression conditions for C-ter gM-SAA3 were 0.1 mM IPTG at an OD_{600} of 0.8 for 1.20 hours, 37°C and LB media supplemented with 100 µg/mL of ampicillin (data not shown). Therefore, low concentrations of inducer and short expression times were optimal for gM-SAA3 protein production, suggesting that the shorter gM-SAA3 isoform might also produce a toxic effect on the bacterial host cells.

Since his-tag proteins have been reported to be highly variable in expression levels and/or purification yield related to the position of the affinity tag (Dyson et al., 2004), a N-terminal his-tag short gM-SAA3 (N-ter gM-SAA3) was constructed. The plasmid containing the N-ter gM-SAA3 construction was named pURAD209 and EcUR210 was the recombinant expression strain. Sequence analyses revealed no differences between the C-ter (pURAD201) and N-ter (pURAD209) his-tagged constructs. EcUR210 recombinant strain was assessed using the optimized conditions described for expression of the C-ter gM-SAA3 construct. Higher amounts of N-ter gM-SAA3 protein were detected by Western-blot analyses compared to the C-ter gM-SAA3. Solubility of the recombinant protein (C-ter and N-ter gM-SAA3) was evaluated by analyzing the soluble and insoluble fractions (SF and IF) by Western-blot (Figure 3). In EcUR206 strain (C-ter gM-SAA3) recombinant protein was localized mainly in the soluble fraction whereas with EcUR210 (N-ter gM-SAA3) it was produced mainly as insoluble protein and formed inclusion bodies.

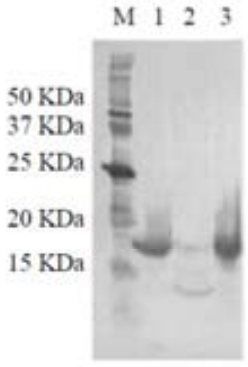


Figure 3. 12% SDS PAGE of soluble or insoluble fractions from the same culture volume followed by Western-blot using anti-his tag of N-ter gM-SAA3. Lane 1: Total cell extract, Lane 2: Soluble fraction, Lane 3: Insoluble fraction

.

C-ter and N-ter short gM-SAA3 were further purified from their respective SF by nickel chelation affinity chromatography. Final eluted sample gave a concentration of 0.73 mg/ml of C-ter gM-SAA3 and 0.18 mg/ml for N-ter gM-SAA3. The eluted samples were visualized by

Coomassie blue staining in order to check the purity (Figure 4a). Note that the molecular weight detected for the monomer of the recombinant C-ter gM-SAA3 was around 15 KDa, while the theoretical molecular weight was 17.8 KDa (entire protein plus the C-ter tag). It has been previously described that SAA3 contains a 18 amino acid leader peptide that is cleaved from the mature form (McDonald et al., 2001). Therefore, the molecular weight here observed could correspond to the protein without signal peptide indicating that C-ter gM-SAA3 protein suffered post-transcriptional changes to reach the mature conformation. In the C-ter construct a band around 30 KDa was seen indicating that the protein may be found as well as a dimer. The N-ter construct showed a main band around 30 KDa and also a weak band around 15 KDa (theoretical molecular weight was 15.7 KDa), indicating a preference for dimer formation. Differently, the molecular weight by SDS-PAGE of the insoluble N-ter protein was around 18 KDa (Figure 3). In summary, the inclusion of the histidine tail at the N-terminal position of the gM-SAA3 was not a good strategy to optimize protein expression since despite increasing protein expression it also drastically affected protein solubility.

The polyclonal antibody generated against gM-SAA3 was able to successfully recognise the C-ter gM-SAA3 protein in a Western-blot assay, obtaining a main band at 15 kDa and secondary bands of higher molecular weight, corresponding to the monomeric and multimeric forms visualized previously (Figure 4b). It also recognized M-SAA3 in bovine colostrums at its predicted molecular weight (data not shown). Since there are no commercially available antibodies against gM-SAA3, specific recognition not only was informative about the protein itself but also it represented a valuable tool for future diagnostic studies.

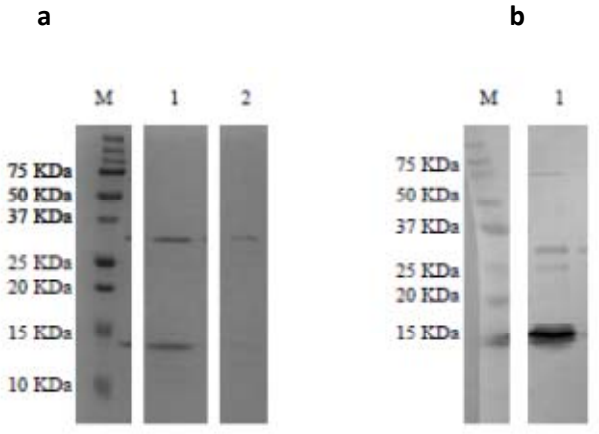


Figure 4. 12% SDS PAGE of the recombinant purified gM-SAA3. a) Coomassie blue staining. Lane 1: C-ter gM-SAA3 protein (9µg), lane 2: N-ter gM-SAA3 recombinant protein (3 µg) b) Western-blot (7.5 µg) using polyclonal anti-goat SAA3 and detection with secondary alkaline phosphatase conjugated antibody.

The folding of SAA1 and SAA2 which have considerable alpha helical content can change dramatically during amyloid formation which is largely beta sheet and conformations can change over time in physiological buffers (Wang et al., 2011) and it is known that purified SAA1 and serum SAA1 associated with HDL and recombinant SAA1 have different immune activating properties (Björkman et al., 2010). As yet little or no thought about this has been applied to research into SAA functional properties but careful thought will be needed in future. It will be important to devise expression systems that ensure correct native folding of the recombinant SAA isoforms. This is likely to apply equally to gMSAA3 with the same domain structure and lack of disulphide bonds.

3.3. Activity of the expressed protein and peptides

Recombinant short isoform of gM-SAA3 and derivative peptides were evaluated for possible effects against the viability of the isolated macrophages and no detrimental effect was observed (data not shown). The activation of phagocytosis by the recombinant protein was firstly evaluated in blood human macrophages. It was also evaluated the activity of the TFLK-like derived peptides, which have been related to protect intestine via mucin overexpression, in order to determine the possible role of the TFLK-like motif in the phagocytosis activation. Results were expressed as association index (AI, number of bacteria per 100 macrophages) and GFP+ (number of macrophages with one or more bacteria, expressed as a percentage). Macrophages isolated from a healthy volunteer (Figure 5) displayed low ability to associate with and take up bacteria (4% ± 2% GFP+ and AI of 10 ± 5). Human serum provided a strong positive control and interestingly, recombinant protein at 3 µg/ml showed similar effects, increasing the GFP+ macrophages to 30% ± 3% ($P<0.001$) and the AI to 63 ± 7 per 100 macrophages ($P<0.001$). The use of higher concentrations of recombinant protein (30 µg/ml) led to an increase of the active macrophages up to 53% ± 3% ($P<0.001$) and to a non-quantifiable AI due to the high number of bacteria associated. On the other hand, derivative SAA3 peptides did not show any effect. To demonstrate that this was not just membrane association we determined the Phagocytic Index (PI) using confocal microscopy. Very similar results were obtained indicating that the association index largely reflected phagocytosis (Human serum: GFP+ cells 67 ± 4%, PI 180 ± 11%; PBS: GFP+ cells 6 ± 2%, PI 12 ± 5%; 3 µg/ml gM-SAA3: GFP+ cells 42 ± 5%, PI 68 ± 11%).

A second donor was used to confirm the results. Again, human serum increased massively the phagocytosis by blood macrophages. In this case, protein at 3 µg/ml did not show effect but at 30 µg/ml produced similar effects to the human serum, increasing the bacterial association. Again, gM-SAA3 derived peptides did not show effect in phagocytosis activation.

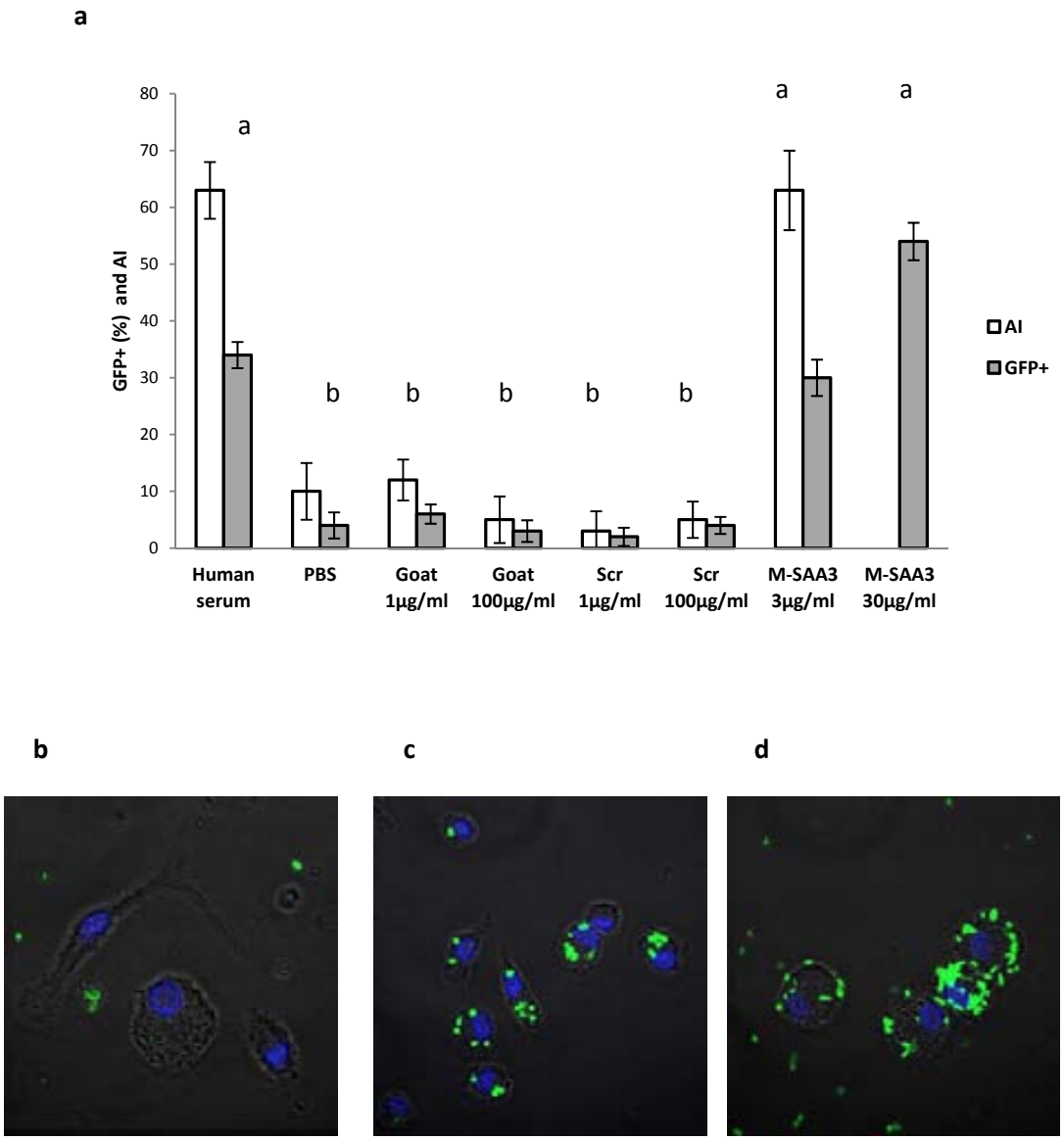


Figure 5. gM-SAA3 but not peptide increases bacterial uptake by human PBMC derived macrophages. a) Association index and GFP+ cells values of donor 1. (*, ** $p<0.05$) in the different treatments (Goat: 10-mer goat peptide, Scr: 10-mer TFLK-like scrambled peptide, SAA3: gM-SAA3 recombinant protein). b, c, d) Example of microscopic view of blood macrophages after incubation with GFP+ bacteria with PBS (b) or opsonised with (c) positive control human serum and (d) recombinant gM-SAA3 at 30 µg/ml from a typical donor.

Goat milk macrophages were also tested for the same purposes in a more physiological context. They were tested against the recombinant protein at 3 and 30 µg/ml, using as a positive control goat serum. Milk derived macrophages were evaluated with confocal microscopy and showed a basal level of macrophages activation of 13% ± 9% GFP + and a PI of 18 ± 15 per 100 macrophages. The 3 µg/ml recombinant protein showed similar levels of

activation, but the 30 µg/ml concentration increased both GFP+ cells (44% ± 9%, P<0.05) and PI (72 ± 17, P<0.05). Goat serum increased both indexes (Figure 6).

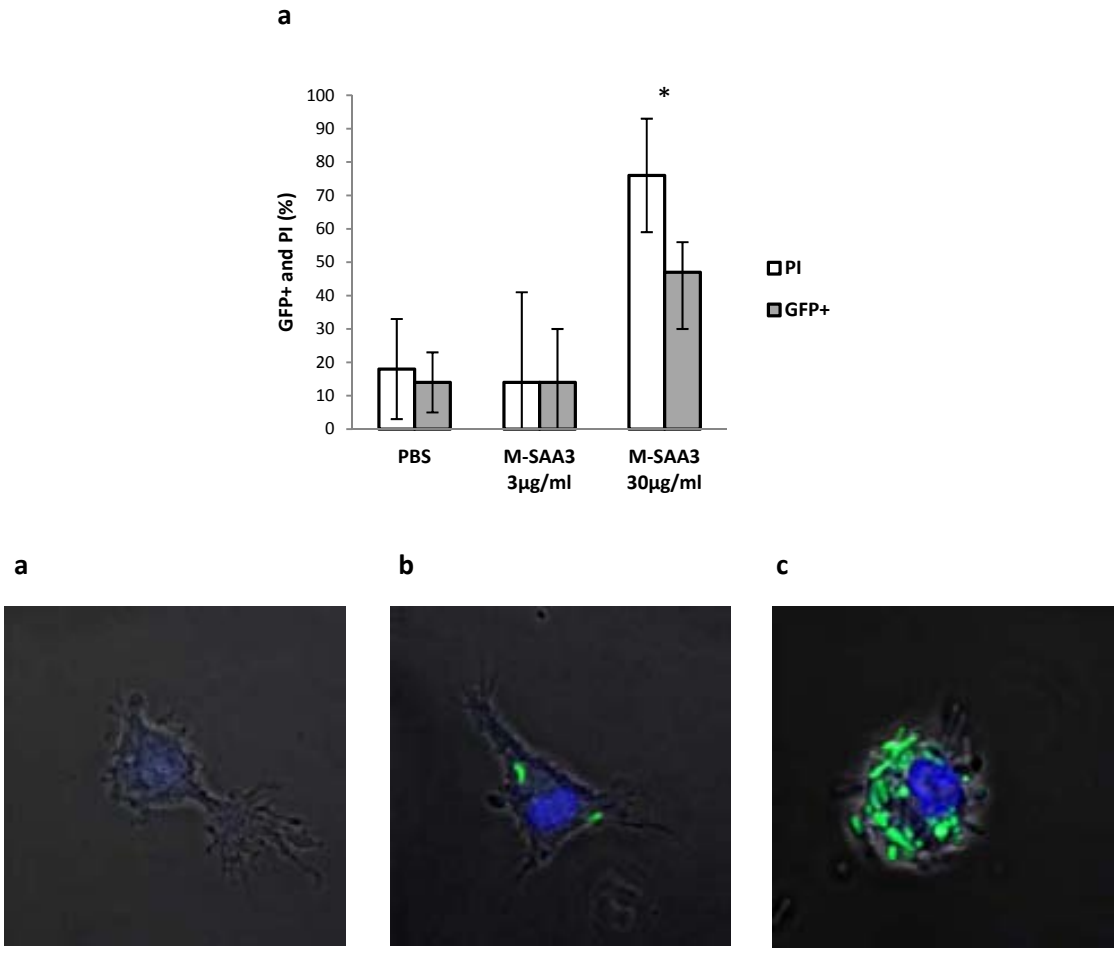


Figure 6. gMSAA3 increases uptake of bacteria by goat milk macrophages a) Phagocytotic index and GFP+ cells of goat milk macrophages (* p<0.05) in the different treatments. b-d) Example of typical microscopic view of goat milk macrophages after incubation with PBS (b), recombinant SAA3 at 30 µg/ml (c) and or positive control goat serum (d).

Therefore, results indicated that extrahepatic gM-SAA3 promotes phagocytosis as previously described for SAA circulating isoforms (Hari-Dass et al., 2005; Shah et al., 2006), indicating that the localized increase in milk SAA3 levels could further promote immune innate responses and help the clearance of bacteria in specific sites of infection. In contrast, gM-SAA3-derived peptides did not show such effect, which would indicate that the TFLK-like region responsible for the up-regulation of mucins in intestine is not the functional part of the protein involved in phagocytosis effects.

4. CONCLUSION

This work is the first attempt of expression of both gM-SAA3 forms and successful production and purification of the 483 bp isoform as a soluble protein gM-SAA3 contains a conserved and not mammary-restricted common N-terminal TFLR motive. Recombinant gM-SAA3, but not its TFLK-like derived peptides in the conditions herein described, activates phagocytosis of blood and milk macrophages, suggesting an important role in the maintenance of tissue health during infective processes.

5. AKNOWLEDGMENTS

The authors thank to Ruminant Production Department for helpful technical assistance and discussions. This study has been funded by INIA-MICINN (RTA2008-00050-C02-01).

Chapter IV – Study 2

Mammary Serum Amyloid A3 (M-SAA3) reduces bacterial translocation and enhances the immune response in epithelial intestinal cells

Anna Domènech[1], Anna Arís[1], Alfred Fernández-Castané[2], Àlex Bach[1,3] and Alícia Serrano

1. Ruminant Department of IRTA, Institute of Research in Agriculture and Technology. Torre Marimón, km 12,1 C-59, Caldes de Montbui, 08140, Barcelona, Spain.
2. Departament d'Enginyeria Química, Escola d'Enginyeria, Unitat de Biocatàlisi Aplicada associada al IQAC (CSIC), Universitat Autònoma de Barcelona, Edifici Q, 08193 Bellaterra (Cerdanyola del Vallés), Spain
3. ICREA, Institució Catalana de Recerca i Estudis Avançats, Barcelona (Spain)

1. INTRODUCTION

Serum amyloid A3 (SAA3) is an acute phase protein, and it is usually referred to be the main extrahepatic isoform of the SAA family (Ramadori et al., 1985; Meek and Benditt, 1986; Upragarin, 2005). Acute phase proteins are mainly produced in the liver, increasing the blood concentration up to 1000-fold during inflammation, infection or damage (Jensen and Whitehead, 1998). The SAA3 isoform is expressed in many tissues others than liver, observing a particularly high concentration in milk during infection (Eckersall et al., 2001 and 2006; Grönlund et al., 2005; Suojala et al., 2008) and after calving (McDonald et al, 2001; Larson et al., 2005; Molenaar et al., 2009).

Mammary SAA3 (M-SAA3) refers to the SAA3 expressed in mammal's mammary gland and which can be found in milk. Bovine M-SAA3 has been expressed recombinantly in both procariotic and eukaryotic systems (Manuell et al., 2007; Molenaar et al., 2009). Moreover, we have recently cloned and expressed successfully the goat M-SAA3 isoform in an *Escherichia coli* system. Two different goat M-SAA3 cDNA sequences in milk were observed, mainly differing in a 3 amino acid deletion whitin a SNARE motif. Only the short isoform was successfully expressed, suggesting that the SNARE motif could affect the bacterial viability (Domènech et al., 2012).

A highly protein conserved sequence is observed in all the members of the SAA family and among different species (Uhlar and Whitehead, 1999). Specifically, sequence analysis of goat M-SAA3 reveals that shares 91.6% of the nucleotides and 87% of the amino acids with the bovine form. In addition, goat M-SAA3 is 70% homologous to the human nucleotide sequence, but this human form is considered to be a pseudogene, as it produces a truncated protein (Kluve-Beckerman et al., 1991).

The functions of SAA family have been widely related to immunological roles. Particularly, goat M-SAA3 along with other SAA proteins, have been described to promote phagocytosis in macrophages and neutrophils (Hari-Dass et al., 2005; Shah et al., 2006; Domènech et al., 2012) helping the immune system to control infectious processes and the clearance of pathogenic bacteria. Bovine M-SAA3 also exhibited direct antimicrobial activity affecting cell viability of a range of bacteria such as *Escherichia coli*, *Streptococcus uberis* and *Pseudomonas aeruginosa* (Molenaar et al., 2009). Finally, synthetic peptides derived from human M-SAA3 protected intestinal cells from enteropathogenic infection in *in vitro* cell culture studies (Larson et al., 2003; Mack et al., 2003 (a); Gardiner et al., 2009). *Larson et al*. and *Gardiner et al.* described that 10- mer and 42-mer synthetic peptides derived from human M-SAA3 inhibited the binding

of *Enteropathogenic Escherichia coli* (EPEC) to HT-29 cell line by activating the Mucin 3 (MUC3) expression. This 42-mer peptide corresponded to a truncated form of the human M-SAA3, caused by the presence of a STOP codon in the codificant sequence. However, up to date, neither the RNA nor the 42-mer peptide have been detected in human tissues (Kluve-Beckerman et al., 1991). Moreover, *Manuell et al*. described that peptides derived from enzymatic digestion of bovine recombinant M-SAA3 also increased MUC3 levels in HT-29 cells (Manuell et al., 2007). These data suggest a clear functional immunological role of the M-SAA3, participating either directly in the protection of the mammary gland against pathogens or indirectly in the newborn gastrointestinal tract after milk intake. However, these studies have been carried out using synthetic peptides or tissue undetectable truncated forms of the M-SAA3 protein. There are no reports about the intestinal protection role of the M-SAA3 using the entire M-SAA3 protein.

Therefore, the aim of this study was to evaluate the role of intact goat M-SAA3 protein in gastrointestinal protection. We evaluated the effect of the protein in front of an enteropathogenic infection using an intestinal cell line. We analyzed the expression of interleukin-8 (IL-8) and MUC3 in the cells as two innate immunity parameters and we evaluated the possible direct effect of M-SAA3 against bacteria cell viability. We had previously successfully expressed recombinant goat M-SAA3 in flask cultures (Domènech et al., 2012) and in this study, we have described for the first time the scale-up production using bench-top bioreactor by performing substrate-limiting fed-batch cultures and subsequent purification of goat M-SAA3..

2. MATERIALS AND METHODS

2.1. Recombinant Expression of M-SAA3

2.1.1. Bacterial strains and culture conditions

Enteropathogenic Escherichia coli CECT 727 (EPEC), *Staphylococcus aureus* CECT 240 and *Lactobacillus rhamnosus* were purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain) and pathogenic *E. coli*, *S. aureus*, *Pseudomonas* and *Klebsiella* were isolated from milk of mastitic goats and kindly provided by Dr. Corrales from Universidad de Murcia (Spain). Pre-inocula were performed from a single colony in Luria Bertani (LB) media (10 g/l peptone, 5 g/l yeast extract and 10 g/l NaCl) or MRS (Difco) for *L. rhamnosus* at 37^{o}C in static conditions overnight (O/N). A serial dilution bank was plated on LB or MRS plates to quantify the CFU/ml in the preinocula. The EcUR206 strain (an *E. coli* BL21 Star (DE3)-pET101/D-TOPO vector containing the goat M-SAA3 sequence) was used in recombinant protein production (Domènech et al., 2012).

2.1.2. Media composition

For bioreactor experiments, the strain grown in LB medium was used to prepare pre-inocula cultures. A Defined medium (DM) composed by glucose 5 g/l, macroelements (K_2HPO_4 2.97 g/l, KH_2PO_4 0.596 g/l, NaCl 0.458 g/l, $(NH_4)_2SO_4$ 0.75 g/l), $MgSO_4 \cdot 7H_2O$ 0.112 g/l, $FeCl_3$ 0.006 g/l, thyamine 0.025 g/l, microelements solution 0.7 ml/l (containing $CaCl_2$ 1.44 g/l, $AlCl_3 \cdot 7H_2O$ 0.04 g/l, $ZnSO_4 \cdot 7H_2O$ 1.74 g/l, $CoCl_2 \cdot 6H_2O$ 0.16 g/l, $CuSO_4 \cdot H_2O$ 1.55 g/l, H_3BO_3 0.01 g/l, $MnCl_2 \cdot 4H_2O$ 1.42 g/l, $NiCl_2 \cdot 6H_2O$ 0.01 g/l, Na_2MoO_4 0.02 g/l) was used for the inocula cultures. The DM for the batch phase was composed of glucose 20 g/l, macroelements (K_2HPO_4 11.9 g/l, KH_2PO_4 2.4 g/l, NaCl 1.8 g/l, $(NH_4)_2SO_4$ 3 g/l), $MgSO_4 \cdot 7H_2O$ 0.45 g/l, $FeCl_3$ 0.02 g/l, thyamine 0.1 g/l, and microelements solution 2.86 ml/l. Ampicillin at 100 µg/ml was used in all cultures. For the fed-batch phase, a high glucose concentrated feeding solution was used (glucose 478 g/l, $MgSO_4 \cdot 7H_2O$ 9.56 g/l, $FeCl_3$ 0.49 g/l, thyamine 0.33 g/l, microelements solution 62.94 ml/l). Phosphates were not included in the feeding solution to avoid salts precipitation and 5 ml of a sterile concentrated solution (K_2HPO_4 500 g/l, KH_2PO_4 100 g/l) were added into the bioreactor every 30 optical density (OD_{600}) increase.

2.1.3. Growing conditions

For the shake-flask production, the process has been explained elsewhere (Domènech et al, 2012). Briefly, BL21/pURAD201 was grown in 400 ml of LB-Amp media were at an initial DO of 0.05 until log phase was achieved. Recombinant expression was induced with IPTG 0.1 mM for 1 hour 20 min. Cell pellet was obtained by centrifugation at 6000g for 10 min, and frozen at -80°C until use.

For the bioreactor experiments, recombinant SAA3 protein was produced in a Biostat® B bioreactor (Sartorius) equipped with a 2 L jar. Detailed bioreactor set up can be found elsewhere (Ruiz et al., 2009). Pre-inocula were grown O/N at 37 ^{o}C at 200 rpm from glycerol stocks. Five ml of the pre-inoculum culture were transferred into 100 ml of defined medium (DM) to prepare inocula cultures and were grown at 37°C and 200 rpm until log phase was achieved (OD_{600} = 1.2). Detailed description of batch phase conditions and feeding strategy has been previously described (Ruiz et al., 2013, accepted for publication). Briefly, 80 ml of the inoculum were transferred into the bioreactor containing 720 ml of DM. Batch phase was finalized after consumption of the carbon source. At this point, substrate-limiting fed-batch phase was initiated by maintaining µ (growth rate) at 0.22 h^{-1}. The specific growth rate (µ) was kept constant by setting an exponential feeding profile (Pinsach et al., 2006). When the culture reached 120 OD_{600}, the growth rate was changed at µ=0.1 and recombinant protein expression

was induced at 140 OD_{600} using IPTG 0.1 mM. Induction phase lasted 2 hours and the broth was harvested and processed by centrifugation at 10000 g for 30 min at 4°C to separate the biomass from the medium. Cell pellet was frozen at -80°C until processing.

