

Role of Calprotectin in Infection and Inflammation.

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Abstract

Calprotectin, an acute phase reactant plays vital physiological roles in infection and inflammation. It serves as a potential diagnostic marker for various inflammatory diseases including Inflammatory bowel disease, Sepsis, and Necrotizing enterocolitis. Once get induced by LPS or chemokines, calprotectin is secreted predominantly by the neutrophils and is translocated to the extracellular fluid where it takes part in major biological processes like signal transduction, tumorigenesis, apoptosis, cell homeostasis and inflammation. Calprotectin acts as an endogenous ligand of TLR4 receptor and further amplifies infection and inflammation. In this article, we review the molecular structure of calprotectin and its roles in disease conditions. Genetic behavior is also an important factor in calprotectin expression and is found to be associated with disease activity. Knock down studies revealed the secrets behind the pathophysiological functions of calprotectin and suggested it as a therapeutic target for inflammatory diseases.

Keywords: Calprotectin, Inflammation, Infection, Apoptosis, Signal transduction, Calcium and zinc binding, Genetic variation

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Introduction

Calprotectin is an interesting peptide, proposed as a biomarker for various inflammatory diseases due its potential role in pathophysiology of inflammation associated outcomes like tissue destruction, apoptosis and growth impairment. As an acute phase reactant, calprotectin increases more than 100 folds during inflamed conditions [1]. Yui *et al* studied the course of intracellular and extracellular calprotectin expression in inflammatory cells using a rat peritonitis model and found that neutrophils, rather than macrophages, were the most important source of extracellular calprotectin [2]. Calprotectin is found abundant in neutrophils [3] and it constitutes 30-60% of the cytosolic proteins [4]. It is also found in monocytes [5], keratinocytes [6], muscle tissue [7] and infiltrating tissue macrophages [8]. Once get stimulated by an injury or cell disruption, neutrophils and monocytes start secreting calprotectin into the extracellular fluid [9].

Physical structure

Calprotectin is a calcium and zinc binding heterodimeric molecule of 36.5 kDa, having two subunits MRP-8 and MRP-14, which are 8.3 kDa and 13.3 kDa respectively [10]. MRP8 and MRP14 are composed of 93 and 114 amino acids respectively [11]. Fagerhol *et al.*, first found calprotectin in 1980 in granulocytes and noted that the

two subunits are non-covalently linked. They named it as L1 protein. MRP-8 constitutes the lighter chain of calprotectin and is designated as L1L and MRP 14 as L1H, which constitutes the heavy chain [12]. MRP8 is the active subunit of MRP8/14 and MRP14 acts as the regulatory subunit preventing early degradation of MRP8 [13]. Calprotectin belongs to the family of S100 calcium binding proteins [14]. Each S100 monomer consist of two helix-loop-helix Ca^{2+} binding domains, EF hands, with hydrophobic regions at both ends separated by a central hinge region [4]. Remarkable conformational changes occurs when calcium binds to the C-terminal EF hand. The C-terminal region contains a His-x-x-x-His motif that binds Zn^{2+} ions [15] and a site for phosphorylation. Various names were given to calprotectin based on its structure and functions but still there is lot of controversies in calprotectin nomenclature (Table 1).

Genome structure

The gene encoding calprotectin subunits, MRP8 (1.16kb) and MRP14 (3.17kb) [22] are located in the gene cluster on human chromosome 1q21 [23]. The HGNC approved names for MRP8 and MRP14 genes are S100A8 and S100A9 respectively. Both MRP8 and MRP14 genes have three exons of different lengths separated by two introns [24]. mRNA of MRP8 and MRP14 contains 532bp, 586bp respectively [25]. In S100A9 mRNA, cal-

cium binding region is situated from 110 to 151 bases, while 242 to 277 bases constitute the phosphorylation site (from NCBI). Promoter of S100A9 has less CpG content

and it may undergo partial or variable methylation [26]. MRP14 protein undergoes post-translational phosphorylation of threonine residue at position 113 [27].