2.2. Protein purification

Cell pellet was treated mechanically or by pressure. For mechanical disruption, cell pellets were resuspended to a OD_{600}=100 in 20 mM Na_2HPO_4 /NaH_2PO_4,0.5 M NaCl, pH 7.4 buffer containing lysozyme (0.2 mg/ml), DNase I and RNase A (20 µg/ml), cocktail inhibitor of proteases (1 mM) and $MgCl_2$ (1 mM) during 30 min at room temperature. The suspension was mixed with pre-weighted 0.1 mm glass beads (range 26-36 mg per ml of sample) (Biospec Product, Inc, Bartlesville, USA). Three disruption cycles of 45 sec each, with one min on ice within cycles, were carried out in the MiniBead Beater (Biospec Product, Inc, Bartlesville, USA).

For high pressure disruption, cell pellet was resuspended in 20 mM Na_2HPO_4 /NaH_2PO_4 buffer with lysozyme (0.2 mg/ml) and a cocktail of proteases inhibitors (1mM). The resuspended fraction was filtered (0.45 µm) and disrupted in a high pressure disruptor at 2.76 kbar in 3 rounds. The disrupted solutions were centrifuged for 15 min at 20000g at 4°C and the supernatant was considered the soluble fraction containing the recombinant protein.

Recombinant protein was purified using two strategies: commercial His Spin Trap columns (GE Healthcare) and Ni Sepharose 6 Fast Flow matrix (GE Healthcare) coupled to a batch/gravity flow purification, following manufacturer instructions. The purified protein was dyalized O/N at 4°C against phosphate buffer saline (PBS) and it was further quantified using spectrophotometry ((A_{280}×Molecular weight)/Extinction coefficient; (mg/ml)).

2.3. SDS-PAGE, Comassie and Western blot

One ml culture samples were collected and centrifuged 5 min at 6000g, 4°C. Cell pellets and supernatant were stored at -20°C until analyses. Proteins were visualized by SDS-PAGE in 12% polyacrylamide gels. Before loading, cell pellets were resuspended with water up to DO_{600} of 0,03 and samples were boiled 10 min with Laemmli 2x (Sigma). Gels were run for 60 min at 200V. Proteins were either stained with Coomassie brilliant blue (1 hour) or transferred to Immuno-Blot PDVF membranes (at 100V during 60 min). In western blot, the membranes were blocked with Tris-buffer solution with 0.05% (v/v) Tween-20 (TBST) and 5% dry skimmed milk. First and second antibodies were a purified caprine anti-SAA3 antibody diluted 1:3000 (Domènech et al., 2012), and an anti-rabbit IgG conjugated to alkaline phosphatase (A3687, Sigma) diluted 1:20000 in TBST.

2.4. Antimicrobial assays

Growing cultures were performed starting at a concentration of $6{\cdot}10^5$ CFU/ml from pre-inocula. Cells were cultured by duplicate in a 96-well plate and were incubated with recombinant M-SAA3 at 50 µg/ml or imidazole (GE Healthcare) at range 6.25 to 200 µg/ml. Growth was measured as the increase of OD_{600}, which was recorded every 30 min, and final cell viability was evaluated after 3 hours of incubation. Serial dilutions were plated in the corresponding media, McConkey (VWR) for *E. coli* and *Klebsiella*, Nutrient (5 g/l beef extract, 10 g/l peptone, 5 g/l NaCl) for *S. aureus* and LB for *Pseudomonas*). Colonies were quantified after 16 hours at 37°C.

2.5. *In vitro* gastrointestinal assays

CaCo-2 cells were purchased from American Tissue Culture Collection (ATCC, Rockwille, MD, USA) and grown at 37°C and 5% CO_2 in DMEM media supplemented with 10% Fetal calf serum (FCS) and 2 mM Glutamax, at an initial concentration of 10^4 cells/well. After 21 days of growth, cells differentiated to a small intestinal phenotype. Differentiated intestinal CaCo-2 cells were incubated in sixtiplicates during 1h with 30 µg/ml of M-SAA3 and then infected for 2h with 10^6 CFU/well of EPEC. PBS and 10^8 CFU/well of *Lactobacillus rhamnosus* were used as negative and positive controls. After infection, cells were washed once with chilled PBS and bacteria were released with Triton 0.1%. EPEC internalization in CaCo-2 cells was quantified by serial diluting and plating the cell extract into McConkey agar media. Colonies were counted after O/N growth at 37 ^{o}C.

2.6. Gene expression analyses

For gene expression analyses, differentiated intestinal CaCo-2 cells were incubated by sixtiplicate during 1h with 30 µg/ml of M-SAA3, and 10^8 CFU/well of *L. rhamnosus* or PBS as positive and negative controls respectively. Then, cells were challenged either with 10^6 CFU/well of EPEC or kept uninfected. After 2 hours, total RNA was extracted using TRIzol® (Invitrogen). One microgram of RNA was retrotranscribed to cDNA using IScript cDNA synthesis kit (Bio-Rad) following manufacturer's instructions. qPCR was performed for human IL-8 and MUC3 using specific primers. A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBER Green Fluorescent (Bio-Rad), and the optimized primer concentration for each gene (see Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10s at 95°C, 15s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the $2^{\Delta Ct}$ method with ACTB as reference gene control.

Gene	Fw	Rv	At	µM
ACTB	CTGGACTTCGAGCAGGAGAT	CCCGTCAGGAAGCTCGTAG	57°C	0.125
IL-8	TTGAGAGTGGACCACACTGC	TGCACCCAGTTTTCCTTGG	55°C	0.5
MUC3	GCTGTGGACCCCTCAGAATG	TGTTCACATCCTGGCTGGCG	57°C	0.125

Table 1 Sequence, annealing temperature (At) and concentration of the primers used for qPCR.

2.7. Statistical analyses

Viable cell counts, MUC3 and IL-8 expression data were analyzed using a mixed effect model (SAS Inst. Inc., Cary, NC). For the viable cell count, the model included treatment as a fixed effect. The MUC3 and IL-8 were distributed in a 2×3 factorial design. The model included the treatment (Control, *L. rhamnosus* and M-SAA3), infection status (infected with EPEC and non-infected) and the interaction between treatment and infection, as fixed effects.

3. RESULTS AND DISCUSSION

3.1. Goat M- SAA3 protein expression in bioreactor and purification

Recombinant goat M-SAA3 was successfully expressed in the bioreactor system using Western-Blot analysis. An increase in the protein production was observed along the time of induction. As can be seen in Figure 1, goat M-SAA3 obtained in the bioreactor had a similar molecular weight (15 KDa) and appearance than goat M-SAA3 obtained in shake-flasks, indicating that in both processes the M-SAA3 was similarly produced. This is relevant considering that the amyloid related protein family often have a high tendency to aggregate and multimerize with the risk to exert different types of activity (Molenaar et al., 2009). By using the fed-batch strategy, recombinant goat M-SAA3 was obtained at a concentration of 13-16 mg/l of culture. In contrast, the obtained concentration was 0.125 mg/l of culture in shake-flasks. Considering differences in final cell concentration (DO_{600}=140 vs DO_{600} =1.8, for bioreactor and flasks respectively), there were no differences in specific cellular protein expression (mg-SAA3/OD_{600}) among the two mechanisms of production which interestingly indicated that the protein was similarly expressed.

Recombinant expression of SAA proteins is widely known for its difficulty (Hirakura et al., 2002; Molenaar et al., 2009) and to our knowledge, no M-SAA3 obtained in bioreactors has been previously described. Our findings described that M-SAA3 could be equally produced in different culturing strategies offering the possibility to scale-up the production in order to obtain higher amounts of protein for further applications. Moreover, the recombinant production of proteins difficult to purify from a natural source (for example milk or sera) is an useful mechanism to study its implication in functional processes.

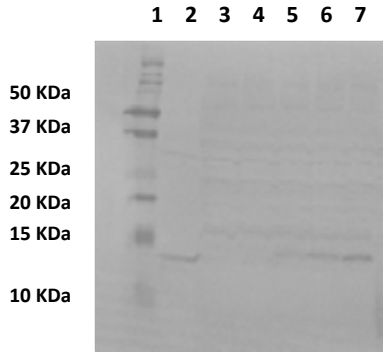


Figure 1. Western blot anti-SAA3 of the cell pellets obtained during the fed-batch process.

The production of the recombinant protein increased along the time, observing a clear band at 2 hours post-induction. Lane 1: molecular weight, Lane 2: positive control, purified erlenmeyer M-SAA3, Lane 3: cell pellet at T=0 (time of induction), Lanes 4 to 7: intervals of 30 min during the induction of the recombinant protein.

3.2. Goat M-SAA3 reduces enteropathogenic infection in epithelial cells

The effect of intact goat M-SAA3 in CaCo-2 cells during an EPEC infection was evaluated. Goat M-SAA3 efficiently reduced the levels of bacteria internalization *in vitro* (Figure 2). After 2 hours of EPEC infection, when goat M-SAA3 was added to the CaCo-2 cells, the levels of EPEC internalized in the epithelial cells were reduced up to 75% ($P < 0.001$). Positive control, *L. rhamnosus* GG, which has been previously described to inhibit gastrointestinal infection (Larson et al., 2003; Mack et al., 2003 (b)), also exhibited a clear reduction of EPEC internalization ($P < 0.001$). These results are in agreement with previous findings that described the functionality of synthetic M-SAA3 derived peptides in the inhibition of EPEC strains to HT-29 epithelial cell line (Larson et al., 2003; Mack et al., 2003 (a); Gardiner et al., 2009).

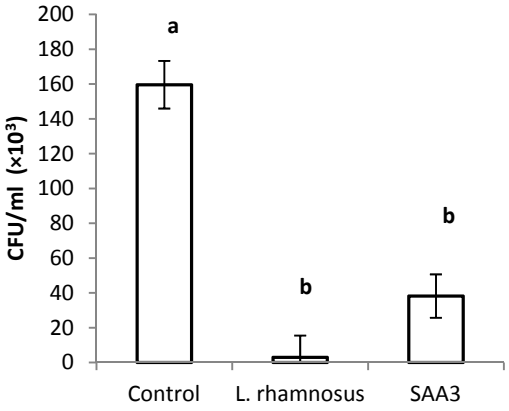


Figure 2. Viable cell counts (CFU/ml) of internalized EPEC in CaCo-2 cells.

Bars represent the means ± SEM in the different treatments. Bars with different superscripts differ ($P < 0.001$). M-SAA3 clearly inhibited the internalization of bacteria (75% of reduction). The probiotic *L. rhamnosus* was used as a positive control.

3.3. Innate immunity during the M-SAA3 treatment

The expression of MUC3 and IL-8 were evaluated after the intestinal cells were incubated with goat M-SAA3. A tendency ($P = 0.06$) in the interaction between treatment and infection status was observed for MUC3 expression results (Figure 3). M-SAA3 presence increased in 2-fold ($P < 0.05$) the levels of MUC3 in infective conditions respect to the negative control and *L. rhamnosus* treatment. The increase of the MUC3 expression was only observed in infective conditions, obtaining similar levels of expression in the three treatments in absence of EPEC. Mucus layer is considered to be one of the first barriers of the innate immune response (Hecht, 1999). The physical contact between the bacteria and enterocytes represents the first step during the infective process. MUC3 is a membrane-associated mucin expressed along the intestine and it has been described to be highly expressed in CaCo-2 cell line (Van Klinken et al., 1996; Bu et al, 2011). The increase in MUC3 levels suggested that the protective role exert by the M-SAA3 could be at the mucus layer level. *Mack et al*. described an increase in the MUC3 levels of HT-29 cells without infection when using bovine M-SAA3 peptides containing a specific N-terminal TFLK domain (Mack et al., 2003 (a)). Despite goat M-SAA3 contains a TFLK-like domain (TFLR) (Domènech et al., 2012) the capacity to overexpress mucin in absence of infection was not observed under the described circumstances. To date, there are no reports that demonstrate that M-SAA3 up-regulates mucin expression during infection. It is important to notice that despite the high inhibitory effect on bacterial translocation, the probiotic *L. rhamnosus* did not increase the mucin expression in any case. In contrast, other authors (Larosn et al., 2003; Mack et al., 2003 (a)) found a direct effect for the *L. rhamnosus* in increasing the MUC3 levels in HT29 cells in non infective conditions. Moreover, other probiotics such as *Lactobacillus casei GG*, have been described to up-regulate mucin expression as a mechanism of defense during intestinal infections (Mattar et al., 2002).

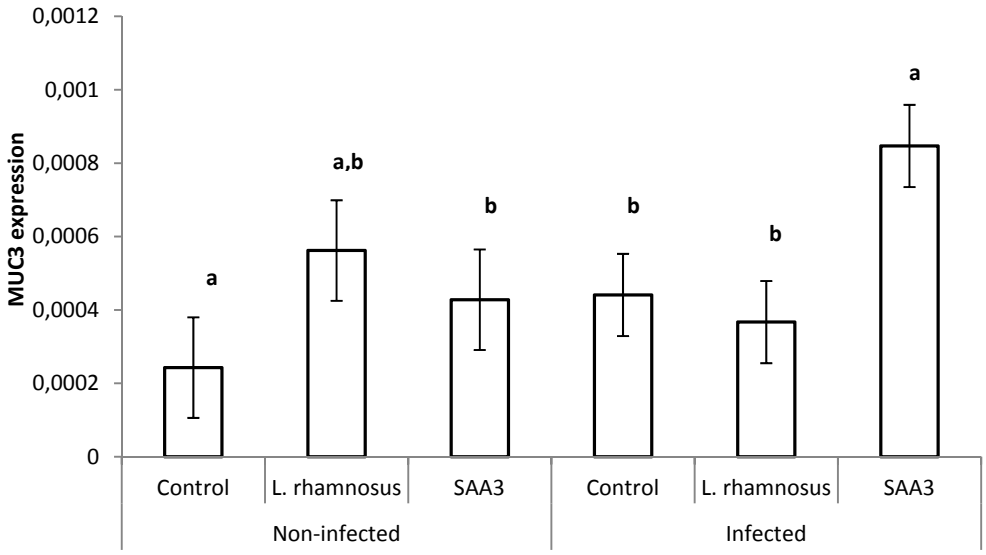


Figure 3. MUC3 expression levels of CaCo-2 epithelial cells.

Bars represent the means ± SEM in the different treatments. A tendency ($P = 0.06$) in the interaction between treatment and infection status were observed. Bars with different superscripts differ ($P < 0.05$). The graph shows the levels of expression in the different treatments (Control, *L. rhamnosus* and M-SAA3) in both infective and non-infective conditions. The M-SAA3 increased in 2-fold the expression of MUC3 in infective conditions.

The IL-8 expresion was affected by treatment ($P < 0.001$) and infection status ($P < 0.001$). An interaction ($P < 0.001$) was observed between treatment and infection. The M-SAA3 increased around 20% the levels of IL-8 during infection (Figure 4). As it was previously observed with MUC3, M-SAA3 did not increase IL-8 expression in absence of EPEC. On the other hand, *L. rhamnosus*, clearly inhibited the expression of IL-8 in infective conditions.

Our study points out the need of infection to obtain the increase in innate immune response mediators, contrasting previous studies which found a direct activity without the need of an infective process (Mack et al., 2003; Jijon et al., 2005).

A reduction of the infection around 75% was observed, and thus, the activation of the IL-8 was expected to be lower as IL-8 is one of the main cytokines activated during an intestinal infection (Eckmann et al, 1993; Hecht et al., 1997). However, the increase in IL-8 expression correlates with previous studies that indicate that members of the family SAA activated the expression of the IL-8 in cells that participate directly in the immune response such neutrophils (He and Ye, 2003; Lee et al., 2009) and monocytes (Ribeiro et al., 2003), but also in non-immune related cells like intestinal epithelial cells (Jijon et al., 2005). The main function of the IL-8 is the recruitment of immune response mediators to the site of inflammation (Hammond

et al., 1995), indicating that apart from reducing directly the infection of enteropathogens, M-SAA3 could also be activating the innate immune response at a local level.

The *L. rhamnosus* reduced the levels of IL-8 expression during infection, due to the ability of some probiotics to balance the innate immune response during infection. This IL-8 decrease can be due either for the reduction of bacteria load or for a direct inhibitory effect of the NF-κB factor of intestinal cells, which is the responsible for the up-regulation of IL-8 among other immune response mediators (Wang et al., 2011).

The gene expression results suggested that the M-SAA3 could be involved in the protection of the gastrointestinal tract by reducing directly the internalization of pathogenic bacteria via mucin overexpression and also by activating pro-inflammatory cytokines and further immune cellular efectors to the site of infection.

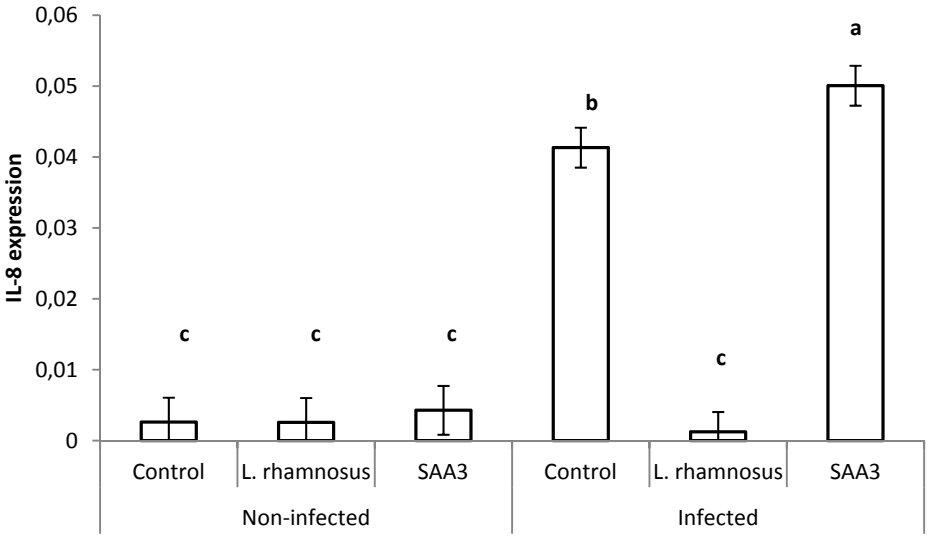


Figure 4. IL-8 expression levels of CaCo-2 epithelial cells.

Bars represent the means ± SEM in the different treatments. IL-8 expression was affected by treatment ($P < 0.001$) and infection status ($P < 0.001$). An interaction ($P < 0.001$) was between treatment and infection was observed. Bars with different superscripts differ ($P < 0.05$). The graph shows the levels of expression in the different treatments (Control, *L. rhamnosus* and M-SAA3) in both infective and non-infective conditions. The M-SAA3 increased the levels of IL-8 in infective conditions.

3.4. Recombinant mammary SAA3 doesn't affect bacteria viability

Recent studies describe a direct antibacterial activity of the SAA proteins (Molenaar et al., 2009). This direct antibacterial activity could be also participating in the reduction of the bacterial translocation observed in the *in vitro* assays. Therefore, cell viability was assessed by recording the increase of the OD_{600} of different pathogens cultures in the presence of goat M-SAA3 and finally plating the bacteria in the corresponding media, as described in material and methods. First of all, it was necessary to discard a possible toxic effect of traces of imidazole in

the final recombinant protein stock. Imidazole is widely used in protein purification processes in the final eluting step but it can present cell toxicity effects (Land et al., 1999; Löfmark et al, 2010). Increasing concentrations of imidazole were incubated with *E. coli* and *S. aureus*, and a clear delay in growth was observed (Figure 5). Moreover, this delay was different between Gram negative and positive bacteria. In *E. coli* it was observed a clear imidazole threshold effect at 25 mM observing an 80% reduction of growth, meanwhile the following lower concentration tested, 12.5 mM, had no effect compared to the negative control (PBS treatment). In contrast, in *S. aureus* it was rather a dose-effect relationship, obtaining a logarithmically correlation ($y = -43.05\ln(x)-84.143$, $R^2=0.99$) between imidazole concentration and OD_{600} decrease. In both species, 25 mM of imidazole was the first concentration in producing detectable differences in growth, but the growth delay reported at 25 mM was smaller in *S. aureus* than in *E. coli*. Since imidazole can be cell-internalized by passive diffusion through the membrane (Löfmark et al., 2010), the different membrane structure among both Gram negative and positive strains could be the reason for the different reported effect. The inhibitory effects of the imidazole were abolished after dyalisis (data not shown), indicating that the recombinant protein could be used for downstream processes only when the dyalisis is performed.

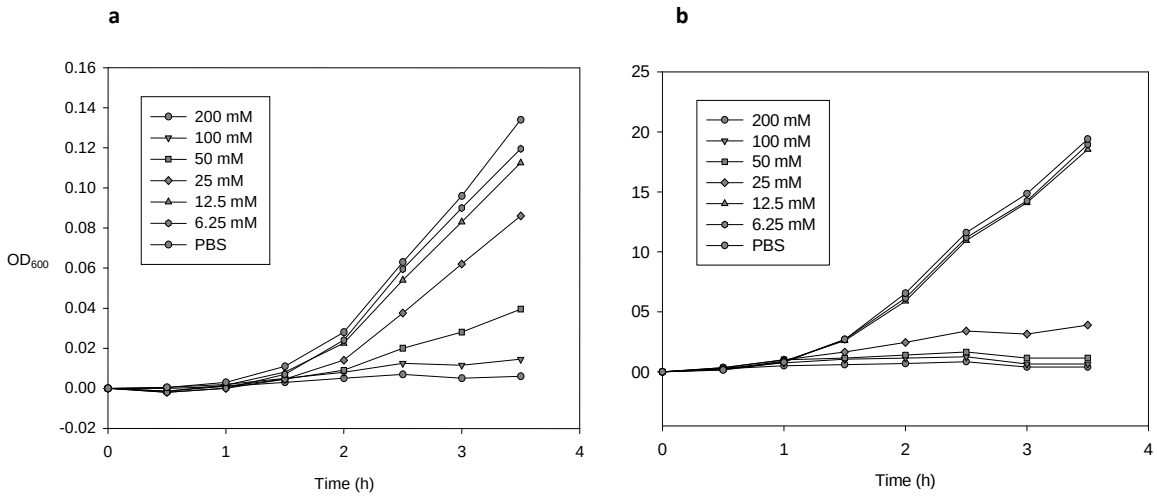


Figure 5. Imidazole concentration effect in bacterial growth.

Graphs represent bacterial OD_{600} increase during 3.5 hours of incubation for *E. coli* (a) and *S. aureus* (b). A dose-effect was observed for gram negative bacteria meanwhile in gram positive it was a threshold effect.

There were no differences between the negative control and the bacteria incubated with the M-SAA3 , neither in the OD_{600} levels nor in the final quantification by colony counting (Figure

6). These results indicated that the protein did not elicit a direct antimicrobial effect. Other authors have also previously described that SAA related proteins did not show any direct antibacterial effect. *Gardiner et al*., working with the human 42-mer protein did not show detrimental effect against EPEC, *S.* Typhimurium UK1 and *L. rhamnosus* (Gardiner et al., 2009). Moreover, *Erman et al*., found that the hepatic commercial SAA1/2 did not affect uropathogenic *E. coli* growth (Erman et al., 2012). However, *Molenaar et al*, found a direct effect of bovine M-SAA3 on bacteria cell viability and showed a clear inhibition of *E. coli, S. uberis* and *P. aeruginosa* when incubated with bovine M-SAA3 (Molenaar et al., 2009). There is a high homology between the bovine protein used by *Moolenar et al*. and goat M-SAA3 (both forms shared the 91.3% of nucleotide and 86.3% of amino acid sequence).

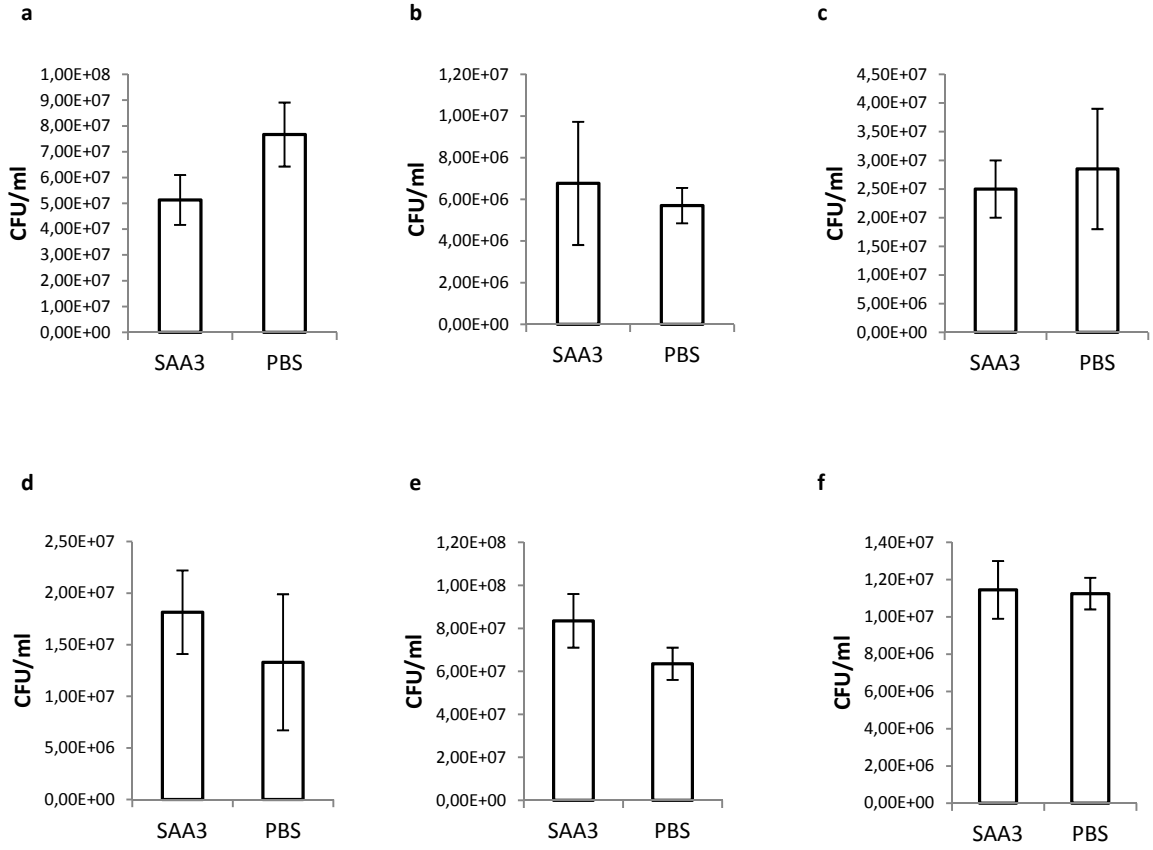


Figure 6. Direct antibacterial activity against pathogenic bacteria.

Bars represent the viable cell count after three hours of incubation with/without the M-SAA3. Laboratory strains EPEC (a), *S. aureus* CECT 240 (b) and pathogenic bacteria isolated from mastitic milk *E. coli* (c), *S. aureus* (d), *Klebsiella* (e) and *Pseudomonas* (f). No differences were observed between treatments.

Interestingly, the main difference between both sequences is a deletion of 3 amino acids within the SNARE domain (78-82 residues of the putative protein) in goat M-SAA3. In this

context, we have previously suggested a potential antibacterial activity of the SNARE-like region. In previous work the cloning of two isoforms of goat M-SAA3 was described (Domènech et al., 2012). These two forms mainly differed in this region, observing a long isoform which contained the SNARE motif and a short isoform without it. The short isoform was successfully expressed recombinantly meanwhile the other produced massive cell lysis. In addition, other authors have described problems in the expression of members of the SAA family. Human SAA1 and mouse SAA2, which contained the SNARE-like, region produced cell lysis but human SAA4 (which did not contain this region) was easily expressed (Hirakura et al., 2002).

4. CONCLUSIONS

These results confirm that recombinant goat M-SAA3 has a gastrointestinal protective role by reducing enteropahtogenic internalization *in vitro*. In addition, the protection is more related to the MUC3 up-regulation rather than a direct effect against bacteria. Furthermore, an activation of internal innate immune response during infection has been described via IL-8 overexpression.

All together, these results confirm that the M-SAA3 exerts an important role during intestinal infections that lead to the clearance of bacteria, either directly inhibiting the bacterial translocation or activating the innate immune response.

5. AKNOWLEDGMENTS

We are indebted to the Cell Culture Unit of SCAC at the UAB. The authors thank to Ruminant Production Department for helpful technical assistance and discussions. This study has been funded by INIA-MICINN (RTA2008-00050-C02-01).

Chapter V – Study 3

Mammary Serum Amyloid A3 (M-SAA3) protection against *Salmonella* Dublin in bovine *ex vivo* intestinal model and dendritic cells

Anna Domènech[1], Alicia Serrano, Àlex Bach[1,2] and Anna Arís[1]

1. Department of Ruminant Production, IRTA, Institute of Research in Agriculture and Technology. Torre Marimón, km 12,1 C-59, Caldes de Montbui, 08140, Barcelona, Spain.
2. ICREA, Institució Catalana de Recerca i Estudis Avançats, Barcelona (Spain)

1. INTRODUCTION

Serum amyloid A (SAA) is a family of proteins which contain acute phase and constitutive members (Uhlar and Whitehead, 1999). Acute-phase proteins (APP) increase their levels in blood up to 1000-fold during inflammation, infection or damage meanwhile constitutive forms remain unaltered (Jensen and Whitehead, 1998). The APPs play an important role in the onset of the innate immune response, activating a series of inflammatory genes that fight the infection (Uhlar and Whitehead, 1999).

The SAA3 isoform is mainly expressed extrahepatically while the other member of the family are produced in the liver and then secreted to the plasma (Ramadori et al., 1985; Meek and Benditt, 1986; Upragarin, 2005). Mammary SAA3 (M-SAA3) is produced in the mammary gland and secreted to milk in high concentrations specially after calving (McDonald et al., 2001; Larson et al., 2005; Molenaar et al., 2009) and during infection (Eckersall et al., 2001 and 2006; Grönlund et al., 2005; Suojala et al., 2008).

The high concentration of M-SAA3 in milk during infections and colostrum, along with the immune related functions, suggest that M-SAA3 plays an important role in both the maintenance of the mammary gland integrity and the gastrointestinal tract protection of the newborn after milk intake.

In this regard, M-SAA3 has been associated to the protection of gastrointestinal tract from infection using commercial intestinal cell lines *in vitro* (Larson et al., 2003; Mack et al., 2003 Gardiner et al., 2009). *Larson et al*. and *Gardiner et al.* described that 10- and 42-mer synthetic peptides derived from human M-SAA3 inhibited the binding of *Enteropathogenic Escherichia coli* (EPEC) to HT-29 cell line by activating the Mucin 3 (MUC3) expression. In addition, SAA members have been described to increase the cytokine expression in cells that participate directly in the immune response such neutrophils (He and Ye, 2003; Lee et al., 2009) and monocytes (Ribeiro et al., 2003), but also in intestinal epithelial cells (Jijon et al., 2005). Most of the data available regarding the functional role of the M-SAA3 at a gastrointestinal level have been conducted using intestinal commercial cell lines (Larson et al., 2003; Mack et al., 2003 Gardiner et al., 2009) such as Caco-2 and HT-29 and using truncated versions of M-SAA3 or small SAA3 peptides. In this work, we were interested in testing the entire protein M-SAA3 protein in a more physiological environment such as intestinal tissue explants where different cell types coexist and perform in a more realistic context.

Intestine is a complex tissue composed by different cell types. In addition, immunological specialized regions called Peyer's Patches (PP), intensively participate in the protection against

enteropathogenic infection. PP are part of the gut-associated lymphoid tissue (GALT) and are distributed throughout the mucosa and submucosa of the gastrointestinal tract but in more density in the jejunum (Cesta, 2006). PP form a dome with lymphoid follicles of B and T lymphocytes. Overlying the follicles a specialized ephithelium which contain M-cells is observed. The M-cells are responsible for the antigen uptake from the intestinal lumen and the transport to the antigen presenting cells scattered around the follicles, mainly referred as dendritic cells (DC) and macrophages (Cesta, 2006). The DC are professional antigen presenting cells, with a key role in the onset of the adaptive immune response. The DC may be the only cell type able to activate naïve T cells (Banchereau and Steinman, 1998). Circulating immature DC in the blood stream enter to tissues, where become resident cells. Immature DC present a high phagocytic activity and are responsible for antigen uptake. Maduration is reached, among other stimulus, by bacterial recognition. Mature DC reduces slightly the phagocytic activity and enhance other features such IL-12 expression, antigen presentation and the capacity to migrate towards secondary lymphatic nodes (Macatonia et al., 1995; Banchereau and Steinman, 1998; Banchereau et al., 2000). Maturation can be detected by an increase of related molecules such as IL-12, CD80 and CCR7 (Zhao et al., 2006; Xia et al., 2009; Facci et al., 2010). The IL-12 activates the INFγ producing cells and direct to the Th1 response. The CD80 is an important co-estimulatory molecule that participates in the antigen presentation along with the major histocompatibility complex II (MHCII). The CCR7 is a receptor that helps the migration of mature DC.

Salmonella enterica subsp. *enterica* serovar Dublin (*Salmonella* Dublin) is considered to be host-adapted to cattle but infrequently infects other mammals including humans (Uzzau et al., 2000). Affects both young and adults but mainly infects calves between 2 weeks and 3 months of age, producing acute enteritis and systemic disease leading to important economical and welfare losses. The most common route of infection is by ingestion (Uzzau et al., 2000). The colonization of the intestinal epithelium is mainly produced at PP level and specifically infecting the epithelial M cells and finally reaching systemic circulation (Jones, 1997).

In this study, we hypostasized that the entire M-SAA3 protein could have a relevant role in the protection of calves gastrointestinal tract against *Salmonella* Dublin infection.

The goal of this study was to evaluate the role of the entire M-SAA3 on bovine gastrointestinal tract using an optimized *ex vivo* intestinal model and an *in vitro* blood derived DC cultures, exploring the protective effect in intestinal infections and the activation of the innate immune response.

2. MATERIALS AND METHODS

2.1. Bacterial culture

Salmonella enteric subsp. enterica serovar Dublin (CECT 4152) was purchased from CECT (Colección Española de Cultivos Tipo, CSIC Valencia, Spain). Single colonies were grown over-night (O/N) with 10 ml of Nutrient agar media at 37°C in static conditions. Cell pellet was obtained by centrifugation at 6000g for 10 min at 4°C. Bacterial concentration was calculated by spectrophotometric quantification (DO_{600}=0.7 correspond to 10^9 CFU/ml). Bacterial doses of infections were obtained by resuspending and diluting the cell pellet with the corresponding media (KREBS or supplemented RPMI).

2.2. Recombinant M-SAA3 production

The EcUR206 strain (an *E. coli* BL21 Star (DE3)-pET101/D-TOPO vector containing the goat M-SAA3 sequence) was used in recombinant protein production. The process has been explained elsewhere (Domènech et al., 2012). Briefly, BL21/pURAD1 was grown in 400 ml of LB-Amp media were at an initial DO of 0.05 until log phase was achieved. Recombinant expression was induced by IPTG 0.1 mM for 1 h and 20 min Cell pellet was obtained by centrifugation at 6000g for 10 min, and frozen at -80°C until use. Cell pellets were resuspended to a DO_{600}=100 in 20 mM Na_2HPO_4/NaH_2PO_4,0.5 M NaCl, pH 7.4 buffer containing lysozyme (0.2 mg/ml), DNase I and RNase A (20 µg/ml), cocktail inhibitor of proteases (1 mM) and $MgCl_2$ (1 mM) during 30 min at room temperature. The suspension was mixed with pre-weighted 0.1 mm glass beads (range 26-36 mg per ml of sample) (Biospec Product, Inc, Bartlesville, USA). Three cycles of beating of 45 sec each, with one min on ice within cycles, were carried out in the MiniBead Beater (Biospec Product, Inc, Bartlesville, USA). The disrupted cell suspension was centrifuged for 15 min at 20000g at 4°C and the supernatant was considered the soluble fraction containing the recombinant protein. Recombinant protein was purified with commercial His Spin Trap columns (GE Healthcare, Uppsala, Sweden), following manufacturer instructions. The purified protein was dialyzed O/N at 4°C against PBS and it was further quantified using spectrophotometry ((A_{280} × Molecular weight)/Extinction coefficient; mg/ml).

2.3. *Ex vivo* bovine intestinal explants

Segments of intestinal tissue were obtained at slaughterhouse from three eleven-month bulls of an average weight of 450 Kg. PP at jejune level (18 meters from pylor) were isolated, gently washed with sterile chilled Phosphate saline buffer (PBS) and immediately transported to the laboratory facilities. Serosa and muscular layers were removed, and the tissue was cut into 0.8 cm^2 diameter segments using veterinarian punchs. The explants were immobilized using pins

in 24-well plates with agarose 4% as bedding and incubated with KREBS media (117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 11 mM Glucose) in 5% CO_2 at 37°C.

2.4. Bovine monocyte isolation and differentiation to dendritic cells

Bovine blood with sodium heparin 50 UI/ml, as anticoagulant, was obtained at the slaughterhouse. The blood was diluted 1:1 with PBS-2% Fetal Calf Serum (FCS) at room temperature (RT). The diluted blood was layered onto Histopaque-1077 (Sigma) at 1:2 ratio (histopaque/diluted blood) and centrifuged at 600g (brakes off) during 30 min at RT. PBMC were isolated from the interphase, washed with media and centrifuged 10 min at 200g. The cell pellet was resuspended and incubated with 10 ml of Red Blood Lysis buffer (Sigma) and incubated for 10 min at RT. After centrifuging at 250 g for 5 min, the process was repeated until a free-erythrocyte pellet was observed. PBMC were resuspended in supplemented media (RPMI-10% FCS, 50 µM β-mercapthoethanol, 1% penicillin/streptomycin) and quantified using an haemocytometer. PBMC were incubated in a 75 cm^2 Falcon in a ratio of 10^6 cells/cm^2, and incubated during 1.5 hours in 5% CO_2 at 37°C. After incubation, adherent monocytes were washed once with PBS and incubated with supplemented media with Interleukin 4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokines (Bovine Dendritic Cell Growht kit, Bionova, 1:20 dilution). Media was changed at day 3 and total cell differentiation by microscopy was observed at day 6. The DC phenotype was checked direct morphology observation at the microscope and by qPCR quantification of CD11c gene. The day of the experiment, cells were scrapped and quantified. $4 \cdot 10^5$ dendritic cells/well were seeded in 24-well plates, and incubated with supplemented RPMI without antibiotics.

2.5. *Salmonella* Dublin infection of ex vivo explants

Tissue explants were incubated for 2 hours with 10-fold increasing concentrations of *Salmonella* Dublin (non-infected, 10^2 CFU/ml up to 10^6 CFU/ml). After infection, tissues were maintained in 1 ml of RNA later (Ambion) at 4°C O/N. Then, the RNA later was removed and tissues were frozen at -80°C until processing for qPCR analyses. Immune response (Interleukin-8 (IL-8), interferon gamma (INFγ) and Interleukin 1 beta (IL-1β) were used as infection markers.

Quantification of *Salmonella* translocation was performed at concentration of 10^2 CFU/well. For this purpose e*x vivo* tissues were thoroughly washed with KREBS media and homogenized in 1 ml of NaCl 0.9% solution at 4°C using a polytron tissue homogenizer. Triton X-100 (Sigma) was added to a final concentration of 0.1% to improve bacterial isolation. After serial dilution

in NaCl 0.9%, bacteria were plated on McConkey agar media and colonies were counted after O/N growth.

2.6. Serum Amyloid A3 (SAA3) assays

Ex vivo explants and dendritic cells were preincubated with 30 µg/ml of M-SAA3 for one hour in 5% of CO_2 at 37°C. Intestinal tissues and dendritic cells were infected with *Salmonella* during 2 hours (*ex vivo* with 10^2 CFU/well and dendritic cells at 10^6 CFU/well corresponding to a MOI of 4), or kept uninfected. After washing with PBS, tissues and cells were processed in different ways. *Ex vivo* tissues for gene expression analyses were maintained in 1 ml of RNA later (Ambion) at 4°C O/N. Then, the RNA later was removed and the tissues were frozen at -80°C until qPCR analyses. *Ex vivo* tissues for bacterial translocation quantification were processed as explained before. DC for gene expression were resuspended with 0.5 ml of TRIzol® and frozen at -80°C until use.

2.7. Retrotranscription and quantitative-PCR (qPCR)

Total RNA was extracted from DC and *ex vivo* tissues using Trizol (Invitrogen). *Ex vivo* tissues were homogenized in 1 ml of Trizol using polytron and they were kept on ice until processed. One microgram of RNA was retrotranscribed to cDNA using IScript cDNA synthesis kit (Bio-Rad, California, USA) following manufacturer's instructions. qPCR was performed using specific primers (Table 1). qPCR conditions for each set of primers were individually optimized. The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8% DNA agarose gels and a single pick in melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which is calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8-2.2 (Chow et al., 2010). A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBER Green Fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10s at 95°C, 15s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the $2^{\Delta Ct}$ method with ACTB as reference gene control.

Gene	Fw	Rv	At	µM	Amplicon
ACTB	CTGGACTTCGAGCAGGAGAT	CCCGTCAGGAAGCTCGTAG	57	0.125	75
IL-8	TTGAGAGTGGGCCACACTGTG	TGCACCCACTTTTCCTTGG	55	0.5	100
INFγ	ATAACCAGGTCATTCAAGG	ATTCTGACTTCTCTTCCGCT	50	0.5	218
IL-1β	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50	0.3125	212
MUC2	CCGTCGTGAGGGAGATTTC	CGAGCACTTGTCGTCCAG	56	0.5	124
MUC3	CCGTCACAAGTACATCTAACAGAG	ACATATTCGAGGCGTTAGCA	60	0.125	150
CLAUDIN-2	CATGCTAGGCCTGCCCGCTG	AAGACTCCGCCCACAACCGC	57	0.5	164
CLAUDIN-4	CATGATCGTGGCCGGCGTG	AGGGCTTGTCGTTGCGGG	60	0.125	226
ZO-1	TTGCCTGCTGCGGCGTACC	GCCCTTCTCCCCAAACACGACA	57	0.250	158
OCCLUDIN	ATCAACCCCGGTGCCGGAAG	GTGGTCTTGCTCTGCCCGCC	57	0.5	162
CCR7	AGCACGTGGAGGCCTTGAT	GCGGATGATGACGAGGTAGC	50	0.5	100
CD80	GAACCGCACCATCACTGACA	TAATGGTCCAGGTCAGGTGC	56	0.25	484
IL-12	GAGGCCTGTTTACCACTGGA	CTCATAGATACTTCTAAGGCACAG	50	0.3125	140
TNFα	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60	0.5	296
iNOS	CACAACGGCAACATCAGG	TAAGCAGGACTAGAGGCAACA	60	0.5	226

Table 1 Sequence, annealing temperature (At), concentration (µM) and amplicon size(bp) of the primers used for qPCR.

2.8. Statistical analyses

Data were analyzed using a mixed-effects model (SAS Inst. Inc., Cary, NC) including period (when indicated) as block and treatment as fixed effect. Data was previously transformed when necessary. Results are expressed as the means of non-transformed data ± SEM.

3. RESULTS

3.1. Settings for *ex vivo Salmonella* infection

Bovine intestinal segments were challenged with increasing doses of *Salmonella* Dublin in order to establish optimal infection conditions for tissue inflammatory response and bacterial translocation quantification. A similar behavior was observed in the expression of all studied cytokines (Figure 1). A dose response was obtained, but surprisingly lower doses of bacteria produced a greater tissue response decreasing gradually as the bacterial load increased. The IL-8 levels raised 3-fold ($P < 0.01$) with the 10^2 and 10^3 CFU/well doses. The INFγ was increased 2-fold ($P < 0.05$) only by the 10^2 CFU/well concentration. The IL-1β expressed a similar curve of expression than IL-8, observing a numerical but not significant increase for both the 10^2 and 10^3 CFU/ml infection concentrations. As the increase in IL-1β was not significative, in following *ex vivo* experiments the immune response markers analysed were IL-8 and INFγ. Similar results were observed with other bacterial strains (data not shown). When intestinal tissues were challenged with bacterial load of 10^7 and 10^8 CFU/well of a three different intestinal pathogens (*Salmonella* Dublin, *Salmonella typhimurium* and EPEC) not only no immune response associated to the infection was observed but levels of the pro-inflammatory cytokines were even lower (data not shown).

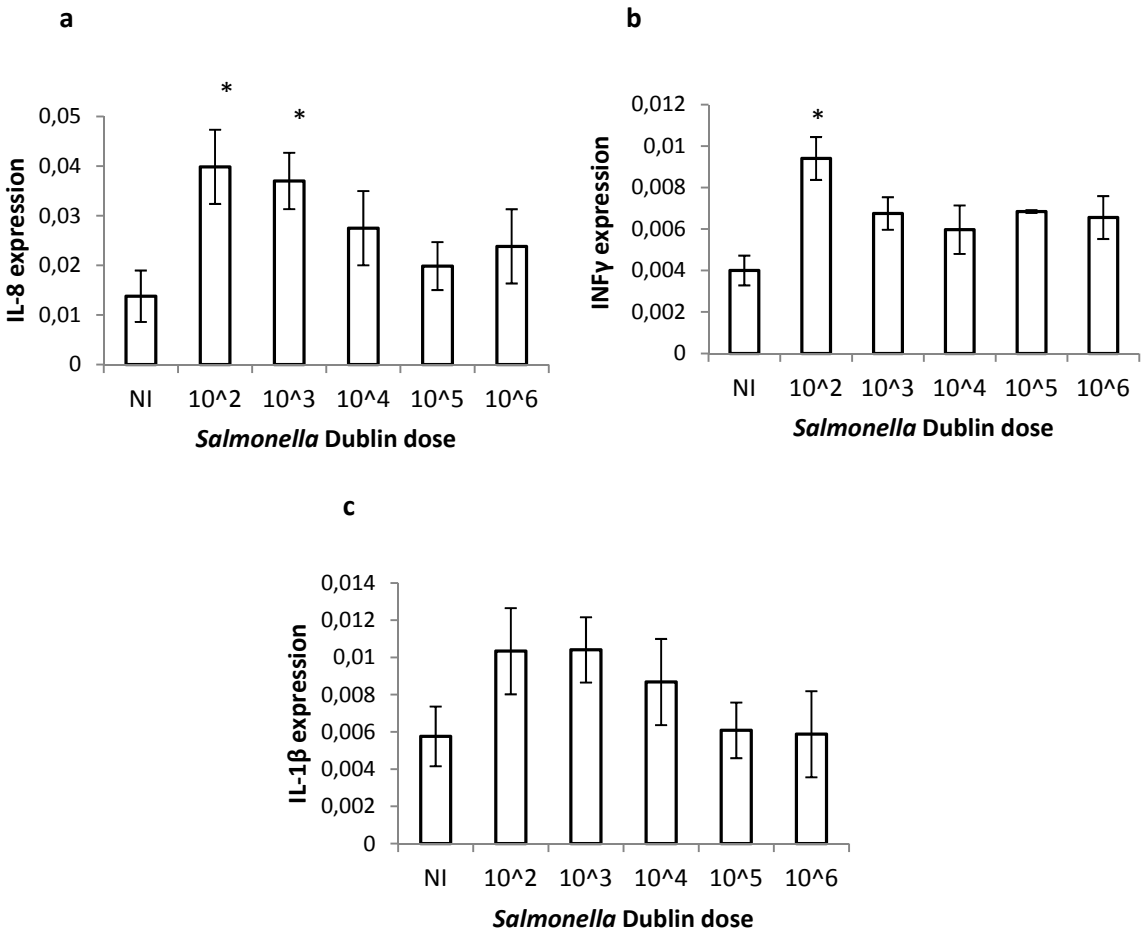


Figure 1. Cytokine expression for the *ex vivo* setting conditions.

Gene expression of IL-8 (a), INFγ (b) and IL-1β (c). Bars represent the means ± SEM for the different *Salmonella* Dublin concentrations from two independent experiments. Bars with asterisk are significatively different ($P < 0.05$). The *Salmonella* infection increases the expression of IL-8 and INFγ when administered at low doses.

Tissue *Salmonella* Dublin translocation was quantified with the previously chosen concentration of 10^2 and 10^3 CFU/well and compared to non-infected (NI) tissues. An increase in translocated bacteria after infection ($P < 0.05$) was clearly observed. Basal levels of bacteria in NI tissues were 162.5 ± 149.29 CFU/ml. The internalized bacteria at initial dose of 10^2 CFU/ml increased ($P < 0.05$) up to 1722 ± 944.01 CFU/ml and up to 2741.25 ± 591.36 CFU/ml with the 10^3 CFU/ml concentration ($P < 0.01$) (Table 2). Bacteria in the media were also quantified and the levels increased ($P < 0.001$) one order of magnitude between each dose (800 ± 639.01, 8550 ± 2064.98, 49500 ± 2327.37 for NI, 10^2 and 10^3 CFU/ml respectively) (Table 2).

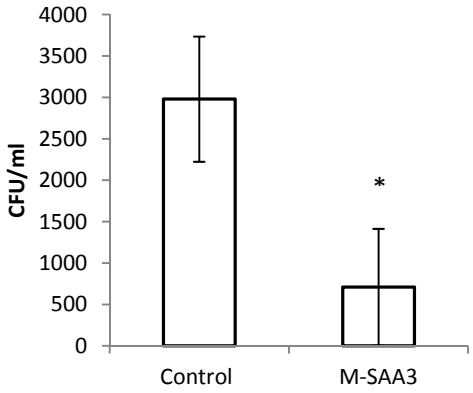
	Tissue	SN
NI	162.5 ± 149.29[b]	800 ± 639.01[c]
100	1722.5 ± 944.01[a]	8550 ± 2064.98[b]
1000	2741.25 ± 591.36[a]	49500 ± 2327.37[a]

Table 2 *Salmonella* Dublin viable cell counts during the ex vivo setting conditions.

Viable cell counts represent the CFU/ml obtained from tissues and in the supernatant (SN) in the three different concentrations of *Salmonella* tested (Non-infected, 100 and 1000 CFU/well). Data is represented as means ± SEM. Data with different superscripts differ ($P < 0.05$).

3.2. SAA3 reduces bacterial translocation in *ex vivo* intestinal tissues and activates pro-inflammatory response

Preincubation with M-SAA3 decreased ($P < 0.05$) the translocation of bacteria in bovine intestinal explants at an infection dose of 10^2 CFU/ml . The M-SAA3 efficiently lowered the bacterial translocation up to 75% (709 ± 705.4 versus 2979.4 ± 755.86 for M-SAA3 treated and not treated cells respectively) (Figure 2).



Fiure 2. Viable cell counts (CFU/ml) of internalized *Salmonella* Dublin in *ex vivo* explants.

Bars represent the means ± SEM in the different treatments from two independent experiments. Bars with asterisk are significatively different ($P < 0.05$).

The pro-inflammatory innate response associated to the infection was evaluated. The IL-8 levels of intestinal tissue pre-incubated with M-SAA raised ($P < 0.001$) 3.6-fold compared to the negative control (gene expression levels of 0.04 ± 0,004 versus 0.011 ± 0.0047 in M-SAA3 and control respectively) (Figure 3a). The INFγ levels were increased ($P < 0.001$) 3-fold (gene expression levels of 0.006 ± 0.0008 versus 0.002 ± 0.0008, for M-SAA3 and negative control respectively) (Figure 3b). The MUC2 and MUC3 genes were not affected by the M-SAA3

preincubation (MUC2, 0.001 ± 0.0002 versus 0.0011 ± 0.0002; MUC3 0.0044 ± 0.0007 versus 0.0035 ± 0.0007 for M-SAA3 and negative control respectively, Figure 3c,d).

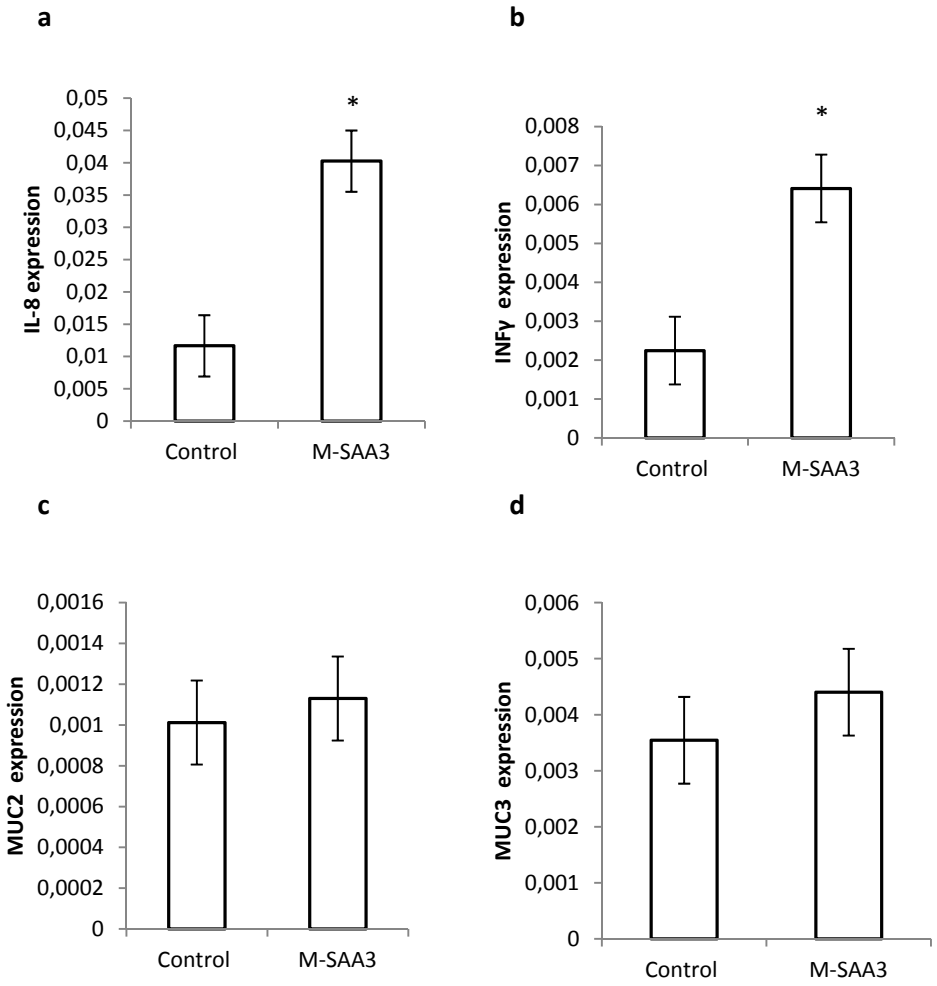


Figure 3. Mammary SAA3 effect on cytokine expression in *ex* vivo intestinal explants.

Gene expression of IL-8 (a), INFγ (b), MUC2 (c) and MUC3 (d). Bars represent the means ± SEM for the different treatments from up to three independent experiments. Bars with asterisk are significatively different ($P < 0.05$). The M-SAA3 up-regulated the expression of pro-inflammatory cytokines such as IL-8 and INFγ but not MUC related genes.

3.3. Mammary SAA3 regulates tight junction gene expression

The M-SAA3 increased Claudin-2 ($P < 0.05$) and Occludin ($P < 0.01$) gene expression levels (Figure 4a,b). Claudin-2 and Occludin levels were 50 and 60 % greater, respectively, in tissues treated with M-SAA3 compared to the negative control (Claudin-2, 0.0037 ± 0.0003 versus 0.0024 ± 0.0003; Occludin 0.00086 ± 0.000071 versus 0.00053 ± 0.000071). On the other hand, Claudin-4 and ZO-1 expression was not affected by treatment (Claudin 4, 0.00025 ± 0.00003 versus 0.0003 ± 0.00003; ZO-1, 0.00048 ± 0.000133 versus 0.00044 ± 0.000133; M-SAA3 treated and non-treated respectively) (Figure 4c,d).

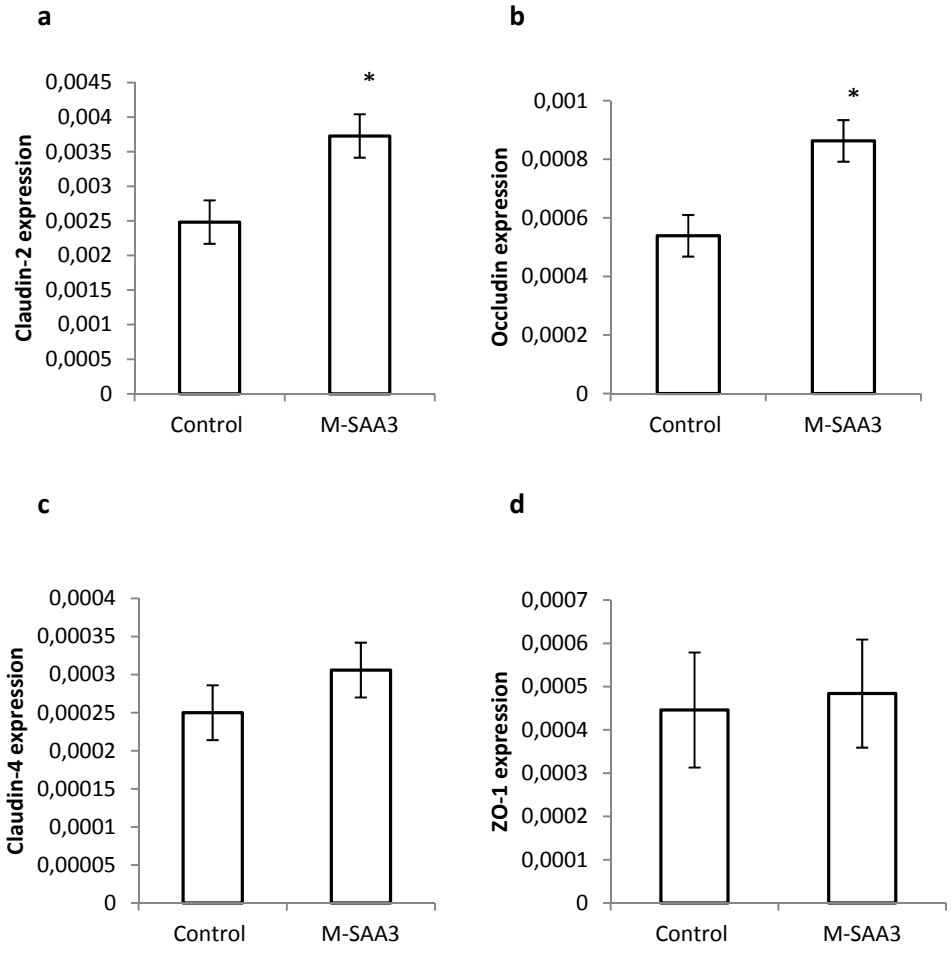


Figure 4. Mammary SAA3 effect on tight junction expression in *ex* vivo intestinal explants.

Gene expression of Claudin-2 (a), Occludin (b), Claudin-4 (c) and ZO-1 (d). Bars represent the means ± SEM for the different treatments from up to three independent experiments. Bars with asterisk are significatively different ($P < 0.05$). The M-SAA3 up-regulated the expression of Claudin-2 and Occludin but had no effect on Claudin-4 and ZO-1 expression.

3.4. Dendritic cell maturation after bacterial challenge

The evolution of the DC morphology was evaluated at the microscope (Figure 5a-c) and the cell phenotype was checked by CD11c quantification. During differentiation, the CD11c gene tended to increase, and was greater after *Salmonella* Dublin challenge (data not shown). Monocytes differentiated to dendritic cell-like phenotype after 6 days incubation with IL-4 and GM-CSF. The CCR7, CD80 and IL-12 expression were quantified as indicator of DC maturation (Figure 5 d,e) . The CCR7 was not increased by *Salmonella* Dublin challenge (0.0074 ± 0.000213 versus 0.0056 ± 0.00135) but CD80 tended ($P = 0.056$) to increase (0.00008 ± 0.00010 versus 0.0004 ± 0.00014) after the *Salmonella* Dublin exposure. The IL-12 levels of non-infected cells

were not detectable by qPCR but after infection IL-12 expression increased up to detectable levels (data not shown).

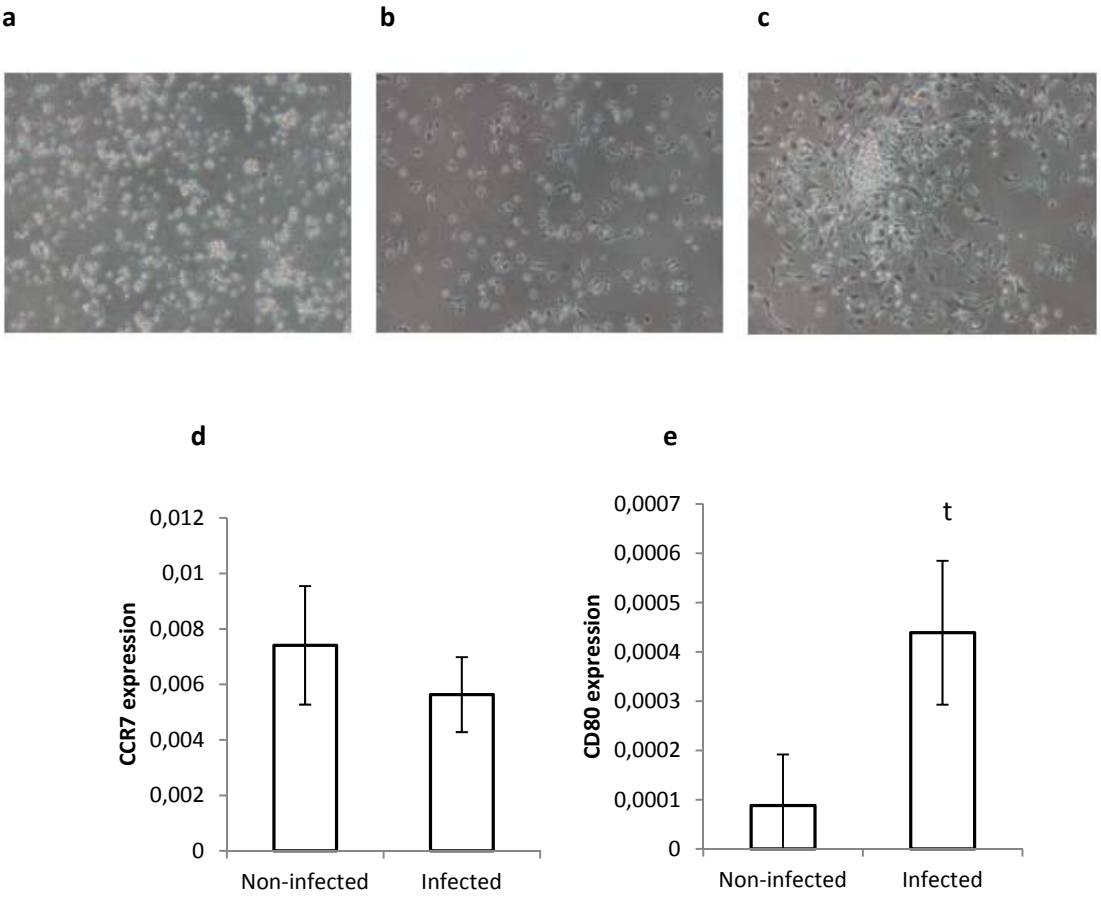


Figure 5. Dendritic cell differentiation and maturation.

Microscopic evaluation of the differentiation of monocytes to dendritic cells at day 1 (a), day 3 (b) and day 6 (c). Gene expression of CCR7 (d) and CD80 (e) markers of mature dendtritic cells after *Salmonella* challenge. Bars represent the means ± SEM for the different treatments. Bars with t presented a tendency ($P < 0.1$). *Salmonella* tended to increase the expression of CD80 maturation marker.

3.5. Mammary SAA3 activates dendritic cell cytokine expression during *Salmonella* Dublin infection

DC treated and non-treated with M-SAA3 were challenged with *Salmonella* Dublin. The response was evaluated by quantification of immunologic parameters such as IL-8, INFγ, TNFα, IL-12, CCR7, CD80 and iNOS. All parameters increased their expression after M-SAA3 preincubation with the exception of TNFα which described a decrease. The IL-8 gene expression levels were increased ($P < 0.05$) in 1.25 fold with the M-SAA3 treatment (1.43 ± 0.104 versus 1.14 ± 0.098). The INFγ increased ($P < 0.0001$) 3.3 fold (0.011 ± 0.0011 versus 0.003 ± 0.0011). The IL-12 levels increased ($P < 0.001$) around 1.6 fold (0.0007 ± 0.000005

versus 0.0004 ± 0.000005). The CCR7 levels increased ($P < 0.001$) in 4.3 fold (0.024 ± 0.0031 versus 0.005 ± 0.0031). The CD80 expression was increased ($P < 0.01$) 2 fold (0.0006 ± 0.00008 versus 0.0003 ± 0.00008). Finally, the TNFα levels decreased ($P < 0.05$) after M-SAA3 treatment (0.08 ± 0.010 versus 0.12 ± 0.010).

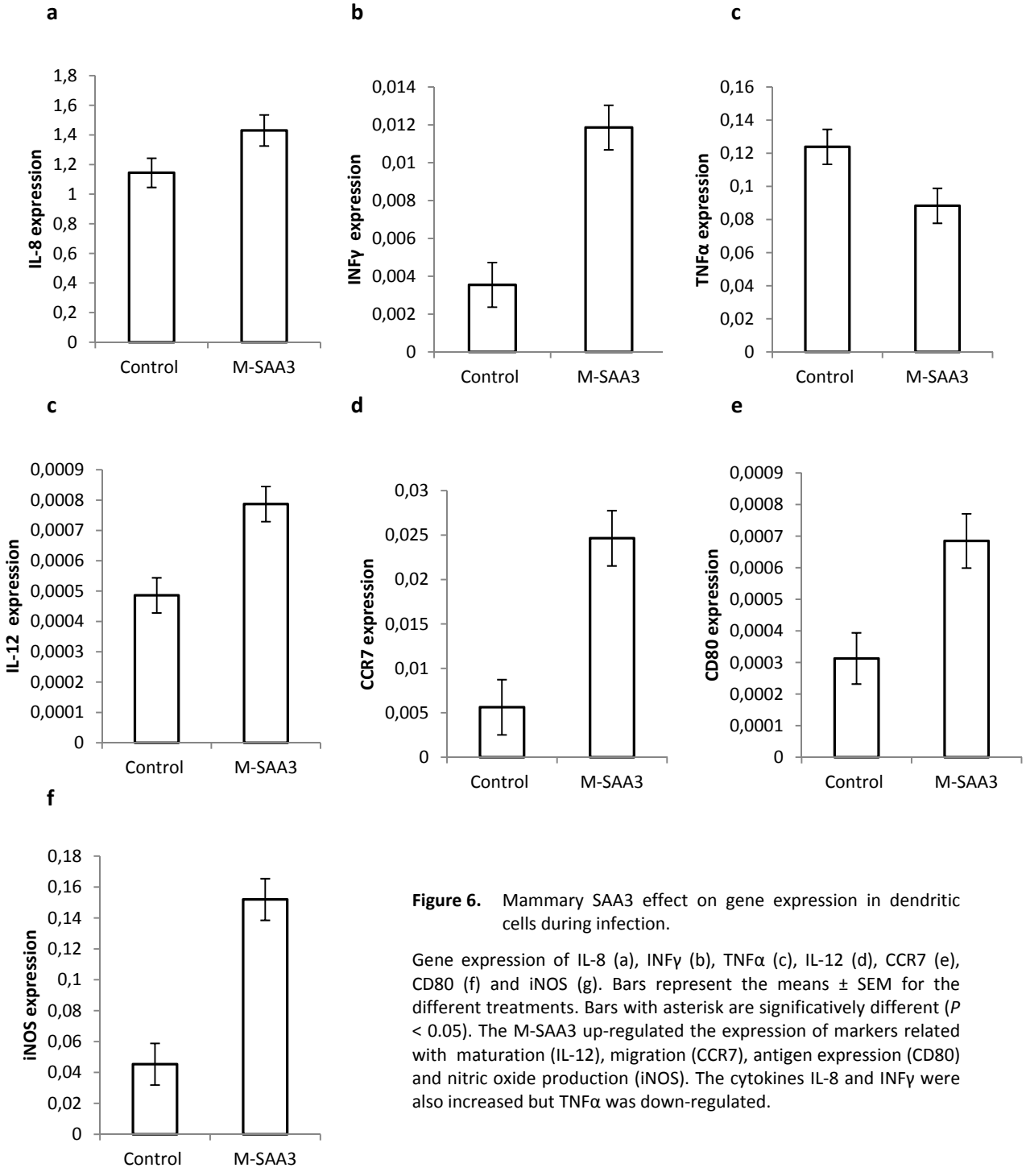


Figure 6. Mammary SAA3 effect on gene expression in dendritic cells during infection.

Gene expression of IL-8 (a), INFγ (b), TNFα (c), IL-12 (d), CCR7 (e), CD80 (f) and iNOS (g). Bars represent the means ± SEM for the different treatments. Bars with asterisk are significatively different ($P < 0.05$). The M-SAA3 up-regulated the expression of markers related with maturation (IL-12), migration (CCR7), antigen expression (CD80) and nitric oxide production (iNOS). The cytokines IL-8 and INFγ were also increased but TNFα was down-regulated.

4. DISCUSSION

This study clearly demonstrated the protective role of the M-SAA3 at gastrointestinal level in *Salmonella* Dublin infection. *Ex vivo* technique was optimized and used as a bovine intestinal infection model. *Salmonella* Dublin at low doses (10^2 and 10^3 CFU/well) activated the immune response of the explants (Figure 1), increasing the INFγ and IL-8 expression. The INFγ is an important link between the innate and adaptive immune response and it is one of the most studied cytokines during *Salmonella* infection (Eckmann et al., 1996; Jones, 1997; Eckmann and Kagnoff, 2001; Schroder et al., 2004; Coburn et al., 2007). The main functions are related with the activation of the cell-mediated immunity, activating macrophages and switching to a Th1 response (Schroeder et al., 2004). The IL-8 is also highly activated during a *Salmonella* challenge (Hyland et al., 2006), and acts as a chemokine, recruiting immune response mediators such neutrophils and macrophages to the site of infection. Surprisingly, in this *ex vivo* intestinal model, higher doses of bacteria produced no increase in cytokine expression. *Zhang et al*. described how oxidative stress down-regulated expression of cytokines such as INFγ, and could partially explain the non-responsivness of *ex vivo* tissues tissues where the basal inflammation is always higher than in cell line cultures (Zhang et al., 2003). The 10^2 and 10^3 CFU/ml doses were used to quantify the translocation of *Salmonella* in the intestinal explants. At both doses there a significant increase in the internalized bacteria was observed, but since there was no difference between 10^2 and 10^3 CFU/ml concentrations in the final translocated bacteria, the lowest dose was chosen in order to maintain the tissue integrity.

M-SAA3 reduced in a 75% the bacterial translocation, which clearly indicates a direct effect in the binding and internalization of *Salmonella* Dublin when using bovine *ex vivo* tissues. These results are in agreement with previous published data performed using commercial intestinal laboratory cell lines. *Larson et al.* described that synthetic peptides derived from the mammary form decreased EPEC infection in the commercial cell line HT-29 (Larson et al., 2003). Moreover*, Gardiner et al.* described similar effects for the human 42-mer protein, which corresponds to a truncated form of the protein due to the presence of a STOP codon in the coding sequence and in consequence is considered to be a pseudogene (Kluve-Beckerman et al., 1991; Gardiner et al., 2009). Previous work in our laboratory also supported this result. We observed that our recombinant M-SAA3 also decreased the EPEC binding and internalization to the commercial intestinal epithelial cell line CaCo-2. In this study, the *ex vivo* technique represented a more physiological approximation to an *in vivo* situation, conserving the mucosa and submucosa structure and permitting a conjunction and coexistence of different cell types. Also, an important pathogenic bacteria specific for cattle and with high economical impact has

been used to evaluate the function and potential of recombinant SAA3 as a fighting agent against gastrointestinal diseases in the bovine sector (Uzzau et al., 2000).

The M-SAA3 increased the pro-inflammatory immune response associated to *Salmonella Dublin* infection, raising INFγ and IL-8 levels around 3-fold. The SAA members have previously been described to produce an increase in IL-8 expression in non infective conditions. This increase was observed either in cells that directly participate in the immune response such neutrophils (He and Ye, 2003; Lee et al., 2009) and monocytes (Ribeiro et al., 2003), and also in non-immune cells like intestinal epithelial cells (Jijon et al., 2005). Our recombinant M-SAA3 has already been tested in CaCo-2 cells under infective conditions but only a moderate increase in IL-8 levels were observed. However in the *ex vivo* treatment the M-SAA3 highly triggers the immune system through a clear IL-8 and INF-γ upregulation. *Yavin et al.* described that carboxi-terminal region of the SAA1 protein (AGLPEKY, SAA_{98-104}) was involved in the INFγ up regulation in T-lymphocytes (Yavin et al., 2000). INFγ is a key effector in immunity against *Salmonella*, however its release is believed to be carried out only by a few cell types. It has been long known that the main source for INFγ production remained in the lymphoid line, mainly referring to NK, B and T cells. More recently, the importance for antigen presenting cells (APC) as INFγ producers is gaining interest (Fructh et al., 2001). Thus, probably the role of M-SAA3 as immune system modulator is highlighted in *ex vivo* explants from PP, because the function of different cell types converges.

In contrast, M-SAA3 did not elicit any effect on mucin expression. The physical contact between the bacteria and enterocytes represents the first step during the infective process. Mucus layer is considered to be one of the first barriers of the innate immune response preventing an infective process (Hecht, 1999). *Larson et a.l* described that the inhibitory effect in bacterial translocation observed with the synthetic derived peptides was achieved by MUC3 overexpression (Larson et al., 2003). Our recombinant M-SAA3 also increased MUC3 expression in CaCo-2 cell lines in infective conditions. However since we also have been observed a decrease in mucin expression in control tissues along the experiment compared to tissues directly frozen (data not shown), we supposed that *ex vivo* explants may not be the best mean to study mucin regulation.

The M-SAA3 also increased the expression of Claudin-2 and Occludin while other tight junction proteins remained unaltered (Claudin-4 and ZO-1). Tight junctions strictly seal the intracellular spaces and contribute to establish an efficient barrier against intracellular infections. A leaking barrier can favor pathogen and other antigen passage (Rosselli et al., 2007). In the tight junctions, Claudins and Occludins are transmembrane proteins that interact with the adjacent

cell, and ZO are cytoplasmatic proteins that help to anchor the transmembrane proteins to the actin cytoskeleton (Miyoshi and Takai, 2005). Pathogenic bacteria efficiently produce defects in the cell-cell contact, delocalizing tight junction proteins (Boyle et al., 2006; Köler et al., 2007; Qin et al., 2009; Roxas et al., 2010). *Salmonella* has been described to decrease the expression of Claudin-1, Occludin and ZO-1 (Boyle et al., 2006; Köler et al., 2007; Roxas et al., 2010). Some authors have described that probiotics may help against intestinal infections by increasing the expression of tight junction proteins. *Qin et al.* described how the probiotic *Lactobacillus plantarum* was able to revert the Enteroinvasive *Escherichia coli* (EIEC) tight junction rearrangements by increasing Claudin-1, Occludin and ZO-1 expression (Qin et al., 2009). Our results suggest that M-SAA3 could be preventing bacterial translocation by maintaining the expression of tight junction proteins, such as Claudin-2 and Occludin.

Finally, in order to elucidate if the highlighted role of M-SAA3 as immune system activator in *ex vivo* explants was related to DC activation, the M-SAA3 effect on DC culture was evaluated. DC phenotype was evaluated by direct visualization at the microscope and the up-regulation of CD11c marker. Conventional DC are identified as $CD11c^{hi}$ $MHCII^{+}$ and can be further divided in subsets depending on the expression of myeloid and lymphoid surface molecules (Tam et al., 2008). In this study immature DC were challenged with *Salmonella* Dublin. Maturation was confirmed by an increase of IL-12 and CD80 expression. The study demonstrated that M-SAA3 enhanced the DC activity since the IL-8, INFγ and IL-12 were increased when treated with the M-SAA3 (Figure 6). In contrast, the pro-inflammatory TNFα levels decreased, fact that should be elucidated in further research. The M-SAA3 also increased other parameters related with migration and antigen presentation such CCR7 and CD80. Finally, the expression of the enzyme oxid nitric synthase (iNOS) was also raised under M-SAA3 treatment. These results are in agreement with published data in which SAA treatment increased Nitric Oxide in macrophages (Sandri et al., 2008), a mediator that participates in the fight against pathogens and it is produced by iNOS (Bogdan, 2001).

In summary, M-SAA3 described a broad range of DC activation functions, upregulating genes that participate in different immune system processes. Also, we corroborate that M-SAA3 acted in different cell types, suggesting that the mechanism of activation should be widely represented. The M-SAA3 binds to Toll like receptor 4 (TLR4) (Bogdan, 2001) which could have a relevant function since it is a receptor that also recognizes bacterial lipoplysaccharide and activates immune response against gram-negative pathogens (Beg, 2002).

5. CONCLUSION

Mammary SAA3 reduces *Salmonella* Dublin translocation to the bovine gastrointestinal tract probably mediated by a set of different processes such as the observed upregulation of tight junction proteins. M-SAA3 activates the innate immune response associated to *Salmonella* Dublin infection through the expression of IL-8 and IFNγ cytokines. The function of MSAA3 is highlighted in the *ex vivo* model probably due to the co-stimulation of different cell types such as dendritic cells which became mature and functionally activated under M-SAA3 incubation.

6. AKNOWLEDGMENTS

We are indebted to the Cell Culture Unit of SCAC at the UAB. The authors thank to Ruminant Production Department for helpful technical assistance and discussions. This study has been funded by INIA-MICINN (RTA2008-00050-C02-01).

Chapter VI – Study 4

Enhancement of early-cow dry period performance by Mammary Serum Amyloid A3

Anna Domènech[1], Sílvia Parès[1], Àlex Bach[1,2], Alicia Serrano and Anna Arís[1]

1. Department of Ruminant Production, IRTA, Institute of Research in Agriculture and Technology. Torre Marimón, km 12,1 C-59, Caldes de Montbui, 08140, Barcelona, Spain.
2. ICREA, Institució Catalana de Recerca i Estudis Avançats, Barcelona (Spain)

1. INTRODUCTION

Milk production is maximized when cows are pregnant during 70% of the time of lactation. A dry period between lactations is necessary to renew senescent epithelial cells of the mammary gland and ensure optimal milk production in the next lactation (Capuco et al., 1997). Involution of the mammary gland in pregnant cows is slower than in other species where it does not coexists with pregnancy. This requires long dry periods of 60 days, which significantly reduce productivity (Noble et al., 1999; Pezeshki et al., 2009).

The dry period is characterized by two main phases of cell turnover: the mammary gland involution, characterized by acute apoptosis, and the cell regeneration before the next lactation (De Vries et al., 2010). A peak of apoptosis is observed during the first 72 h after milk cessation, continuing along the rest of the dry period and combined with an increase in cell proliferation (Annen et al., 2007). Physiologically, milk stasis is initiated by stopping milk removal from the mammary gland and promoted by protein and hormonal factors, such as a lactogenic hormones drop that induce active gland involution and in turn stimulate contraction of the parenchyma and cessation of milk secretion (Wilde et al., 1999; Accorsi et al., 2002, Pezeshki et al., 2009).

Mammary gland tissue is composed by epithelial secretory cells and stromal tissue with different cell types such as fibroblast and immune cells (Muschler et al., 2010). Both tissues are separated by basement membrane, a type of extracellular matrix (ECM), which influences the development and biology of the mammary gland (Lin et al., 1993; Streuli et al., 2003). The ECM directly establishes and maintains the mammary gland differentiated state and prevents apoptosis (Talhouk et al., 1992; Bodreau, 1995). The proteolytic destruction of the basement membrane induces apoptosis and involution of mammary gland (Bodreau, 1995). There are several proteases involved in the extracellular matrix degradation but basically the matrix metalloproteinases (MMP) are the key enzymes of this process (Rabot et al., 2007). Moreover, other functions have been assigned to MMP such as the release of growth factors and cytokines, which modulate other key aspects of the dry period such as the immune system activation and cell growth (Rabot et al., 2007).

In the early dry period there is an increased risk of intramammary infections. Milk stasis increases pressure within mammary gland promoting milk leakage and susceptibility to bacterial infection (Burvenich et al., 2007). It has been proposed that animals with high milk yields are more vulnerable to contract intramammary infections since they have a delayed formation of the keratin plug and an increased time of teat canal occlusion. The intramammary

infections contracted during the dry period have been associated with increased infection prevalence in the next lactation, which causes loss of milk production and decreases milk quality (Hernandez et al., 2011).

The activation of the immune system that concurs with the beginning of the dry period recruits blood leukocytes but they do not reach the mammary gland until 6 days after the onset of drying and their concentrations are still below protective levels at day 8 (Burvenich et al., 2007). To avoid the high risk of mastitis along this period, antibiotics are infused into the mammary gland routinely. However, this practice is associated with the risk of developing antimicrobial resistance to antibiotics which are also used in humans, with undesirable consequences. Stimulation of animal immunity at the beginning of the dry period may be relevant not only to improve the efficacy of antibiotics specially in cases of infection with bacteria escaping spectrum antibiotics (Erskine et al 2003), but also to avoid the use of antibiotics as a preventive agent. Moreover, reducing the extent of the dry period under 60 days without losing milk yield has been an active area of discussion during the last years, as it would greatly increase the efficiency of dairy farms, shortening the unproductive days (De Vries et al., 2010).

Mammary serum Amyloid A3 (M-SAA3) is an acute phase protein that participates in the innate immune response. Differently to other members of the family, it is specifically produced in the mammary gland, observing high concentrations in colostrum and in milk during infections processes and variable levels along the lactation period (McDonald et al, 2001; Molenaar et al., 2009). This mammary SAA3 isoform (M-SAA3) has been related to several immunological functions in the organism such as chemotaxis of immune cells (Badolato, 1994; de Jesus Rodriguez et al., 2009), cytokine modulation (Patel et al., 1998; He et al., 2003) and organism protection against infection (Larson et al,2003; Mack et al., 2003; Gardiner et al., 2009, Molenaar et al., 2009). Moreover, other SAA members have been described to activate metalloproteinase expression in immune cells (Lee et al., 2005).

The aim of this study was to evaluate the potential of the M-SAA3 protein as an activator ofthe involution of the mammary gland during the early dry period. The effects of the intramammary infusion of the recombinant protein were evaluated at different levels, including the ability to increase the recruitment of immune cells and the activation of metalloproteinase activity. *In vitro* studies were carried out to determine the effect of the M-SAA3 in epithelial mammary cells and DC.

2. MATERIALS & METHODS

2.1. Recombinant M-SAA3 production

The EcUR206 strain (an *E. coli* BL21 Star (DE3)-pET101/D-TOPO vector containing the goat M-SAA3 sequence) was used in recombinant protein production. The process has been explained elsewhere (Domènech et al., 2012). Briefly, BL21/pURAD1 was growth in 400 ml of LB-Amp media at an initial OD_{600} of 0.05 until log phase was achieved. Recombinant expression was induced by IPTG 0.1 mM for 1 h and 20 min Cell pellet was obtained by centrifugation at 6000g for 10 min, and frozen at -80°C until use. Cell pellets were resuspended to a OD_{600}=100 in 20 mM Na_2HPO_4 /NaH_2PO_4,0.5 M NaCl, pH 7.4 buffer containing lysozyme (0.2 mg/ml), DNase I and RNase A (20 µg/ml), cocktail inhibitor of proteases (1 mM) and $MgCl_2$ (1 mM) during 30 min at RT. The suspension was mixed with pre-weighted 0.1 mm glass beads (range 26-36 mg per ml of sample) (Biospec Product, Inc, Bartlesville, USA). Three cycles of beating of 45 sec each, with one min on ice within cycles, were carried out in the MiniBead Beater (Biospec Product, Inc, Bartlesville, USA). The disrupted cell suspension was centrifuged for 15 min at 20000g at 4°C and the supernatant was considered the soluble fraction containing the recombinant protein. Recombinant protein was purified with commercial His Spin Trap columns (GE Healthcare, Uppsala, Sweden), following manufacturer instructions. The purified protein was dialyzed O/N at 4°C against PBS and it was further quantified using spectrophotometry ((A_{280} × Molecular weight)/Extinction coefficient; mg/ml). Lypopolysaccharide (LPS) traces in the eluted fraction were quantified by endoLISA endotoxin assay (Hyglos). Final LPS levels corresponded to 0.08 ng LPS/µg recombinant protein. This level was similar to the traces observed in the recombinant commercial Apo-SAA (ProSci incorporated, endotoxin levels less than 0.1 ng/µg protein).

2.2. Mammary gland M-SAA3 infusions

Two quarters of 9 cows were intramammary infused the first day of the drying off after the last milking (T=0). The infusion was performed using mammary cannulas with 1 mg of M-SAA3 or 80 ng *E. coli* LPS (Sigma) to reproduce the possible effect of LPS traces in purified recombinant SAA3 fraction. The same volume (10 ml) of saline solution was infused in the respective control quarters (front or back quarters). Immediately after treatment all quarters were treated intramammary with routine antibiotic (Cloxacillin benzathine, 0.6g; Orbenin extra dry cow, Pfizer). Front and back quarters were treated independently statistically. Milk samples were taken at 8 a.m before intrammamary infusion (T=0) and every day at 8 a.m during 3 consecutive days (T=1, T=2 and T=3). 10 ml of milk were frozen for metalloproteinases

analyses and fresh milk was analyzed for somatic cell count (SCC), fat, and protein content in ALLIC (Laboratori interprofessional lleter de Catalunya, Cabrils, Spain).

2.3. Metalloproteinases quantification by zymography gel

Frozen milk was thawed and centrifuged at 2700g for 10 min to obtain skimmed milk. Diluted 1:20 skimmed milk was mixed 1:1 with loading buffer (0.125M Tris pH=6.8 with 0.005% bromophenol blue, 20% glycerol, 4% SDS) and run in a 10% polyacrylamide gel with 1 mg/ml porcine gelatin. Samples were run for 1 h at 150V in running buffer (192mM Gly, 25mM Tris, 0.1% SDS). After running, gel was washed with 2.5% Triton X-100 solution at RT with gentle agitation for 30 min Then, the gel was incubated with developing buffer (50mM Tris pH 7.6 with 0.2M NaCl, 5mM $CaCl_2$, 0.02% Brij) during 30 min, and reincubated in fresh developing buffer for 48 hours at 37°C without agitation. Gels were stained with Comassie Blue R-250 (Bio-Rad) during 30 min Destaining buffer (50% methanol, 10% acetic acid) was used to visualize the bands corresponding to the metalloproteinases. Gels were digitalized and bands were analyzed with the Quantity One software. The metalloproteinase activity was defined by the ratio INT/mm^2, calculated by the Quantity One software regarding the intensity and size data of each band.

2.4. Mammary gland primary cultures

Mammary gland tissue was obtained at slaughterhouse and transported in chilled PBS with 100 µg/ml streptomycin, 100 U/ml penicillin and 2.5 µ/ml amphotericin B. In the laboratory, tissue was cut into small pieces and incubated in Hanks balanced salt solution with 0.1 mM EDTA and 0.1 mM DTT for 30 min at 37°C in 10% CO2 at 150 rpm. Then, supernatant was removed and RPMI 1640 media with 0.05 % collagenase was added and incubated for 30 min The media contained epithelial cells, which were centrifuged at 800g for 5 min This process was repeated 3 times. Final cell pellets were resuspended in F-12 media with 8 µg/ml bovine insulin, 10 µg/ml gentamycin, 50 µg/ml hydrocortisone, 100 µg/ml streptomycin, 100 U/ml penicillin and 2.5 µg/ml amphotericin. Cells were quantified by haemocytometer counting and incubated at 80.000 cells/cm^2 in flasks until differentiation. Epithelial cell phenotype was confirmed by immunofluorescence staining against anti-cytokeratin antibodies (Sigma) as previously described in literature (Hashim et al., 2004). Mammary gland primary cells were grown on coverslips and fixed with 4% paraformaldehyde (diluted in PBS). HeLa cells were used as positive control and macrophage differentiated THP-1 cells as negative control. Coverslips were blocked with 150 µl of PBS containing goat serum (0.05 % v/v) and Triton 0.2%, for 30 min at RT. Primary antibody (monoclonal anti-cytokeratin pan antibody produced

in mouse, Sigma, 1:500 dilution) was incubated in blocking buffer for 2 h at RT. After 3 washes with PBS, secondary antibody (antimouse-FITC, Sigma, 1:1000) was incubated for 1 h at RT. Finally, cells were washed and dried coverslips, mounted into glass slides using Fluoroprep and observed in the fluorescence microscope.

2.5. Bovine monocyte isolation and differentiation to dendritic cells

Bovine blood with sodium heparin 50 UI/ml, as anticoagulant was obtained at slaughterhouse. The blood was diluted 1:1 with PBS-2% Fetal Calf Serum (FCS) at RT. The diluted blood was layered onto Histopaque-1077 (Sigma) at 1:2 ratio (histopaque/diluted blood) and centrifuged at 600 g (brakes off) during 30 min at RT. PBMC were isolated from the interphase, washed with media and centrifuged 10 min at 200 g. The cell pellet was resuspended and incubated with 10 ml of Red Blood Lysis buffer (Sigma) and incubated for 10 min at RT. After centrifuging at 250 g for 5 min, the process was repeated until a free-erythrocyte pellet was observed. PBMC were resuspended in supplemented media (RPMI-10% FCS, 50 µM β-mercapthoethanol, 1% penicillin/streptomycin) and quantified using a haemocytometer. PBMCs cells were incubated in a 75 cm^2 Falcon in a ratio of 10^6 cells/cm^2, and incubated during 1.5 hours in 5% CO_2 at 37°C. After incubation, adherent monocytes were washed once with PBS and incubated with supplemented media with Interleukin 4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) cytokines (Bovine Dendritic Cell Growht kit, Bionova, 1:20 dilution). Media was changed at day 3. The DC phenotype was checked by direct morphology observation at the microscope and by qPCR quantification of CD11c gene. The day of the experiment, cells were scrapped and quantified. An amount of $4 \cdot 10^5$ dendritic cells/well wee seeded in 24-well plates, and incubated with supplemented RPMI without antibiotics.

2.6. *Staphylococcus aureus* infection of mammary gland primary cultures

Pathogenic *Staphylococcus aureus* was isolated from mastitic milk and kindly provided by ALLIC (Laboratori interprofessional lleter de Catalunya, Cabrils, Spain). The *S. aureus* were grown in Nutrient media and plated onto mannitol salt agar. Single colonies were grown in 10 ml of Nutrient media over-night at 37°C in static conditions. Cell pellet was obtained by centrifugation at 6000g for 10 min at 4°C. Bacterial concentration was calculated by spectrophotometric quantification (DO_{600}= 0.4 corresponds to 10^7 CFU/ml). Bacterial doses of infections were obtained by resuspending and diluting the cell pellet with the corresponding media without antibiotics.

Mammary epithelial cells were seeded in 24-well plates at 44,000 cells/well. Cells were preincubated with 30 µg/ml of recombinant M-SAA3 during 1 h. After preincubation,

mammary cells were infected with *S. aureus* at 10^6 CFU/ml for 2 h. Cells were washed thoroughly with PBS and internalized *S. aureus* was released from cells using Triton 0.1%. Bacteria were serially diluted and plated onto mannitol salt agar. Colonies were counted after O/N growth at 37°C.

2.7. M-SAA3 treatment on dendritic cells and mammary epithelial cells

Mammary epithelial cells from primary cultures (44,000 cells/well) and dendritic cells ($4 \cdot 10^5$ cells/well) were seeded in 24-well plates. Cells were incubated with the corresponding media without antibiotics, with and without 30 µg/ml of recombinant M-SAA3 during 3 h. After washing with PBS, cells were resuspended with 0.5 ml of Trizol (invitrogen) and frozen at -80°C until gene expression analyses by quantitative RT-PCR were performed.

2.8. Quantitative RT-PCR

Total RNA was extracted from epithelial mammary cells and dendritic cells using Trizol® (Invitrogen) following manufacturer's instruction and quantified using Nanodrop. One microgram of RNA was retrotranscribed to DNA using IScript cDNA synthesis kit (Bio-Rad, California, USA). qPCR was performed using iQ5 Termocycler (Bio-Rad). qPCR conditions for each set of primers were individually optimized (Table 1). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in DNA agarose gel and a single peak in the qPCR melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which is calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8-2.2 (Chow et al, 2010). A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBER Green Fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10s at 95°C, 15s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the $2^{\Delta Ct}$ method with ACTB as reference gene.

Gene	Fw	Rv	At	µM	Amplicon
ACTB	CTGGACTTCGAGCAGGAGAT	CCCGTCAGGAAGCTCGTAG	57	0.125	75
IL-8	TTGAGAGTGGGCCACACTGTG	TGCACCCACTTTTCCTTGG	55	0.5	100
INFγ	ATAACCAGGTCATTCAAGG	ATTCTGACTTCTCTTCCGCT	50	0.5	218
CCR7	AGCACGTGGAGGCCTTGAT	GCGGATGATGACGAGGTAGC	50	0.5	100
CD80	GAACCGCACCATCACTGACA	TAATGGTCCAGGTCAGGTGC	56	0.25	484
IL-12	GAGGCCTGTTTACCACTGGA	CTCATAGATACTTCTAAGGCACAG	50	0.3125	140
TNFα	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60	0.5	296
iNOS	CACAACGGCAACATCAGG	TAAGCAGGACTAGAGGCAACA	60	0.5	226

Table 1 Sequence, annealing temperature (At), concentration (µM) and amplicon size (bp) of the primers used for qPCR.

2.9. Statistical analyses

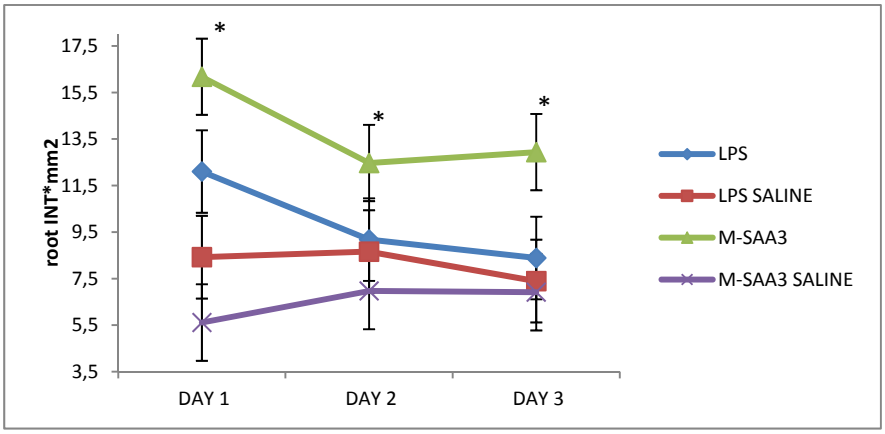
Data pertaining to SCC, fat, protein, and metalloproteinase activity, and gene expression were analyzed using a mixed-effects linear model (SAS Inst. Inc., Cary, NC). For the *in vivo* experiment, treatment, quarter, day and the interaction between treatment and dat and treatment and quarter were used as fixed effects. Time 0 was used as covariate and day as a repeated measure. The model also accounted for the random effects of animal within treatment. Data were previously transformed when necessary to achieve a normal distribution. Results are expressed as the means of non-transformed data ± SEM (except otherwise stated).

3. RESULTS

3.1. Mammary SAA3 effect on Somatic Cell Counts (SCC), metalloproteinase activity and fat and protein content after mammary gland infusion

Milk metalloproteinase activity for MMP-9 and MMP-2 was quantified through zymography gel. Clear bands were observed at 92 KDa and 72 KDa, respectively. These bands represent the enzymatic activity, since they reflect the amount of substrate digested by the enzymes present in the sample. A significant M-SAA3 treatment effect was observed in the MMP-9 activity ($P < 0.0001$) (Figure 1a). The M-SAA3 increased ($P < 0.0001$) the activity of MMP-9 during the 3 days of experiment compared with the saline solution (data represented as root INT*mm^2; day 1: 16.1 ± 1.63 vs 5.6 ± 1.64; day 2: 12.4 ± 1.63 vs 6.9 ± 1.64; day 3: 12.9 ± 1.63 vs 6.91 ± 1.64, for M-SAA3 and saline solution, respectively). The M-SAA3 infusion also increased ($P < 0.001$) the levels of MMP-9 activity compared with LPS (day 1: 16.1 ± 1.63 vs 12.1 ± 1.77; day 2: 12.4 ± 1.63 vs 9.1 ± 1.77; day 3: 13.9 ± 1.63 vs 8.3 ± 1.77, for M-SAA3 and LPS, respectively) (Figure. 2). In contrast, there were no differences between LPS and their negative control (day 1: 12.1 ± 1.77 vs 8.4 ± 1.77; day 2: 9.1 ± 1.77 vs 8.6 ± 1.77; day 3: 8.3 ± 1.77 vs 7.3 ± 1.77, for LPS and saline solution, respectively). On the other hand, the MMP-2 activity remained unaltered after treatment either with M-SAA3 or LPS (Figure 1b).

a



b

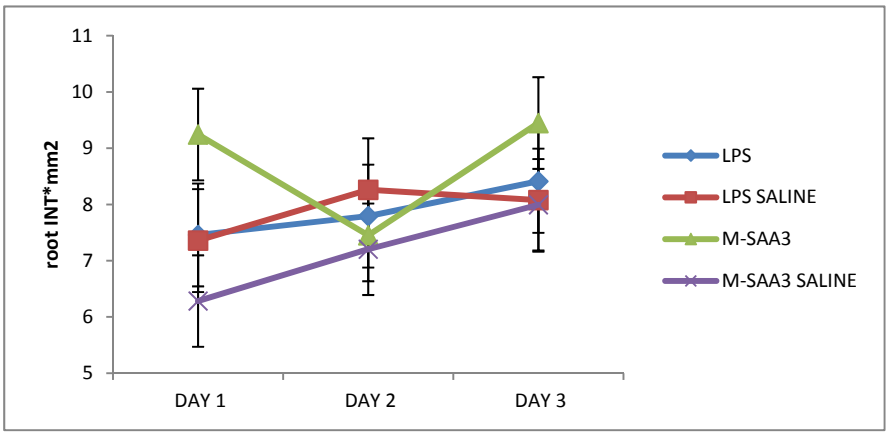


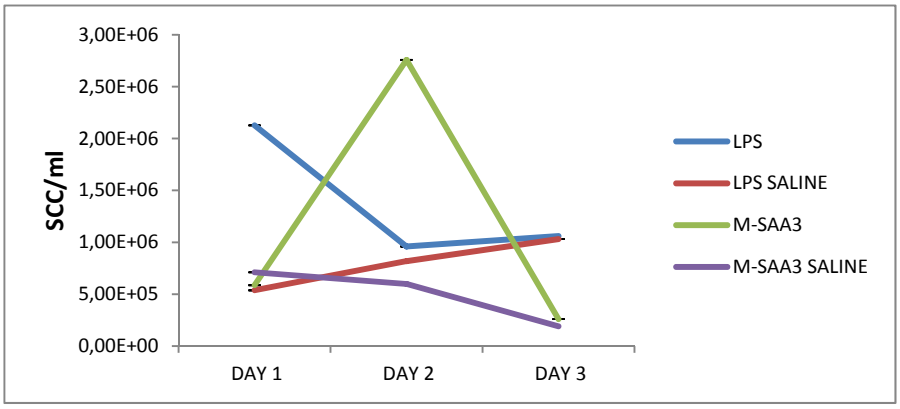
Figure 1. Metalloproteinase activity during the early dry period.

Metalloproteinase activity is reperesented by the root of INT*mm^2 ± SEM. Data were obtained from band quantification of zymography gels using the Quantity One software during the 3 d after dry off, for MMP-9 (a) and MMP-2 (b).Treatments with asterisk differ ($P < 0.05$).

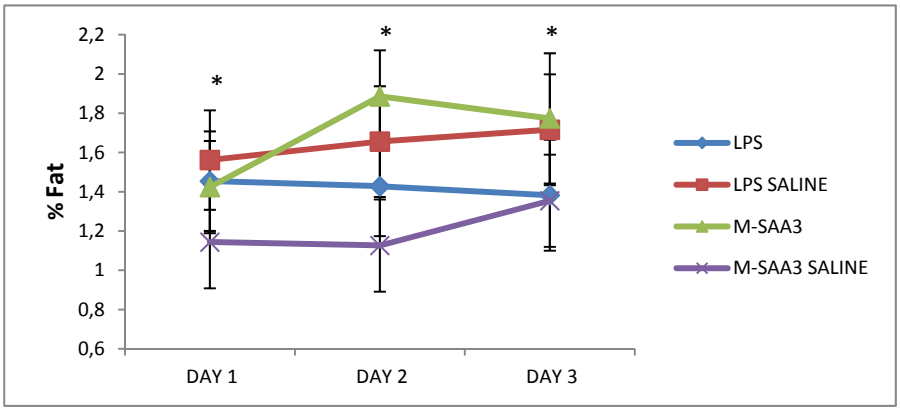
The M-SAA3 numerically increased SCC in milk (Figure. 2a) at day 2 compared with the saline solution (data represented as SCC/ml ± SEM where SEM corresponded to the standard error mean of ln transformed data; day 2: $2.76 \cdot 10^6$ ± 0.673 vs $5.98 \cdot 10^5$ ± 0.664, for M-SAA3 and saline solution) and LPS (day 2: $2.76 \cdot 10^6$ ± 0.673 vs $9.60 \cdot 10^5$ ± 0.726, for M-SAA3 and LPS respectively). In contrast, LPS showed a more moderate numeric increase during the first day (day 1: $2.13 \cdot 10^6$ ± 0.993 vs $5.37 \cdot 10^5$ ± 0.994, for LPS and saline solution respectively) and then the SCC decreased to the negative control levels.

Milk fat and protein contents were evaluated. A treatment effect was observed ($P < 0.05$) for fat content (Figure 2b). The M-SAA3 increased ($P < 0.01$) milk fat levels compared with the respective negative control (data represented as percentage of fat; day 1: 1.42 ± 0.234 vs 1.14 ± 0.234; day 2: 1.88 ± 0.234 vs 1.12 ± 0.234, day 3: 1.77 ± 0.234 vs 0.13 ± 0.234, for M-SAA3 and saline solution, respectively). In addition, fat content was not increased by LPS treatment compared to its negative control. A tendency ($P = 0.051$) in the treatment effect was observed for protein content (Figure 2c). The M-SAA3 increased ($P < 0.01$) milk protein concentration during the 3 d of the experiment compared with the negative control (data represented as percentage of protein; day 1: 0.66 ± 0.027 vs 0.58 ± 0.027; day 2: 0.74 ± 0.027 vs 0.67 ± 0.027, day 3: 0.80 ± 0.027 vs 0.73 ± 0.027, for M-SAA3 and saline solution, respectively). Protein content in M-SAA3 treated quarters tended ($P = 0.06$) to be greater than in the LPS quarters (day 1: 0.66 ± 0.027 vs 0.60 ± 0.030; day 2: 0.74 ± 0.027 vs 0.70 ± 0.030, day 3: 0.80 ± 0.027 vs 0.74 ± 0.035, for M-SAA3 and LPS, respectively). Protein content with LPS did not differ from its negative control.

a



b



(Figure 2 continues in the next page)

c

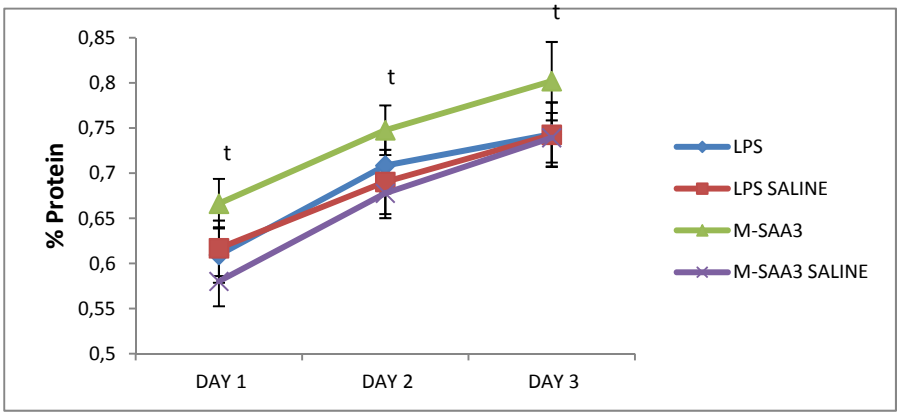


Figure 2. SCC, fat and protein content during the early dry period.

The SCC (a) are represented as SCC/ml ± SEM (transformed data). Fat (b) and protein (c) concentration (data represented as percentage ± SEM) during the 3 d after dry off).Treatments with asterisk differ ($P < 0.05$) and *t* ($P < 0.1$).

3.2. The M-SAA3 increased IL-8 and TNFα expression and inhibited the *S. aureus* translocation in mammary gland primary cultures

The effect of M-SAA3 on IL-8 expression as a main cytokine recruiting neutrophils was evaluated on epithelial primary cultures from cow mammary gland. The epithelial origin of the cultured cells was demonstrated by immunofluorescence staining with a monoclonal anti-pan cytokeratin (Sigma) (Figure 3 a-c). A 15-fold increased ($P < 0.0001$) expression of IL-8 was observed after 3 h incubation with the M-SAA3 (data represented as relative gene expression, 0.006 ± 0.0003 vs 0.00039 ± 0.00038, for M-SAA3 and negative control respectively) (Figure 3d). Furthermore, TNFα levels were increased from non detectable levels to clear detectable levels (data not shown).

Mammary gland primary cultures preincubated with or without M-SAA3 were challenged with a pathogenic strain of *S. aureus* isolated from mastitic milk. The M-SAA3 decreased ($P < 0.05$) the bacterial translocation up to 25% (data represented as CFU/ml; $2.4 \cdot 10^5 \pm 2.9 \cdot 10^4$ vs $3.3 \cdot 10^5$ vs $2.9 \cdot 10^4$, for M-SAA3 and control, respectively) (Figure 3e).

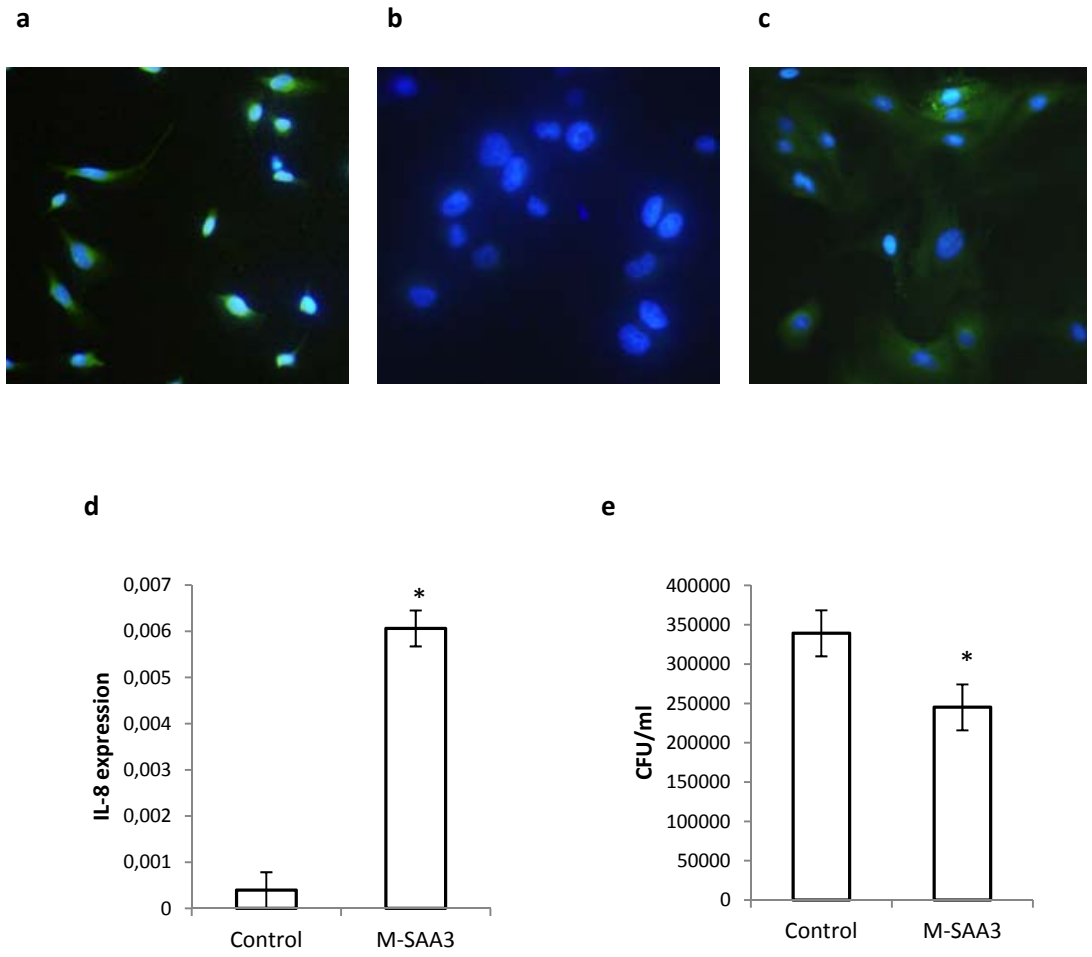


Figure 3. Primary mammary gland cultures.

Immunofluorescence of primary mammary cultures with pan-cytokeratin. HeLa cells (a) as positive control, THP-1 macrophages (b) as negative control and mammary cultures (c). Graphs represent gene expression of IL-8 (a) and viable cell counts of internalized *S. aureus* (b). Bars represent the means ± SEM. Bars with asterisk are different ($P < 0.05$).

3.3. The M-SAA3 promoted Dendritic Cells activation and maturation

The evolution of the DC morphology was evaluated at the microscope and the cell phenotype was checked by CD11c quantification. During differentiation from monocytes, the CD11c gene tended to increase, and was greater after *Salmonella* Dublin challenge (data not shown). DC were incubated with or without M-SAA3. A set of genes related to activation and maturation were evaluated (Figure 4 a-f). Expression of IL-8 increased ($P < 0.0001$) 2-fold (data represented as relative gene expression, 1.06 ± 0.084 vs 0.48 ± 0.0084, for M-SAA3 and negative control respectively) after treatment with M-SAA3. Expression of INFγ was highly upregulated ($P < 0.0001$), obtaining levels 28-fold greater (0.002 ± 0.0004 vs 0.00007 ±

0.000412, for M-SAA3 and negative control). The expression of TNFα increased ($P < 0.0001$) by 7-fold (0.07 ± 0.007 vs 0.01 ± 0.007, for M-SAA3 and negative control respectively). The CCR7 marker increased ($P < 0.01$) 2.5-fold (0.018 ± 0.0016 vs 0.007 ± 0.0016, for M-SAA3 and negative control respectively). The CD80 marker tended ($P = 0.053$) to increase 3-fold (0.00045 ± 0.000096 vs 0.00014 ± 0.000096, for M-SAA3 and negative control respectively). Last, iNOS expression tended (P = 0.054) to increase 3-fold (0.09 ± 0.019 vs 0.03 ± 0.021).

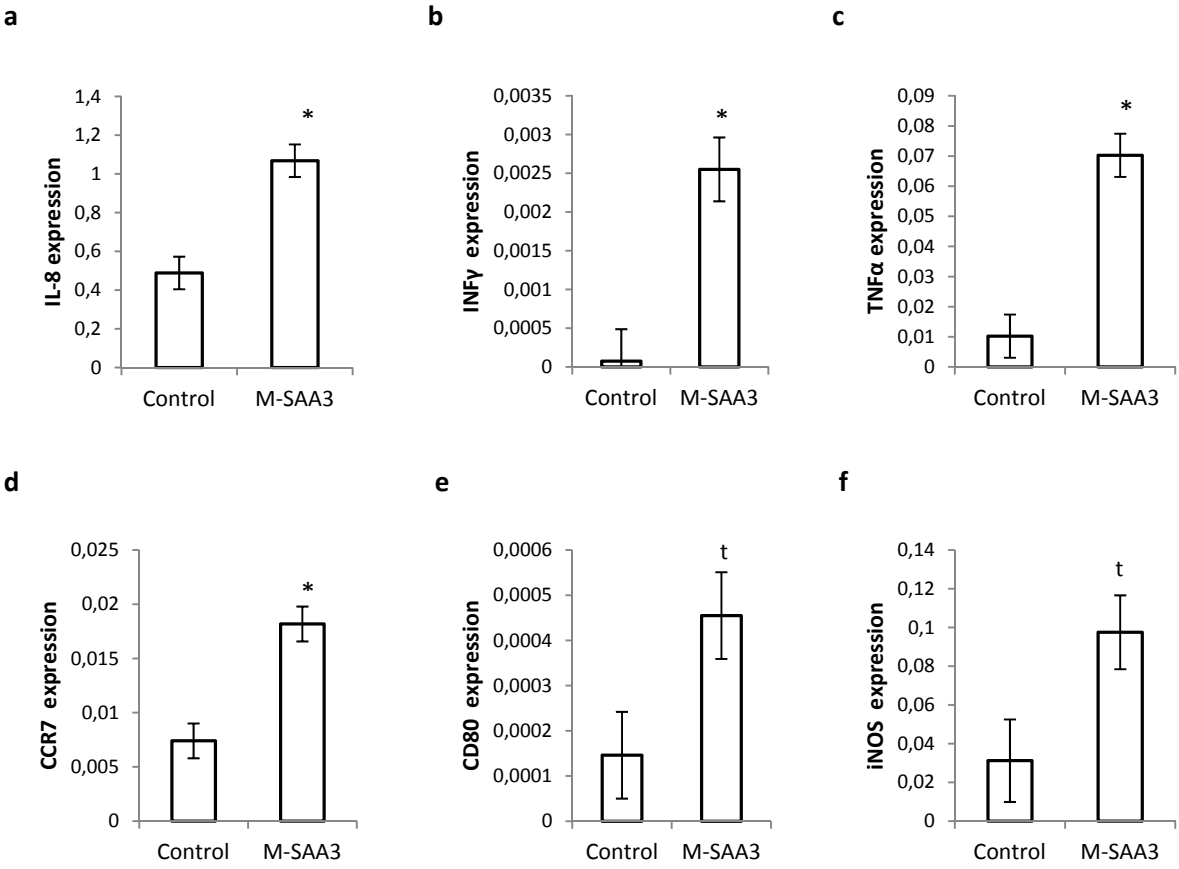


Figure 4. Mammary SAA3 effect on gene expression in dendritic cells.

Gene expression of IL-8 (a), INFγ (b), TNFα (c), CCR7 (d), CD80 (e) and iNOS (f). Bars represent the means ± SEM for the different treatments. Bars with asterisk are different ($P < 0.05$) and *t* ($P < 0.1$).

4. DISCUSSION

This study points out a potential role of the M-SAA3 during the mammary gland involution. The infusion of M-SAA3 clearly increased the MMP-9 activity on milk during early dry off. The activity levels of MMP-9 (Figure 1a) were greater after 24 h post infusion and remained beyond the basal levels during all the experiment. The LPS or endotoxin, is a major constituent of the Gram negative cell wall, and highly activates immune response. During the M-SAA3 production, residual LPS traces were detected in the final eluted protein. Thus, the LPS traces

had to be evaluated, as the bovine mammary gland is highly sensitive to low doses of LPS (Burvenich et al., 2007). However, infusion of pure traces of LPS did not increase the MMP-9 activity, observing similar levels to its respective negative control. On the other hand, MMP-2 activity remained unaltered during all the experiment (Figure 1b). Metalloproteinases play a key role during the onset of the early involution, as they promote the degradation of the basement membrane of the mammary gland (Rabot et al., 2007). MMP-9 has been described as the most active metalloproteinase participating in the involution process of the mammary gland in cows and the main source of MMP-9 is considered to be the neutrophil fraction (Yu et al, 2012). Levels of MMP-2 have been described to increase during mammogenesis and also during the late involution, being the endothelial cells the major MMP-2 producers in the bovine (Rabot et al., 2007).

Interestingly, a numerical increase in the SSC counts was observed in the M-SAA3 (Figure 2a) infused quarters during the second day of the experiment. Contrary, the effect of the LPS traces in the SCC was different. A lower numerical increase was observed only in the first day of the experiment. The somatic cell counts represent the quantification of the immune response cells that are present in milk, mainly referred as neutrophils, macrophages, and other cells types such epithelial cells. Somatic cells normally increase during the involution process (Yu et al., 2012), as immune response mediators are attracted from blood vessels. An increase in immune response mediators would help to the protection of the mammary gland against infections, and also to increase the activity of the MMP-9.

Fat and milk protein concentrations (Figure 2b,c) were both increased in the M-SAA3 treatment in the early dry period. Although expression of milk-specific proteins is decreased during drying off, the total protein content increases. This is due in part because of water reabsorption but mainly to a direct passage of serum-derived proteins. During the early dry off, there is an increased permeability of the epithelial cells barrier which facilitates the passage of high concentrations of lactoferrin, serum albumin and immunoglobulins (Smith and Todhunter, 1982; Hurley and Rejman, 1993). The amount of M-SAA3 innoculated would not increase itself the concentrations obtained in the secretions since only 1 mg is added and the protein levels are much higher. For instance, levels of lactogenic proteins in the first day of drying off vary among 4-17 mg/ml (Watanabe et al., 2007).

Concentrations of milk fat decline slowly during the first 2-3 wk of the dry period but it is possible that during the early dry period samples we still detect fat accumulation which could be greater in M-SAA3 quarters due to the lipid binding capacity of the protein (Liang and Sipe, 1995).

The expression profile of endogenous M-SAA3 in cow mammary gland has been described to be similar to that of lactoferrin and inversely related to lactogenic related proteins like lactoalbumin High expression levels are observed during pregnancy and after calving and low levels during lactation. Then, the M-SAA3 levels increase 72 h after milking cessation (Moolenar et al., 2009). These data suggest that under normal conditions, M-SAA3 would be already participating in the mammary gland integrity. According to this, an intrammamary infusion of exogenous M-SAA3 at the onset of the dry period would facilitate the involution process.

The numerical SCC increase during the day 2 of the experiment (Figure 2a) suggested that M-SAA3 could be promoting cell chemotaxis. This is in agreement with previous data that indicated that hepatic SAA forms activated migration of monocytes and polimorphonuclear cells to the site of inflammation (Badolato et al., 1994). This chemotactic attraction could be mediated by IL-8, which main function is related to the attraction of immune response mediators (Baggiolini et al., 1992). The expression of IL-8 in mammary epithelial cells increased dramatically (Figure 3a), indicating that the mammary gland epithelial cells were able to release a chemotactic cytokine after M-SAA3 exposure. Other cell types such monocytes and neutrophils (He et al., 2003; Lee et al., 2009; Ribeiro et al., 2003) have been also described to produce IL-8 in response to members of the SAA family.

Moreover, primary mammary gland cultures were challenged with a pathogenic strain of *S. aureus* isolated from mastitic milk. Interestingly, M-SAA3 decreased the bacterial infection by 25% (Figure 3e). Mastitis is the main cause of economical losses in dairy farms, over $2 billion per year in United States (Rainard, 2005). In addition, *S. aureus* infection is one of the major producing mastitis, and one of the most difficult to control by antibiotic treatment (Rainard, 2005). We have previously reported that M-SAA3 inhibited the internalization of Enteropathogenic *Escherichia coli* in intestinal CaCo-2 cell line. Other authors have described that recombinant SAA1/2 inhibited the biofilm formation of Uropathogenic *Eschericia coli* (Erman et al., 2012). Adhesion to the epithelium is a critical step in mastitis produced by *S. aureus*. Most of the pathogenic *S. aureus* produce biofilms, which are indeed related to decreased antibiotic sensitivity (Vasudevan et al., 2003).

Additionally, M-SAA3 effects were tested in DC. The DC are effective antigen presenting cells, with a key role in the onset of the adaptive immune response. The DC may be the only cell type able to activate naïve T cells (Banchereau et al., 1998). Circulating immature DC in the blood stream enter to tissues, where become resident cells. In mammary gland, DC populations have been identified among alveoli, epithelia and interalveolar tissue (Maxymiv et

al, 2012). Immature DC present a high phagocytic activity and are responsible for antigen uptake. Maturation is reached, among other stimulus, by bacterial recognition. Mature DC reduce slightly the phagocytic activity and enhance other features such antigen presentation and the capacity to migrate towards secondary lymphatic nodes (Banchereau et al., 1998 and 2000). Maturation can be detected by an increase of related molecules such as CD80 and CCR7 (Xia et al., 2009; Marina et al., 2010; Zhao et al., 2006). The CD80 is an important co-stimulatory molecule that participates in the antigen presentation along with the major histocompatibility complex II (MHCII). The CCR7 is a receptor that helps the migration of mature DC. The infused M-SAA3 increased several maturation markers related to DC activation, migration and antigen presentation (Figure 4a-f). Proinflammatory cytokines such as IL-8, INFγ, and TNFα were upregulated. The CCR7 and CD80 markers were also increased. Finally, the expression of the enzyme nitric oxide synthase (iNOS) was also raised under M-SAA3 treatment. These results are in agreement with published data in which SAA treatment increased nitric oxide in macrophages (Sandri et al., 2008), a mediator that participates in the fight against pathogens and it is produced by iNOS (Bogdan et al., 2001).

In summary, M-SAA3 promotes the increase of agents that participate in the mammary involution process, such as MMP-9 activity and results in a numerical increase in SCC, which could be partially explained by the up-regulation of IL-8 cytokine observed in primary mammary gland cultures. Moreover, M-SAA3 inhibits *S. aureus* invasion, helping to control the increased risk of intramammary infections during early dry off and directly participating in the immune response by activating the maturation of dentritic cells.

5. CONCLUSION

Recombinant M-SAA3 infusion was proved to act as an activator of the mammary involution process and increase immune response mediators. Therefore, this could be a possible strategy to effectively accelerate mammary involution in cows and potentially shorten the dry period.

6. AKNOWLEDGMENTS

We are indebted to the Cell Culture Unit of SCAC at the UAB. The authors thank to Ruminant Production Department for helpful technical assistance and discussions. This study has been funded by INIA-MICINN (RTA2008-00050-C02-01).

Chapter 7

General Discussion

The goal of this thesis was to efficiently produce recombinant goat mammary SAA3 and explore the functional roles of this particular mammary protein in the organism. Biological functions of acute phase proteins are not totally elucidated and M-SAA3 is of particular interest because of its site-specific production in the mammary gland and also its uptake via colostrum and milk intake by the new-born, a distinctive and unique immune scenery.

SAA purification from milk is a complex and tedious process (McDonald et al., 2003) and many authors have previously reported difficulties and limitations in SAA expression studies (Yamada et al., 1994; Hirakura et al., 2002). *Yamada et al.*, described a decrease on bacterial growth after 2 hours of human SAA expression as a result of cell lysis directly related to the toxicity of the recombinant protein. The success in the protein expression fell on the use of high concentrations of antibiotic and short periods of expression. Moreover, *Hirakura et al.* also described complications in the expression of human SAA1, hamster SAA1 and mouse SAA2 isoforms associated with bacterial lysis.

In **Chapter III** two different M-SAA3 sequences were cloned. They mainly differed in a 3 amino acid deletion within a SNARE region of their putative proteins. This fact has been only reported in *Capra hircus*. In other mammals, only the long M-SAA3 isoform is described. Interestingly, only the recombinant expression of the short M-SAA3 isoform succeeded. The long M-SAA3 isoform containing the SNARE sequence, produced cell lysis of the *E. coli* carrying the recombinant plasmid and an increased growth of plasmid lacking bacterial cells, suggesting that the protein, even when expressed at basal levels, produced bacterial killing. In this context, all SAA reported forms that produced cell lysis in literature contained a SNARE-like region except for the human constitutive SAA4 form (see figure 1).

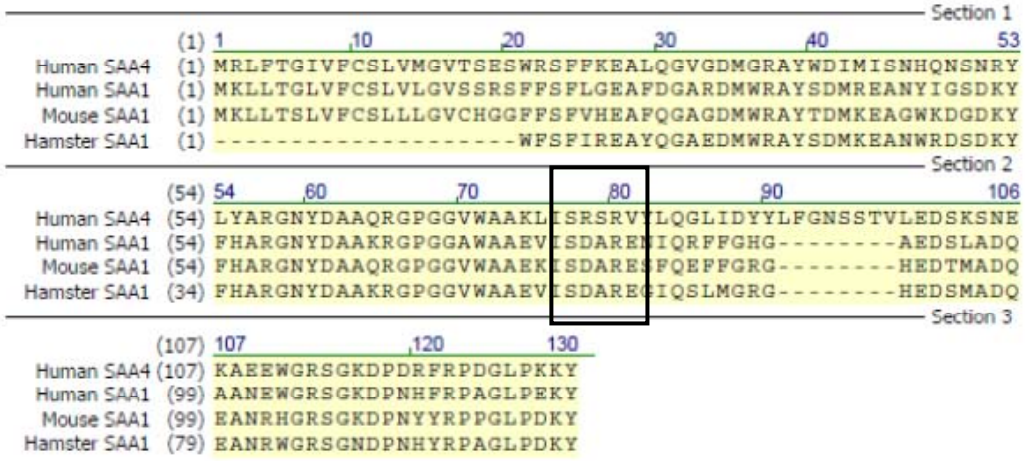


Figure 1. Amino acid alignment of SAA isoforms (Human SAA1 and SAA4, Mouse SAA1 and Hamster SAA2).

Other different regions related to secondary protein structure could also be responsible for the bacterial toxicity. SAA4 is a constitutive form and it is the most divergent compared to the acute phase forms, which remain more homologous within them (Hirakura et al., 2002). However, in our case, two forms of the same acute SAA3 with a high sequence homology (88.5%) and mainly differing in the SNARE region were obtained. Interestingly, only the long form offered a similar behavior than the observed by *Hirakura et al.*, suggesting not only that this region could be implicated in the direct bacterial toxicity, but also that the lack of it could be related to the success of bacterial recombinant production. The non-containing SNARE isoform was efficiently produced and purified from a bacterial expression system as in Chapter III was described.

In **Chapter III** the first functional role of M-SAA3 was assessed. Recombinant M-SAA3 and derived peptides were evaluated regarding their capability of affecting phagocytic activity of macrophages. The whole recombinant M-SAA3 protein increased the number of active milk and blood macrophages and their phagogytic capacity, which is consistent with results reported for serum SAA forms (Hari-Dass et al., 2005; Shah et al., 2006). However, this capability was not related to the N-terminal region of the M-SAA3 protein containing the specific TFLK motif, previously described by *McDonald et al.*, which was related to other biological functions at the gastrointestinal level. The exact mechanism of the increased phagocytic activity is not clear and it was not possible to elucidate it in the present work. The M-SAA3 could be acting directly to bacteria, directly to the macrophages or more possibly, a combination of both processes.

Related to the effect on bacteria, serum A-SAA binds a large number of Gram-negative bacteria through the OmpA protein, present in the cell membrane, and acts as an opsonin facilitating the clearance by phagocytosis (Hari-Dass et al., 2005; Shah et al., 2006).

Regarding the effect on macrophages, there are several studies that relate the serum SAA with an increase in the cytokines expression in immune related cells. *Patel et al.* and *Leow et al.*, described an up-regulation of several pro-inflammatory cytokines such as CCL4, CCL1, CCL3, IL-23 and IL-8 after incubation with SAA proteins. Other immune cells such as neutrophils increase IL-8 and TNFα expression by SAA (He et al., 2003). Moreover, SAA up-regulate the surface adhesion proteins CD18/CD11b and CD18/CDS11c in neutrophils and monocytes, increasing the adhesion to endothelial cells (Badolato et al., 1994). Finally, *Shah et al.*, described an increase in TNFα expression in macrophages challenged with bacteria. The cytokine up-regulation of immune cells indicates an activated status of these cells. Specifically, activated macrophages are characterized by an increased expression of TNFα (Lacy and Stow,

2011; Shah et al., 2006). In this context, we described in **Chapter V** the effect of M-SAA3 in DC during infective processes. DC are important immune cells that lay in the edge of the innate and the adaptive immune response. DC are mainly derived from the myeloid lineage, and share common features with monocytes and macrophages (Satpathy et al., 2012). Maturation is reached, among other stimulus, by bacterial recognition. During maturation, DC reduces the phagocytic activity and enhance other characteristics such as the antigen presentation and the capacity to migrate towards secondary lymphatic nodes (Banchereau et al., 1998 and 2000). CCR7 is a receptor that participates in the migration of mature DC and CD80 is an important co-stimulatory molecule that participates in the antigen presentation along with the MHCII (Zhao et al., 2006; Xia et al., 2009; Facci et al., 2010). The levels of both markers increased in presence of M-SAA3. Moreover, other genes related to immune response, such as IL-8, INFγ and iNOS were also up-regulated, indicating that M-SAA3 not only participated in the DC maturation but also in their activation and function.

The TFLK function was not relevant in macrophages activation, but *McDonald et al.* described the TLFK-like motif as responsible of up-regulating the mucus secretion in intestinal cells and indeed, decreasing the adherence of pathogenic enterobacteria (Larson et al., 2003; Mack et al., 2003). In this context, the protective role of the whole M-SAA3 was tested *in vitro* in **Chapter IV**. The internalization of EPEC was decreased up to 75% in cells pre-incubated with M-SAA3. *Larson et al.*, attributed the reduction of infective bacteria to an increased MUC3 expression. The mucous layer is considered one of the first innate immune barriers (Hecht, 2003), directly interfering the penetration of pathogenic bacteria. In support, we also observed an increased MUC3 expression.

The reduced infective bacteria, though, could also be explained by a direct antibacterial effect. Several studies describe how SAA impairs bacterial viability. *Moolenar et al.*, described a direct effect against *E.coli*, *S. uberis* and *P.aeruginosa*. In addition, *Hirakura et al.* described channel formation in bilayer membranes by A-SAA forms, which would be directly affecting the viability. In support, murine SAA2 has been described to from hexamers with a central channel (Wang et al., 2002). To clarify if the reduced infective bacteria were either produced by a blockade of the bacterial interaction to the intestinal cells or rather a viability impairment, we tested the effect of the recombinant M-SAA against a broad range of laboratory and field pathogenic bacterial strains. No effect in bacterial viability was observed. This result was surprising, as the protein sequence of the recombinant bovine M-SAA3 used by *Moolenar et al.* shared a high homology with the short goat M-SAA3 form (Figure 2). Bovine M-SAA3 showed a 93.1% of identity with long goat M-SAA3 and a 86.3% with the short one. Interestingly, the

bovine protein with antibacterial activity contained in its sequence the SNARE motif, meanwhile the short goat M-SAA3 did not. This result supports the possible antibacterial activity of the SNARE motif, although further research must be done to confirm it.

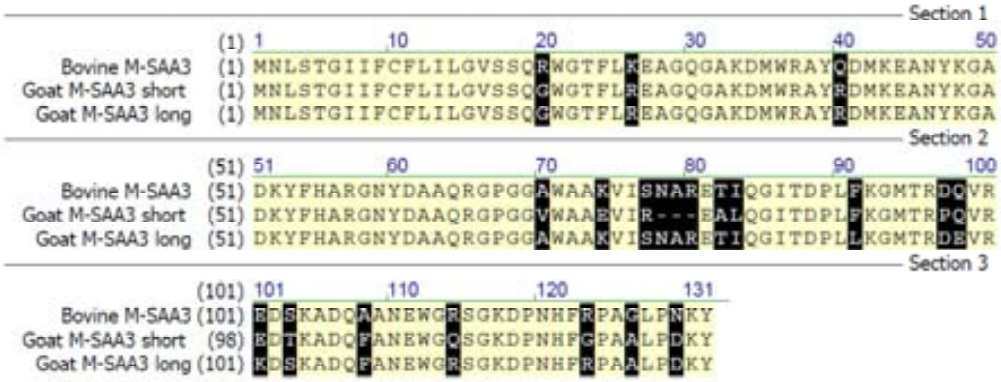


Figure 2. Amino acid alignment of goat and bovine M-SAA3 proteins.

These results indicated that the inhibition observed in intestinal cells was not due to an effect on the bacteria itself. The MUC3 up-regulation would favor the intestinal protection, although other mechanisms could also be participating in this protection. In fact, SAA3 is also able to inhibit bacterial infection in mammary epithelial cells where mucus is not participating. In **Chapter VI**, the M-SAA3 functionality against *S. aureus* was tested in primary mammary gland cultures. The bacterial translocation was reduced in a 25%. This is especially important since *S. aureus* is one of the major bacteria that produce mastitis (Pitkala et al., 2004; Nam et al., 2010), and more importantly, one of the most difficult to control (Rainard, 2005). They mainly occur as chronic subclinical infections with occasional acute episodes (Hogeveen et al., 2005). Moreover, deep reservoirs are produced complicating the antibiotic therapy, directly damaging neutrophils and impairing phagocytosis (Jain, 1979).

The immune response in intestinal epithelial cells was also evaluated. The M-SAA3 increased the IL-8 during infection. The IL-8 function is related to the recruitment of immune response mediators to the site of infection (Hammond et al., 1995). On the other hand, no effect neither in the MUC3 nor the IL-8 expression was observed in absence of infection.

Increased cytokine expression by M-SAA3 produced in epithelial cells of primary mammary gland cultures was also assessed in **Chapter VI**. M-SAA3 increased IL-8 and TNFα expression.

So far, M-SAA3 directly stimulates immune cells (such as macrophages and DC), activates the innate immune response at epithelial level and inhibits bacterial infection. The need to test these mechanisms simultaneously encouraged us to test an *ex vivo* intestinal model. In **Chapter V**, the protective effect in bovine intestinal PPs explants was evaluated during *Salmonella* Dublin infection. An intestinal infection model was optimized with low levels of

infective *Salmonella*. Higher doses of bacteria produced tissue unresponsiveness, probably due to the oxidative stress and accumulation of ROS (Zhang et al., 2003). PP are an important part of the GALT tissue and represent the main infection site for *Salmonella*. PP are formed by a heterogeneous niche of immune and non-immune cells, which offer a more complex view of the immune responses at gastrointestinal level. Specifically, macrophages and epithelial cells (cell types studied in **Chapter III** and **IV**) are present in PP and play a synergistic role during infections, providing a more physiological approximation than the commercial cell lines. The M-SAA3 reduced bacterial translocation in PP similar levels than observed with the solely intestinal cells. In contrast, no increased expression of MUC3 was found, but some evidences indicated that *ex vivo* PP explants might not be a good mean to study the mucin regulation.

As MUC3 was not responsible for the anti-pathogenic properties in PP, an alternative mechanism was studied. Several studies indicate that intestinal infections cause tight junctions rearrangement and decrease tight junctions protein expression (Miyoshi, 2005; Boyle et al., 2006; Köhler et al., 2007; Qin et al., 2009; Roxas et al., 2010). Tight junctions effectively seal the intercellular space, preventing the bacterial entrance (Roselli et al., 2007). The M-SAA3 increased the expression of Claudin-2 and Occludin, suggesting that bacterial translocation could be decreased by enhancing the tight junction network.

The immune response was also exacerbated, observing increased levels of IL-8 and INFγ. Specifically, INFγ is a key effector in *Salmonella* infections but is only produced by a few cell types such as lymphocytes and some antigen presenting cells (Frucht et al., 2001). Moreover, INFγ is also an important cell activator. Macrophages, for instance, need to be primed by INFγ (Mosser, 2003). DC are also an important cell type present in PP. As explained previously, M-SAA3 activated DC during infection, increasing maturation markers such as CD80 and IL-12, cytokine expression of IL-8 and INFγ (but not TNFα), migration markers (CCR7) and iNOS, indicating that DC can participate actively in the cytokine expression profile. In **Chapter VI** the effect of M-SAA3 was tested in DC in non-infective conditions. To this regard, similar results were obtained. All parameters analyzed were increased, observing a clear rise in cytokines and maturation markers. The activation of DC is important due to their key role between the innate and adaptive immune response. The INFγ up-regulation contributes indeed, to macrophage activation. Moreover, the IL-12 and the CD80 are key effectors that participate in the CD4 T lymphocytes proliferation and differentiation towards the Th1 response (also responsible for macrophages activation) (Ni and O'Neill, 1997).

At this point, we have clearly demonstrated that M-SAA3 has an important role stimulating the innate immune system and inhibiting bacterial infection at both mammary gland and

gastrointestinal tract levels. This is consistent with the presence of M-SAA3 in mammary gland secretion under mastitis and in colostrum to feed newborn animals and protect gastrointestinal tract during the first days after calving.

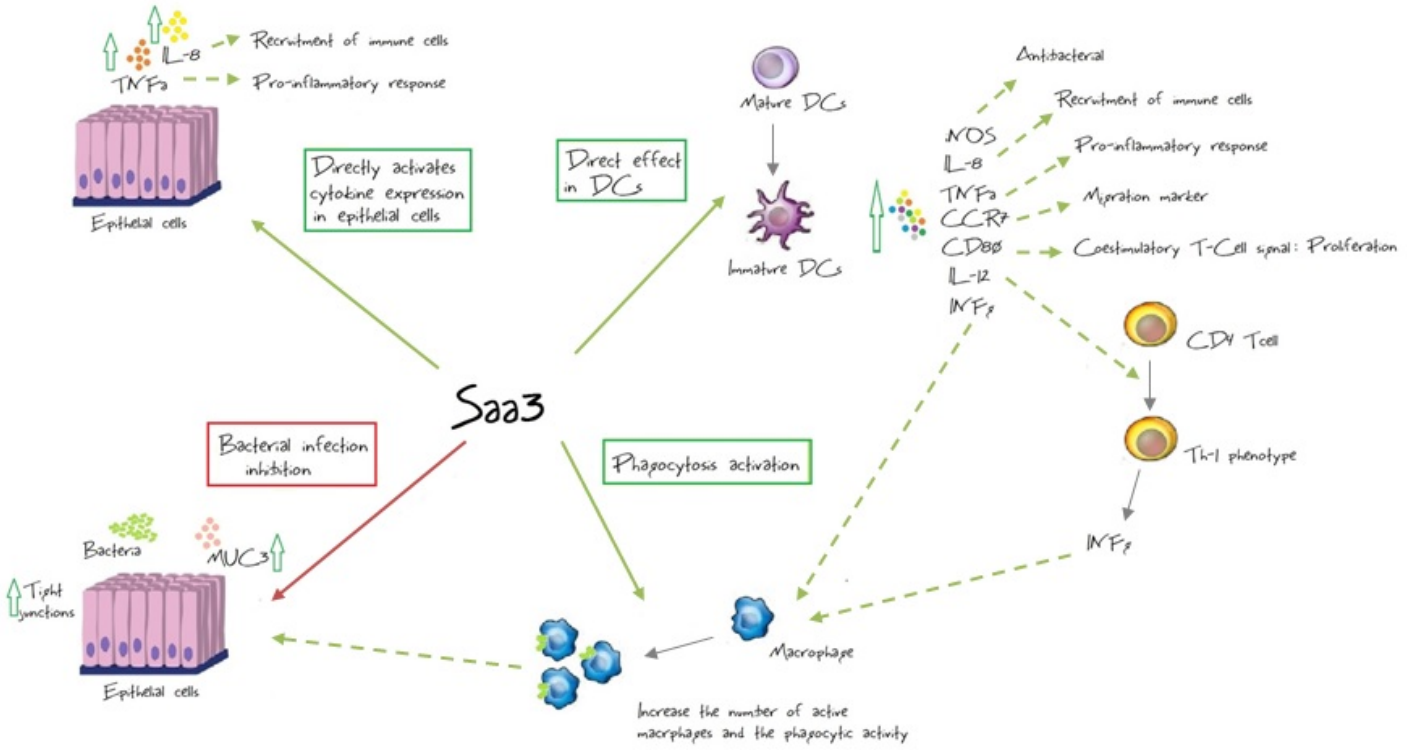


Figure 3. SAA3 related functions.

Green and red continuous lines indicate direct activation or inhibition of the pathway, respectively. Dotted lines indicate an indirect effect, as a consequence of the activation of secondary mediators. SAA3 directly activates cytokine expression in epithelial cells. The levels of IL-8 and TNFα are raised which promote immune cells recruitment and enhance the pro-inflammatory response. SAA3 also decreases bacterial translocation in epithelial cells, via MUC3 and tight junction over-expression. SAA3 clearly activates innate immune cells, such macrophages and DC. Macrophages increase the phagocytic capacity and DC express maturation and activation markers. Among these stimulus, maturation markers such CD80 and IL-12 enhance the T cell response (indirect action on adaptive immune response); INFγ activates macrophage activity and iNOS participates in the fight against bacteria.

All these functions previously exposed, encouraged us to study the effect of M-SAA3 during the bovine dry period. During this period it is necessary to prevent the high risk of intramamary infections, to stimulate the immune system and renew senescent epithelial cells of the mammary gland to ensure optimal production of milk in the next lactation (Capuco et al., 1997). Moreover, as cows are pregnant, the involution and regeneration of mammary gland is slow because of the presence of lactogenic hormones and requires long dry periods to be achieved, usually representing the 60 last days before calving which causes a great impact in the productive period and so, in the economy (Noble et al., 1999; Pezeshki et al., 2009).

The expression profile of endogenous M-SAA3 in cow mammary gland has been described to be similar to that of lactoferrin and inversely related to lactogenic related proteins like lactoalbumin High expression levels are observed during pregnancy and after calving and low levels during lactation. Then, the M-SAA3 levels increase in dry period 72 h after milking cessation (Moolenar et al.., 2009). These data suggest that under normal conditions, M-SAA3 would be yet participating in the mammary gland integrity. According to this, an intrammamary infusion of exogenous M-SAA3 at the onset of the dry period would accelerate and facilitate the involution process and what it is even more important will help to prevent intramammary infections.

In **Chapter VI** we demonstrated that the infusion of M-SAA3 clearly increased the activity of metalloproteinase MMP-9 on milk during early dry off. The activity levels of MMP-9 were greater after 24 h post infusion and remained beyond the basal levels during all the experiment. Metalloproteinases play a key role during the onset of the early involution, as they promote the degradation of the basement membrane of the mammary gland (Rabot et al., 2007). MMP-9 has been described as the most active metalloproteinase participating in the involution process of the mammary gland in cows and the main source of MMP-9 is considered to be the neutrophil fraction (Yu et al., 2012).

A numerical increase in the SCC was observed in M-SAA3 infused quarters during the second day. SCC represent the immune cells present in milk and they would help to the protection against infections and to increase the MMP-9 concentration (also present in neutrophils).

The numerical SCC increase suggested that M-SAA3 could be promoting cell chemotaxis. This is in agreement with previous data that indicated that hepatic SAA forms activated migration of monocytes and polimorphonuclear cells to the site of inflammation (Badolato et al., 1994). The chemotactic attraction could be mediated by IL-8. As described previously, M-SAA3 increases IL-8 expression in primary mammary gland cultures and helps to control *S. aureus* infection.

At the light of these results, M-SAA3 could be exerting an important role in mammary gland, directly participating in the tissue remodeling and protection (Figure 4).

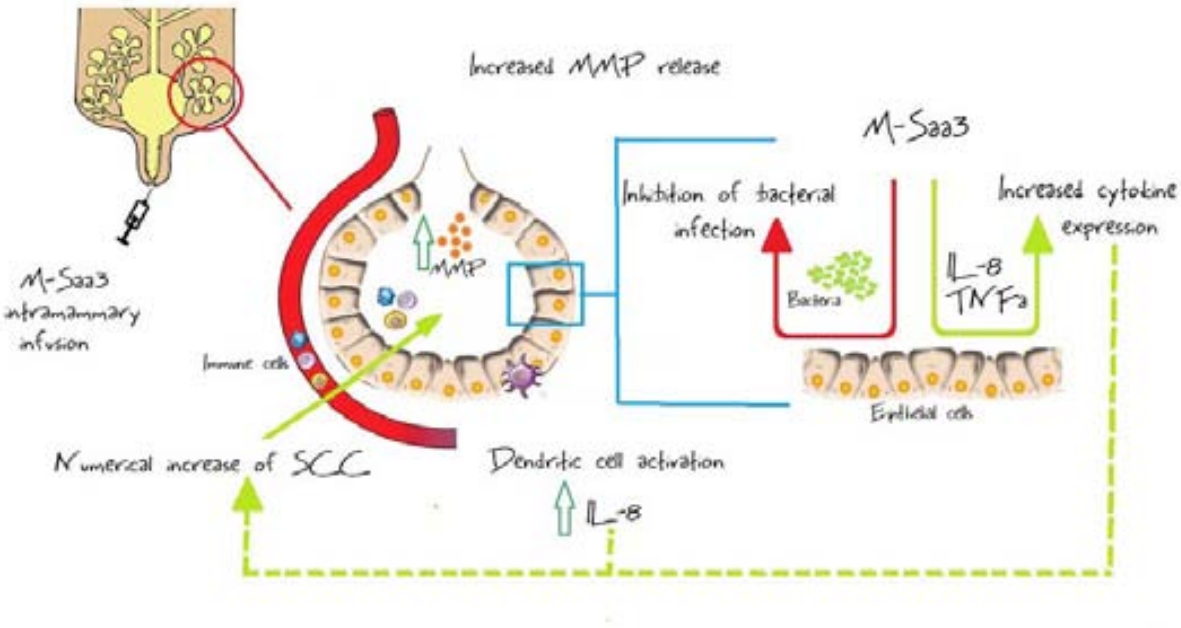


Figure 4. M-SAA3 effect during the cow dry period.

Green and red continuous lines indicate direct activation or inhibition of the pathway, respectively. Dotted lines indicate an indirect effect, as a consequence of the activation of secondary mediators. M-SAA3 infused in the mammary gland numerically increased the metalloproteinase release in milk and raised the SCCs number. In epithelial cells, M-SAA3 up-regulated the IL-8 which could be related to the increased SCCs. Bacterial infection was inhibited. DC present on the mammary gland, become active and enhance the innate immune response.

In summary, this thesis contributes to the knowledge about the SAA3 protein functionality, resulting to be a key regulator of innate immunity and a direct inhibitor of bacterial infections at mammary and gastrointestinal level, showing at the same time, potential applications of the recombinant forms in critical periods of ruminant production such as the cow dry, a period where the mammary gland health is highly compromised and there are important economical losses for the sector.

Chapter VIII

Conclusions

1. Two forms of M-SAA3 are expressed in *Capra hircus*, which mainly differ in a 3 amino acid deletion within the SNARE motif of their putative proteins. The non-containing SNARE isoform has not been reported in any other mammal specie. This motif seems to be related to a direct antibacterial activity, as it produces massive cell lysis during the recombinant protein production.
2. M-SAA3 activates blood and milk macrophages, increasing both the number of active macrophages and their phagocytic activity. The TFLK motif of the M-SAA3 protein does not participate in macrophage activation.
3. M-SAA3 efficiently reduces EPEC bacterial translocation in intestinal epithelial cells and increases MUC3 expression but does not directly affect bacterial viability.
4. M-SAA3 increases the expression of IL-8 in intestinal epithelial cells during infection, which links the M-SAA3 functionality to an innate immune activation.
5. It has been established a useful bovine intestinal *ex vivo* infection model based in tissue explants derived from Peyer's Patches. The model gives a precise approach to an *in vivo* situation.
6. M-SAA3 reduces *Salmonella* Dublin infection in the bovine *ex vivo* model, corroborating the results obtained in epithelial cell lines. This protection is related to an increased expression of Claudin-2 and Occludin, proteins that participate in the tight junctions sealing of epithelial cells.
7. M-SAA3 increases the pro-inflammatory innate response in *ex vivo* explants during infection, raising the levels of important cytokines such as IL-8 and INFγ.
8. Dendritic cells, key link between the innate and adaptive immune response, become activated by M-SAA3, increasing maturation markers such as CD80 and CCR7; cytokine genes such as IL-8, INFγ, TNFα and IL-12; and other enzymes such as iNOS both in infective and non-infective conditions.
9. M-SAA3 appears to play a key role in the newborn gastrointestinal tract protection through milk intake.
10. M-SAA3 also decreased *S. aureus* translocation and increased the pro-inflammatory response in epithelial mammary gland primary cultures suggesting an important protective role in the mammary gland protection in the adulthood.

11. M-SAA3 increases the MMP levels and the numerical number of SCC in milk when administered intramammary after milk cessation, suggesting that it actively promotes the mammary gland involution. These results, along with the immune system stimulation and bacterial protection in mammary epithelial cultures indicate a clear potential of M-SAA3 in the optimization of the cow dry period.

Chapter IX

Literature cited

Abbas KA, Lichtman AH, Pober JS. Inmunología cellular y molecular. 4th Edition, 2002, McGraw-Hill Interamericana de España ISBN 8448604059.

Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat. Rev. Immunol., 2010 Feb 10(2):131-44.

Accorsi PA, Pacioni B, Pezzi C, Forni M, Flint DJ, Seren E. Role of prolactin, growth hormone and insulin-like growth factor 1 in mammary gland involution in the dairy cow. J Dairy Sci., 2002 Mar;85(3):507-13.

Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, Boyson JE, Fitzgerald KA, Flavell RA, Eisenbarth SC, Poynter ME. Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. J. Immunol., 2011 Jul 1;187(1):64-73.

Badolato R, Wang, JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, Kelvin DJ, Oppenheim JJ. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polimorphonuclear leukocytes. J. Exp. Med., 1994; 180(1), 203-9.

Baggiolini M and Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett.,1992 Jul 27;307(1):97-101.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu. Rev. Immunol., 2000; 18:767-811.

Banchereau J and Steinman RM. Dendritic cells and the control of immunity. Nature, 1998 Mar 19;392(6673):245-52.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu. Rev. Immunol., 2001; 18:767-811.

Banka CL, Yuan T, de Beer MC, Kindy M, Curtiss LK, de Beer FC. Serum amyloid A (SAA): influence on HDL-mediated cellular cholesterol efflux. J. Lipid Res., 1995 May;36(5):1058-65.

Baranova IN, Vishnyakova TG, Bocharov AV, Kurlander R, Chen Z, Kimelman ML, Remaley AT, Csako G, Thomas F, Eggerman TL, Patterson AP. Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. J. Biol. Chem., 2005 Mar 4;280(9):8031-40.

Baranova IN, Bocharov AV, Vishnyakova TG, Kurlander R, Chen Z, Fu D, Arias IM, Csako G, Patterson AP, Eggerman TL. CD36 is a novel serum amyloid A (SAA) receptor mediating SAA binding and SAA-induced signaling in human and rodent cells. J. Biol. Chem., 2010 Mar 12;285(11):8492-506.

Bartels CJ, Holzhauer M, Jorritsma R, Swart WA, Lam TJ. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev. Vet. Med., 2010 Feb 1;93(2-3):162-9.

Beg AA. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol., 2002 Nov;23(11):509-12.

Benditt EP, Eriksen N. Amyloid protein SAA is associated with high density lipoprotein from human serum. Proc. Natl. Acad. Sci. USA, 1977 Sep;74(9):4025-8.

Bendit EP, Eriksen N, Hanson RH. Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein. Proc. Natl. Acad. Sci. USA, 1979 August; 76(8): 4092–4096.

Benditt, EP. Amyloid A gene family expression in different mouse tissues. J. Exp. Med., 1986; 164(6), 2006-17.

Benditt EP and Meek RL. Expression of the third member of the serum amyloid A gene family in mouse adipocytes. J. Exp. Med., 1989 May 1;169(5):1841-6.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med., 1993;4(2):197-250.

Björkman L, Raynes JG, Shah C, Karlsson A, Dahlgren C, Bylund J. The proinflammatory activity of recombinant serum amyloid A is not shared by the endogenous protein in the circulation. Arthritis Rheum., 2010; 62, 1660-5.

Blanchard PC. Diagnostics of dairy and beef cattle diarrhea. Vet. Clin. North Am. Food Anim. Pract., 2012 Nov;28(3):443-64.

Bogdan C. Nitric oxide and the immune response. Nat. Immunol., 2001 Oct;2(10):907-16.

Boudreau N, Sympson CJ, Werb Z, Bissell MJ. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science, 1995 Feb 10;267(5199):891-3.

Boutinaud M, and Jammes H. Potential uses of milk epithelial cells: a review. Reprod. Nutr. Dev., 2002; 42(2):133-47.

Boyle EC, Brown NF, Finlay BB. Salmonella enterica serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function. Cell Microbiol., 2006 Dec;8(12):1946-57.

Bradley A. Bovine mastitis: an evolving disease. Vet. J., 2002 Sep;164(2):116-28.

Bu XD, Li N, Tian XQ, Huang PL. CaCo-2 and LS174T cell lines provide different models for studying mucin expression in colon cancer. Tissue Cell., 2011 Jun;43(3):201-6.

Burvenich C, Bannerman DD, Lippolis JD, Peelman L, Nonnecke BJ, Kehrli ME Jr, Paape MJ. Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. J. Dairy Sci., 2007 90 Suppl 1:E39-54.

Capuco AV, Akers RM, Smith JJ. Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. J. Dairy Sci., 1997 Mar;80(3):477-87.

Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue Toxicol. Pathol., 2006;34(5):599-608.

Cheng N, He R, Tian J, Ye PP, Ye RD. Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. J. Immunol., 2008 Jul 1;181(1):22-6.

Chow AW, Liang JF, Wong JS, Fu Y, Tang NL, Ko WH. Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o-cells in response tocationic polypeptide challenge. PLoS One, 2010 Aug 12;5(8).

Coburn B, Grassl GA, Finlay BB. *Salmonella*, the host and disease: a brief review. Immunol. Cell Biol., 2007 Feb-Mar;85(2):112-8.

Coetzee GA, Strachan AF, van der Westhuyzen DR, Hoppe HC, Jeenah MS, de Beer FC. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. J. Bio.l Chem., 1986 Jul 25;261(21):9644-51.

Coombes JL and Powrie F. Dendritic cells in intestinal immune regulation. Nat. Rev. Immunol., 2008 Jun;8(6):435-46.

Cray C, Zaias J, Altman NH. Acute Phase Response in Animals: A Review. Comp Med., 2009 December; 59(6): 517–526.

Croxen MA, and Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat. Rev. Microbiol., 2010 Jan;8(1):26-38.

De Beer MC, Yuan T, Kindy MS, Asztalos BF, Roheim PS, de Beer FC. Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. J. Lipid Res., 1995 Mar;36(3):526-34.

De Beer MC, de Beer FC, Gerardot CJ, Cecil DR, Webb NR, Goodson ML, Kindy MS. Structure of the mouse Saa4 gene and its linkage to the serum amyloid A gene family. Genomics, 1996 May 15;34(1):139-42.

De Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE. A review of the importance of *cryptosporidiosis* in farm animals. Int. J. Parasitol., 1999 Aug;29(8):1269-87.

De Jesus Rodriguez B, Chevaleyre C, Henry G, Mollé D, Virlogeux-Payant I, Berri M, Boulay F, Léonil J, Meurens F, Salmon H. Identification in milk of a serum amyloid A peptide chemoattractant for B lymphoblasts. BMC immunology, 2009, 10:4.

De Vliegher S, Fox LK, Piepers S, McDougall S, Barkema HW. Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J. Dairy Sci., 2012 Mar;95(3):1025-40.

De Vries LD, Dover H, Casey T, VandeHaar MJ, Plaut K. Characterization of mammary stromal remodeling during the dry period. J. Dairy Sci., 2010 Jun;93(6):2433-43.

Denis M, Parlane NA, Lacy-Hulbert SJ, Summers EL, Buddle BM, Wedlock DN. Bactericidal activity of macrophages against Streptococcus uberis is different in mammary gland secretions of lactating and drying off cows. Vet. Immunol. Immunopathol., 2006,114(1-2), 111-20.

Desmarchelier P and Fegan N. Pathogens in Milk. *Escherichia coli* Encyclopedia of Dairy Sciences. 2nd Second, 2011, Academic Press, San Diego, Pages 60-66, ISBN 9780123744074.

Domènech A, Raynes JG, Rodríguez EG, Arís A, Bach A, Serrano A. Recombinant expression of goat milk serum amyloid A: preliminary studies of the protein and derived peptides on macrophage phagocytosis. Protein Pept. Lett., 2012 Mar;19(3):299-307.

Dyson MR, Shadbolt SP, Vincent KJ, Perera RL, McCafferty J. Production of soluble mammalian proteins in Escherichia coli: identification of protein features that correlate with successful expression. BMC Biotechnol., 2004, 4: 32.

Eckersall PD, Young FJ, McComb C, Hogarth CJ, Safi S, Weber A, McDonald T, Nolan AM, Fitzpatrick JL. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet. Rec., 2001 Jan 13;148(2):35-41.

Eckersall PD, Young FJ, Nolan AM, Knight CH, McComb C, Waterston MM, Hogarth CJ, Scott EM, Fitzpatrick JL. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. J. Dairy Sci., 2006 May;89(5):1488-501.

Eckersall PD, Bell R.Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet. J., 2010 Jul;185(1):23-7

Eckhardt ER, Witta J, Zhong J, Arsenescu R, Arsenescu V, Wang Y, Ghoshal S, de Beer MC, de Beer FC, de Villiers WJ. Intestinal epithelial serum amyloid A modulates bacterial growth *in vitro* and pro-inflammatory responses in mouse experimental colitis. BMC Gastroenterol., 2010, 10, 10-133.

Eckmann L, Fierer J, Kagnoff MF. Genetically resistant (Ityr) and susceptible (Itys) congenic mouse strains show similar cytokine responses following infection with *Salmonella* Dublin. J. Immunol., 1996 Apr 15;156(8):2894-900.

Eckmann L and Kagnoff MF. Cytokines in host defense against *Salmonella.* Microbes Infect., 2001 Nov-Dec;3(14-15):1191-200.

Eckmann L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect. Immun., 1993 Nov;61(11):4569-74.

Eliezer D., Amyloid Ion Channels: A Porous Argument or a Thin Excuse? J. Gen. Physiol., 2006 December; 128(6): 631–633.

Erman A, Lakota K, Mrak-Poljsak K, Blango MG, Krizan-Hergouth V, Mulvey MA, Sodin-Semrl S, Veranic P. Uropathogenic *Escherichia coli* induces serum amyloid A in mice following urinary tract and systemic inoculation. PLoS One, 2012;7(3):e32933.

Erskine RJ, Wagner S, DeGraves FJ. (2003) Mastitis therapy and pharmacology. Vet. Clin. North Am. Food Anim. Pract., 2003; 19(1):109-38.

Facci MR, Auray G, Buchanan R, Kessel J, Thompson DR, Mackenzie-Dyck S, Babiuk L A, Gerdts V. A comparison between isolated blood dendritic cells and monocyte-derived dendritic cells in pigs. Immunology, 2010 March; 129(3): 396–405.

Foster DM and Smith GW. Pathophysiology of diarrhea in calves. Vet. Clin. North. Am. Food Anim. Pract., 2009 Mar;25(1):13-36, xi.

Frucht DM, Fukao T, Bogdan C, Schindler H, O'Shea JJ, Koyasu S. IFN-gamma production by antigen-presenting cells: mechanisms emerge. Trends Immunol., 2001 Oct;22(10):556-60.

García A, Ruiz-Santa-Quiteria JA, Orden JA, Cid D, Sanz R, Gómez-Bautista M, de la Fuente R. Rotavirus and concurrent infections with other enteropathogens in neonatal diarrheic dairy calves in Spain. Comp. Immunol. Microbiol. Infect. Dis., 2000 Jul;23(3):175-83.

Gardiner GE, O'Flaherty S, Casey PG, Weber A, McDonald T, Cronin M, Hill C, Ross RP, Gahan CG, Shanahan F. Evaluation of colostrum-derived human mammary-associated serum amyloid A3 (M-SAA3) protein and peptide derivatives for the prevention of enteric infection: in vitro and in murine models of intestinal disease. FEMS Immunol. Med. Microbiol., 2009, 55(3), 404-413.

Gazi U and Martinez-Pomares L. Influence of the mannose receptor in host immune responses. Immunobiology, 2009 Jul;214(7):554-61.

Green MJ, Green LE, Medley GF, Schukken YH, Bradley AJ. Influence of dry period bacterial intramammary infection on clinical mastitis in dairy cows. J. Dairy Sci., 2002 Oct;85(10):2589-99.

Gribar SC, Anand RJ, Sodhi CP, Hackam DJ. The role of epithelial Toll-like receptor signaling in the pathogenesis of intestinal inflammation. J. Leukoc. Biol., 2008 Mar;83(3):493-8.

Grönlund U, Hallén Sandgren C, Persson Waller K. Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. Vet. Res., 2005 Mar-Apr;36(2):191-8.

Gruys E, Toussaint MJM, Niewold TA, Koopmans S J. Acute phase reaction and acute phase proteins. J Zhejiang Univ. Sc.i B., 2005 November; 6(11): 1045–1056.

Gulliksen SM, Jor E, Lie KI, Hamnes IS, Løken T, Akerstedt J, Osterås O. Enteropathogens and risk factors for diarrhea in Norwegian dairy calves. J. Dairy Sci., 2009 Oct;92(10):5057-66.

Guo JT, Aldrich CE, Mason WS, Pugh JC. Characterization of serum amyloid A protein mRNA expression and secondary amyloidosis in the domestic duck. Proc, Natl, Acad, Sci, USA, 1996 Dec 10;93(25):14548-53.

Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, Giedlin MA, Mullenbach G, Tekamp-Olson P. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. J. Immunol., 1995 Aug 1;155(3):1428-33.

Hari-Dass R, Shah C, Meyer DJ, Raynes, JG. Serum amyloid A protein binds to outer membrane protein A of gram-negative bacteria. J. Biol. Chem., 2005, 280(19), 18562-18567.

Hashim A, Clyne M, Mulcahy G, Akiyoshi D, Chalmers R, Bourke B. Host cell tropism underlies species restriction of human and bovine Cryptosporidium parvum genotypes. Infect. Immun., 2004 Oct; 72 (10):6125-31.

Hathaway LJ and Kraehenbuhl JP. The role of M cells in mucosal immunity. Cell Mol. Life Sci., 2000 Feb;57(2):323-32.

He R, Sang H, Ye RD. Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. Blood, 2003 Feb 15;101(4):1572-81.

Hecht G. Innate mechanisms of epithelial host defense: spotlight on intestine. Am. J. Physiol., 1999 Sep;277(3 Pt 1):C351-C358.

Hecht G, Savkovic SD. Effector role of epithelia in inflammation--interaction with bacteria. Aliment Pharmacol. Ther., 1997 Dec;11 Suppl 3:64-8; discussion 68-9.

Heikkilä AM, Nousiainen JI, Pyörälä S. Costs of clinical mastitis with special reference to premature culling. J. Dairy Sci., 2012 Jan;95(1):139-50.

Hernandez LL, Collier JL, Vomachka AJ, Collier RJ, Horseman ND. Suppression of lactation and acceleration of involution in the bovine mammary gland by a selective serotonin reuptake inhibitor. J. Endocrinol., 2011 209(1):45-54.

Hirakura Y, Carreras I, Sipe JD, Kagan BL. Channel formation by serum amyloid A: a potential mechanism for amyloid pathogenesis and host defense. Amyloid, 2002 Mar;9(1):13-23.

Hogeveen H. Mastitis in Dairy Production: Current Knowledge and Future Solutions. 4th IDF International Dairy Conference] Wageningen Academic Pub, 2005 ISBN 9789076998701.

Hurley WL, Rejman JJ.(1993) Bovine lactoferrin in involuting mammary tissue. Cell Biol. Int. 1993 Mar;17(3):283-9.

Hyland KA, Brown DR, Murtaugh MP. *Salmonella enterica serovar Choleraesuis* infection of the porcine jejunal Peyer's patch rapidly induces IL-1beta and IL-8 expression. Vet. Immunol. Immunopathol.,2006 Jan 15;109(1-2):1-11.

Hyland KA, Kohrt L, Vulchanova L, Murtaugh MP. Mucosal innate immune response to intragastric infection by *Salmonella enterica serovar Choleraesuis*. Mol. Immunol., 2006 Apr;43(11):1890-9.

Jensen LE and Whitehead AS. Regulation of serum amyloid A protein expression during the acute-phase response. Biochem. J., 1998 Sep 15;334 (Pt 3):489-503.

Jain NC. Common mammary pathogens and factors in infection and mastitis. J. Dairy Sci., 1979 Jan;62(1):128-34.

Jijon HB, Madsen KL, Walker JW, Allard B, Jobin C. Serum amyloid A activates NF-kappaB and proinflammatory gene expression in human and murine intestinal epithelial cells. Eur. J. Immunol., 2005 Mar;35(3):718-26.

Johansson C and Kelsall BL. Phenotype and function of intestinal dendritic cells. Semin Immunol., 2005 Aug;17(4):284-94.

Jones BD. Host responses to pathogenic *Salmonella* infection. Genes Dev., 1997 Mar 15;11(6):679-87.

Kaisho T, Akira S. Toll-like receptors and their signaling mechanism in innate immunity. Acta Odontol. Scand., 2001 Jun;59(3):124-30.

Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity, 2011 May 27;34(5):637-50.

Kindy MS, King AR, Yu J, Geradot C, Whitley J, de Beer FC. Adenoviral expression of murine serum amyloid A proteins to study amyloid fibrillogenesis. Biochem. J., 1998 June 15; 332(Pt 3): 721–728.

Kisilevsky R, Subrahmanyan L. Serum amyloid A changes high density lipoprotein's cellular affinity. A clue to serum amyloid A's principal function. Lab. Invest., 1992 Jun;66(6):778-85.

Kisilevsky R and Manley PN. Acute-phase serum amyloid A: perspectives on its physiological and pathological roles. Amyloid, 2012 Mar;19(1):5-14.

Kluve-Beckerman B, Drumm ML, Benson MD. Nonexpression of the human serum amyloid A three (SAA3) gene. DNA Cell Biol., 1991 Nov;10(9):651-61.

Köhler H, Sakaguchi T, Hurley BP, Kase BA, Reinecker HC, McCormick BA. *Salmonella enterica serovar Typhimurium* regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. Am. J. Physiol. Gastrointest. Liver Physiol., 2007 Jul;293(1):G178-87.

Kovačević-Filipović M, Ilić V, Vujčić Z, Dojnov B, Stevanov-Pavlović M, Mijačević Z, Božić T. Serum amyloid A isoforms in serum and milk from cows with *Staphylococcus aureus* subclinical mastitis. Vet. Immunol. Immunopathol., 2012 Jan 15;145(1-2):120-8.

Kumon Y, Hosokawa T, Suehiro T, Ikeda Y, Sipe JD, Hashimoto K. Acute-phase, but not constitutive serum amyloid A (SAA) is chemotactic for cultured human aortic smooth muscle cells. Amyloid, 2002 Dec;9(4):237-41.

Lachmann HJ, Goodman HJ, Gilbertson JA, Gallimore JR, Sabin CA, Gillmore JD, Hawkins PN. Natural history and outcome in systemic AA amyloidosis. N. Engl. J. Med., 2007 Jun 7;356(23):2361-71.

Lacy P and Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood, 2011 Jul 7;118(1):9-18.

Land KM, Johnson PJ. Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa. Drug. Resist., 1999; 2(5) 289-294.

Larson MA, Weber A, Weber AT, McDonald TL. Differential expression and secretion of bovine serum amyloid A3 (SAA3) by mammary epithelial cells stimulated with prolactin or lipopolysaccharide. Vet Immunol. Immunopathol., 2005 Sep 15;107(3-4):255-64.

Larson M, Wei Shu H, Weber A, Mack D, McDonald T. Human serum amyloid A3 peptide enhances intestinal MUC3 expression and inhibits EPEC adherence. Biochem. Biophys. Res. Commun., 2003;300 531–540.

Lee HY, Kim MK, Park KS, Bae YH, Yun J, Park JI, Kwak JY, Bae YS. Serum amyloid A stimulates matrix-metalloproteinase-9 upregulation via formyl peptide receptor like-1-mediated signaling in human monocytic cells. Biochem. Biophys. Res. Commun., 2005 May 13;330(3):989-98.

Lee HY, Kim SD, Shim JW, Lee SY, Yun J, Bae YS. LL-37 inhibits serum amyloid A-induced IL-8 production in human neutrophils. Exp. Mol. Med., 2009 May 31;41(5):325-33.

Leow KY, Goh WW, Heng CK. Effect of serum amyloid A1 treatment on global gene expression in THP-1-derived macrophages. Inflamm. Res., 2012 Apr;61(4):391-8.

Liang JS and Sipe JD. Recombinant human serum amyloid A (apoSAAp) binds cholesterol and modulates cholesterol flux. J. Lipid Res., 1995 Jan;36(1):37-46.

Lin CQ and Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J., 1993 Jun;7(9):737-43.

Lindhorst E, Young D, Bagshaw W, Hyland M, Kisilevsky R. Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. Biochim. Biophys. Acta., 1997 Apr 25;1339(1):143-54.

Löfmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. Clin. Dis. Infect., 2010;50 Suppl 1 (2010) S16-23.

Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J. Immunol., 1995 May 15;154(10):5071-9.

Mack D, McDonald T, Larson M, Shu W, Webber, A. The conserved TFLK motif of mammary-associated serum amyloid A3 is responsible for up-regulation of intestinal Muc3 mucin expression *in vitro.* Pediatr. Res., 2003, 53(1), 137-142. (a)

Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. Gut, 2003 Jun;52(6):827-33. (b)

Mak TW and Saunders ME. The Immune Response: Basic and Clinical Principles. 1th edition, September 2005, Chapter 4. Academic Press; ISBN 9780120884513.

Manley PN, Ancsin JB, Kisilevsky R. Rapid recycling of cholesterol: the joint biologic role of C-reactive protein and serum amyloid A. Med. Hypotheses., 2006;66(4):784-92.

Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, Weber A, McDonald TL, Mayfield SP. Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. Plant Biotechnol. J., 2007 May;5(3):402-12.

Marhaug G, Hackett B, Dowton SB. Serum amyloid A gene expression in rabbit, mink and mouse. Clin. Exp. Immunol., 1997 Feb;107(2):425-34.

Mattar AF, Teitelbaum DH, Drongowski RA, Yongyi F, Harmon CM, Coran AG. Probiotics up-regulate MUC-2 mucin gene expression in a CaCo-2 cell-culture model. Pediatr. Surg. Int., 2002 Oct;18(7):586-90.

Maxymiv NG, Bharathan M, Mullarky IK. Bovine mammary dendritic cells: a heterogeneous population, distinct from macrophages and similar in phenotype to afferent lymph veiled cells. Comp. Immunol. Microbiol. Infect. Dis., 2012 Jan;35(1):31-8.

McAdam KP and Sipe JD. Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. J. Exp. Med., 1976 Oct 1;144(4):1121-7.

McAdam KPWJ, Elin RJ, Sipe JD, Wolff SM. Changes in Human Serum Amyloid A and C-Reactive Protein after Etiocholanolone-Induced Inflammation. J. Clin. Investv.61(2); Feb 1978 PMC372549.

McDonald TL, Larson MA, Mack DR, Weber A. Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. Vet. Immunol. Immunopathol., 2001 Dec;83(3-4):203-11.

Meek RL and Benditt EP. Amyloid A gene family expression in different mouse tissues. J. Exp. Med., 1986 Dec 1;164(6):2006-17.

Meek RL and Benditt EP. Rat tissues express serum amyloid A protein-related mRNAs. Proc. Natl. Acad. Sci. USA, 1989 Mar;86(6):1890-4.

Meeker AK and Sack GH Jr. A fusion protein between serum amyloid A and staphylococcal nuclease--synthesis, purification, and structural studies. Proteins, 1998 Mar 1;30(4):381-7.

Mitchell TI, Coon CI, Brinckerhoff CE. Serum amyloid A (SAA3) produced by rabbit synovial fibroblasts treated with phorbol esters or interleukin 1 induces synthesis of collagenase and is neutralized with specific antiserum. J. Clin. Invest., 1991 Apr;87(4):1177-85.

Miyoshi J, Takai Y. Molecular perspective on tight-junction assembly and epithelial polarity. Adv. Drug Deliv. Rev., 2005 Apr 25;57(6):815-55.

Molenaar AJ, Harris DP, Rajan GH, Pearson ML, Callaghan MR, Sommer L, Farr VC, Oden KE, Miles MC, Petrova RS, Good LL, Singh K, McLaren RD, Prosser CG, Kim KS, Wieliczko RJ, Dines MH, Johannessen KM, Grigor MR, Davis SR, Stelwagen K. The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. Biomarkers, 2009, 14(1), 26-37.

Montero-Vega MT. A new era for innate immunity. Allergol. Immunopathol., 2008;36:164-75.

Morrow JF, Stearman RS, Peltzman CG, Potter DA. Induction of hepatic synthesis of serum amyloid A protein and actin. Proc. Natl. Acad. Sci. USA, 1981 Aug;78(8):4718-22.

Mosser DM. The many faces of macrophage activation. J. Leukoc. Biol., 2003 Feb;73(2):209-12.

Murphy K. Janeway's Immunobiology, 8th edition, July 2011, Chapter 2. Garland Science ISBN: 9780815342434.

Muschler J and Streuli CH. Cell-matrix interactions in mammary gland development and breast cancer. Cold Spring Harb. Perspect. Biol., 2010 Oct;2(10):a003202.

Nam HM, Kim JM, Lim SK, Jang KC, Jung SC. Infectious aetiologies of mastitis on Korean dairy farms during 2008. Res. Vet. Sci., 2010 Jun;88(3):372-4.

Ni K and O'Neill HC. The role of dendritic cells in T cell activation. Immunol. Cell Biol., 1997 Jun;75(3):223-30.

Nickerson SC. Immunological aspects of mammary involution. J. Dairy. Sci., 1989 Jun;72(6):1665-78.

Noble MS and Hurley WL. Effects of secretion removal on bovine mammary gland function following an extended milk stasis. J. Dairy Sci., 1999Aug;82(8):1723-30.

Olsson N, Siegbahn A, Nilsson G. Serum amyloid A induces chemotaxis of human mast cells by activating a pertussis toxin-sensitive signal transduction pathway. Biochem. Biophys. Res. Commun., 1999 Jan 8;254(1):143-6.

Oram JF. Does serum amyloid A mobilize cholesterol from macrophages during inflammation? Amyloid, 1996, Vol. 3, No. 4 , Pages 290-293.

Patel H, Fellowes S, Coade S, Woo P. Human Serum Amyloid A has Cytokine-Like. Properties Scand. J. Immunol., 1998; 47, 410-418.

Peiser L, Mukhopadhyay S, Gordon S. Scavenger receptors in innate immunity. Curr. Opin. Immunol., 2002 Feb;14(1):123-8.

Petrovski KR, Trajcev M, Buneski G. A review of the factors affecting the costs of bovine mastitis. J. S. Afr. Vet. Assoc. 2006 Jun;77(2):52-60.

Pezeshki A, Capuco AV, De Spiegeleer B, Peelman L, Stevens M, Collier RJ, Burvenich C. An integrated view on how the management of the dry period length of lactating cows could affect mammary biology and defence. J. Anim. Physiol. Anim. Nutr. (Berl)2010;94(5):e7-30.

Philpott DJ and Girardin SE. Nod-like receptors: sentinels at host membranes. Curr. Opin. Immunol., 2010 Aug;22(4):428-34.

Pinsach J, de Mas C, Lopez-Santín J. A simple feedback control of *Escheriachia coli* growth for recombinant aldolase production in fed-batch mode. Biochem. Eng. J., 2006, 29, pp 235-42.

Pitkälä A, Haveri M, Pyörälä S, Myllys V, Honkanen-Buzalski T. Bovine mastitis in Finland 2001--prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy. Sci., 2004 Aug;87(8):2433-41.

Pollard TD, Earnshaw WC, Lippincott-Schwartz J. Cell biology. 2nd edition 2007, Elsevier Health Sciences ISBN 9781437700633.

Preciado-Patt L, Levartowsky D, Prass M, Hershokoviz R, Lider O, Fridkin M. Inhibition of cell adhesion to glycoproteins of the extracellular matrix by peptides corresponding to serum amyloid A. Toward understanding the Physiological role of an enigmatic protein. Eur. J. Biochem., 1994, 223, 35-42.

Pruzanski W, de Beer FC, de Beer MC, Stefanski E, Vadas P. Serum amyloid A protein enhances the activity of secretory non-pancreatic phospholipase A2. Biochem J., 1995 Jul 15;309 (Pt 2):461-4.

Rabot A, Sinowatz F, Berisha B, Meyer HH, Schams D. Expression and localization of extracellular matrix-degrading proteinases and their inhibitors in the bovine mammary gland during development, function, and involution. J Dairy Sci., 2007; 90(2):740-8.

Rainard P. Tackling mastitis in dairy cows. Nat Biotechnol. 2005 Apr;23(4):430-2.

Ramadori G, Sipe JD, Colten HR. Expression and regulation of the murine serum amyloid A (SAA) gene in extrahepatic sites. J Immunol., 1985 Dec;135(6):3645-7.

Ribeiro FP, Furlaneto CJ, Hatanaka E, Ribeiro WB, Souza GM, Cassatella MA, Campa A. mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. Mediators Inflamm., 2003 Jun;12(3):173-8.

Roselli M, Finamore A, Britti MS, Konstantinov SR, Smidt H, de Vos WM, Mengheri E. The novel porcine *Lactobacillus sobrius* strain protects intestinal cells from enterotoxigenic *Escherichia coli* K88 infection and prevents membrane barrier damage. J. Nutr., 2007 Dec;137(12):2709-16.

Roxas JL, Koutsouris A, Bellmeyer A, Tesfay S, Royan S, Falzari K, Harris A, Cheng H, Rhee K J, Hecht G. Enterohemorrhagic *E. coli* alters murine intestinal epithelial tight junction protein expression and barrier function in Shiga toxin independent manner. Lab. Invest., 2010 August; 90(8): 1152–1168.

Rubino SJ, Selvanantham T, Girardin SE, Philpott DJ. Nod-like receptors in the control of intestinal inflammation. Curr. Opin. Immunol. 2012 Aug;24(4):398-404.

Ruiz J, Pinsach J, Álvaro G, González G, de Mas C, Resina D. Alternative production process strategies in *E. coli* improving protein quality and downstream yields. Process. Biochem., 2009, 44, pp 1039-1045.

Ruiz J, Fernández-Castané A, de Mas C, González G, Lopez-Santin J. From Laboratory to Pilot Plant. *E. coli* fed-batch Cultures: Optimizing the Cellular Environment for Protein Maximization, 2013. Accepted for publication in J. Ind. Microbiol. Biotechnol.

Rummele FM, Beaulieu JF, Dionne S, Levy E, Seidman EG, Cerf-Bensussan N, Lentze MJ. Lipopolysaccharide modulation of normal enterocyte turnover by toll-like receptors is mediated by endogenously produced tumour necrosis factor alpha. Gut, 2002 Dec;51(6):842-8.

Rygg M., Husby G. and Marhaug G. (1993). Differential expression of rabbit serum amyloid A genes in response to various inflammatory agents. Scand. J. Immunol., 1993 Nov; 39(5):417-22.

Qin H, Zhang Z, Hang X, Jiang Y. *L. plantarum* prevents enteroinvasive *Escherichia coli*-induced tight junction proteins changes in intestinal epithelial cells. BMC Microbiol., 2009 Mar 31;9:63.

Shah C, Hari-Dass R, Raynes JG. Serum amyloid A is an innate immune opsonin for Gram-negative bacteria. Blood, 2006, 108(5), 1751-1757.

Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A. Is serum amyloid A an endogenous TLR4 agonist? J. Leukoc. Biol., 2008 May;83(5):1174-80.

Santiago-Cardona PG, Berríos CA, Ramírez F, García-Arrarás JE. Lipopolysaccharides induce intestinal serum amyloid A expression in the sea cucumber *Holothuria glaberrima*. Dev. Comp. Immunol., 2003 Feb;27(2):105-10.

Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat. Immunol., 2012 Dec;13(12):1145-54.

Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol., 2004 Feb;75(2):163-89.

Schukken YH, Tikofsky LL, Zadoks RN. Environmental control for mastitis prevention, milk quality and food safety. Wageningen, The Netherlands: Wageningen Academic Publishers 2005 ISBN 90-76998-70-1.

Sipe JD. Induction of the acute-phase serum protein SAA requires both RNA and protein synthesis. Br. J. Exp. Pathol., 1978 Jun;59(3):305-10.

Sipe JD. Serum amyloid A: from fibril to function. Current status. Amyloid, 2000 Mar;7(1):10-2.

Smith KL and Todhunter DA. The physiology of mammary glands during the dry period and the relationship to infection. In Proc. 26th Annu. Mtg. Natl. Mastitis Counc. Arlington, VA 1982;Vol. 87, p. 100.

Stevens FJ. Hypothetical structure of human serum amyloid A protein. Amyloid, 2004 Jun;11(2):71-80.

Steel DM and Whitehead AS. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunol. Today., 1994 Feb;15(2):81-8.

Steinmetz A, Hocke G, Saïle R, Puchois P, Fruchart JC. Influence of serum amyloid A on cholesterol esterification in human plasma. Biochim. Biophys. Acta, 1989 Nov 28;1006(2):173-8.

Streuli CH. Cell adhesion in mammary gland biology and neoplasia. J. Mammary Gland Biol. Neoplasia, 2003 Oct;8(4):375-81.

Strissel KJ, Girard MT, West-Mays JA, Rinehart WB, Cook JR, Brinckerhoff CE, Fini ME. Role of serum amyloid A as an intermediate in the IL-1 and PMA-stimulated signaling pathways regulating expression of rabbit fibroblast collagenase. Exp. Cell Res., 1997 Dec 15;237(2):275-87.

Su SB, Gong W, Gao JL, Shen W, Murphy PM, Oppenheim JJ, Wang JM. A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. J. Exp. Med., 1999 Jan 18;189(2):395-402.

Suojala L, Orro T, Järvinen H, Saatsi J, Pyörälä S. Acute phase response in two consecutive experimentally induced *E. coli* intramammary infections in dairy cows. Acta Vet. Scand., 2008 Jun 13;50:18.

Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu. Rev. Immunol., 2003;21:335-76.

Talhouk RS, Bissell MJ, Werb Z. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J. Cell. Biol., 1992 Sep;118(5):1271-82.

Tam MA, Rydström A, Sundquist M, Wick MJ. Early cellular responses to *Salmonella* infection: dendritic cells, monocytes, and more. Immunol. Rev., 2008 Oct;225:140-62.

Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol. Rev., 2012 Sep;249(1):158-75.

Tietge UJ, Maugeais C, Lund-Katz S, Grass D, de Beer FC, Rader DJ. Human secretory phospholipase A2 mediates decreased plasma levels of HDL cholesterol and apoA-I in response to inflammation in human apoA-I transgenic mice. Arterioscler. Thromb. Vasc. Biol., 2002 Jul 1;22(7):1213-8.

Turner JR. Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol., 2009 Nov;9(11):799-809.

Uhde FL, Kaufmann T, Sager H, Albini S, Zanoni R, Schelling E, Meylan M. Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. Vet. Rec., 2008 Sep 20;163(12):362-6.

Uhlar CM and Whitehead AS. Serum amyloid A, the major vertebrate acute-phase reactant. Eur. J. Biochem., 1999 Oct;265(2):501-23.

Upragarin N. Extrahepatic production of acute phase serum amyloid A. Histol. Histopathol., 2005 Oct;20(4):1295-307.

Urieli-Shoval S, Meek RL, Hanson RH, Eriksen N, Benditt EP. Human serum amyloid A genes are expressed in monocyte/macrophage cell lines. Am. J. Pathol., 1994;145, 650-660

Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, Casadesús J, Platt DJ, Olsen JE. Host adapted serotypes of *Salmonella enterica.* Epidemiol. Infect., 2000 October; 125(2): 229–255.

Van der Westhuyzen DR, Cai L, de Beer MC, de Beer FC. Serum amyloid A promotes cholesterol efflux mediated by scavenger receptor B-I. J. Biol. Chem., 2005 Oct 28;280(43):35890-5.

Van Klinken BJ, Oussoren E, Weenink JJ, Strous GJ, Büller HA, Dekker J, Einerhand AW. The human intestinal cell lines CaCo-2 and LS174T as models to study cell-type specific mucin expression. Glycoconj. J., 1996 Oct;13(5):757-68.

Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of Staphylococcus aureus for biofilm formation. Vet, Microbiol., 2003 Mar 20;92(1-2):179-85.

Veterinary Investigation Diagnosis Analysis database – VIDA 2001 report –United Kingdom (http://www.defra.gov.uk/ahvla-en/files/pub-vida11-intro.pdf).

Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R. Mastitis detection: current trends and future perspectives. Trends Biotechnol. 2009 Aug;27(8):486-93.

Virtala AM, Mechor GD, Gröhn YT, Erb HN. Morbidity from nonrespiratory diseases and mortality in dairy heifers during the first three months of life. J. Am. Vet. Med. Assoc., 1996 Jun 15;208(12):2043-6.

Wang L, Lashuel HA, Walz T, Colon W. Murine apolipoprotein serum amyloid A in solution forms a hexamer containing a central channel. Proc. Natl. Acad. Sci. USA. 2002 Dec 10;99(25):15947-52.

Wang Y, Srinivasan S, Ye Z, Aguilera J, Lopez MM, Colón W. Serum amyloid A 2.2 refolds into a octameric oligomer that slowly converts to a more stable hexamer. Biochem. Biophys. Res. Commun., 2011, 407,725-9.

Wang Z, Wang J, Cheng Y, Liu X, Huang Y. Secreted factors from *Bifidobacterium animalis* subsp*. lactis* inhibit NF-κB-mediated interleukin-8 gene expression in CaCo-2 cells. Appl. Environ. Microbiol., 2011 Nov;77(22):8171-4.

Watanabe A, Yagi Y, Shiono H, Yokomizo Y, Inumaru S. Effects of intramammary infusions of interleukin-8 on milk protein composition and induction of acute-phase protein in cows during mammary involution. Can. J. Vet. Res. 2008 Apr;72(3):291-6.

Webb CF, Tucker PW, Dowton SB. Expression and sequence analyses of serum amyloid A in the Syrian hamster. Biochemistry, 1989 May 30;28(11):4785-90.

Wilde CJ, Knight CH, Flint DJ. Control of milk secretion and apoptosis during mammary involution. J. Mammary Gland Biol. Neoplasia, 1999 Apr;4(2):129-36.

Wittum TE, Salman MD, Odde KG, Mortimer RG, King ME. Causes and costs of calf mortality in Colorado beef herds participating in the National Animal Health Monitoring System. J. Am. Vet. Med. Assoc., 1993 Jul 15;203(2):232-6.

Xia H, Liu H, Zhang G, Zheng Y. Phenotype and function of monocyte-derived dendritic cells from chinese rhesus macaques. Cell Mol. Immunol., 2009 Jun;6(3):159-65.

Yamada T, Kluve-Beckerman B, Liepnieks J, Benson M. Fibril formation from recombinant serum amyloid A. Biochimica et Biophysica Acta, 1994, 1226, 323-329.

Yavin EJ, Preciado-Patt L, Rosen O, Yaron M, Suessmuth RD, Levartowsky D, Jung G, Lider O, Fridkin M. Serum amyloid A-derived peptides, present in human rheumatic synovial fluids, induce the secretion of interferon-gamma by human CD(4)(+) T-lymphocytes. FEBS Lett., 2000 Apr 28;472(2-3):259-62.

Yu TC, Chen SE, Ho TH, Peh HC, Liu WB, Tiantong A, Nagahata H, Chang CJ. Involvement of TNF-α and MAPK pathway in the intramammary MMP-9 release via degranulation of cow neutrophils during acute mammary gland involution. Vet. Immunol. Immunopathol., 2012 Jun 30;147(3-4):161-9

Zhang G, Nichols RD, Taniguchi M, Nakayama T, Parmely MJ. Gamma interferon production by hepatic NK T cells during *Escherichia coli* infection is resistant to the inhibitory effects of oxidative stress. Infect. Immun., 2003 May;71(5):2468-77.

Zhao C, Wood MW, Galyov EE, Höpken UE, Lipp M, Bodmer HC, Tough DF, Carter RW. *Salmonella typhimurium* infection triggers dendritic cells and macrophages to adopt distinct migration patterns *in vivo*. Eur. J. Immunol., 2006 Nov;36(11):2939-50.