Table 1. Various synonyms of calprotectin and description for naming the protein

Names	Description for naming	Reference
L1 Antigen	Leukocyte derived proteins released by mechanisms like immunological injury.	Fagerhol <i>et al.</i> [12]
27E10 Antigen	Monoclonal antibody 27E10 detects a surface antigen that is found on 20% of peripheral blood monocytes.	Zwadlo <i>et al.</i> [16]
Cystic Fibrosis Antigen	Elevated serum levels of calcium binding protein in cystic fibrosis patients.	Dorin <i>et al</i> [14]
MRP8 and MRP14	Specific expression in cells of myeloid origin namely granulocytes, monocytes and macrophages	Odink <i>et al</i> [11]
L1 light and heavy chain	Two polypeptide chains of 13.3 & 8.3, heavy and light chains	Anderson <i>et al</i> [10]
Calgranulin A (CAGA) and Calgranulin B (CAGB)	Protein with two subunits of 11 kDa and 14 kDa which contains the cystic fibrosis antigen	Wilkinson <i>et al</i> [17]
p8,14	Complex of two proteins of molecular mass 8 kDa and 14 kDa under non-reducing conditions	Hogg <i>et al.</i> [18]
60B8-A and B antigen	Differentiation antigen which induce differentiation of HL-60 cells into macrophage like cells	Tobe <i>et al</i> [19]
Calprotectin	Calcium binding protein with antimicrobial properties	Steinbak <i>et al.</i> [20]
S100A8 and S100A9	Based on the arrangement in S100 gene cluster on 1q21 chromosome	Schafer <i>et al</i> [21]

Calcium Binding

MRP14 shows higher affinity for calcium than MRP8, and the Ca²⁺ affinity of C-terminal EF2 is higher than that of N-terminal EF1 [28], which are composed of 12 and 14 amino acids respectively [29]. Calcium plays vital role in the formation of different protein complexes (dimer, trimer and tetramer) by MRP8 and MRP14 [30]. Calprotectin can bind 6 calcium ions and it leads to the exposure of hydrophobic surfaces that allow calprotectin to interact with target proteins like, casein kinase, which is inhibited by calprotectin [31]. Calcium binding allows MRP14 phosphorylation by p38 MAPK, while in the absence of calcium; MRP8 inhibits p38 preventing MRP14 phosphorylation [32]. Calcium regulates the translocation of S100A8/A9 from cytosol to plasma membrane which subsequently causes NOX2 activation leading to phagosomal phenomenon [33, 34]. Calprotectin binds polyunsaturated fatty acids like arachidonic acid (AA) in a calcium dependent manner. Maximum AA binding occurs when 3 calcium ions binds to each EF hand [29]. The studies conducted by Reza *et al* indicate that calcium at higher concentrations

induces aggregation of human calprotectin. Calcium at concentration of 6 mM or higher induces aggregation of human calprotectin in K562 Chronic myelogenous leukemia cell lines at pH 7.0 [35]. Akiyama *et al* reported the high expression of calprotectin in brain tissue in Alzheimer's disease, which suggests that calprotectin aggregation may be an important pathological condition in Alzheimer's disease [36]. Reza *et al* explained that this is possible because calcium binding to calprotectin may lead to the development of a toxic form of calprotectin with sticky hydrophobic surface that interact with plasma membrane to kill cells [35]. Amita Quadros suggested that increase in calcium influx may be important in the pathogenesis of Alzheimer's disease [36], supporting the studies of Reza *et al.*

Zinc Binding

Zinc binding capacity of calprotectin is higher than that of other proteins in S100 family [31]. The zinc-binding capacity of calprotectin is not affected by calcium binding, because both chains of calprotectin contain distinct motifs

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(HEXXH motif) for binding to zinc [38]. Like calcium, zinc also induces the formation of calprotectin tetramer [39]. Zinc binding is the key behind microbiostatic property of calprotectin against *B.burgdorferi* [40], *C.albicans* [40, 41]. When zinc is supplemented, antimicrobial property of calprotectin get reversed [42]. Properties of calprotectin like calcium mediated AA binding capacity of calprotectin [29], cytotoxicity [44] are also get reversed by zinc. 10µM zinc is enough to neutralize the activities of 100µg/ml of calprotectin [31].

Ghavami *et al* in their study suggested that decrease in intracellular zinc level following extracellular zinc chelation by calprotectin might be responsible for caspase-3 activation, which may lead to apoptosis [45, 46]. Calprotectin inhibits the activity of matrix metalloproteinases (MMPs) by sequestration of zinc in K562 chronic myelogenous leukemia cells [35]. Calprotectin over-expression can cause dysregulation of zinc homeostasis with severe clinical symptoms [47]. Recent study showed that zinc deficiency may lead to S100A8/A9 mediated inflammation-associated progression of tumors [48].

Calprotectin Detection in Body Fluids

Faecal Calprotectin serves as a promising biomarker for Inflammatory Bowel Syndrome and is used to distinguish it from Irritable Bowel Syndrome [49]. It is widely used for diagnosing diseases related to intestinal inflammation. Serum calprotectin is also used in diagnosing some diseases like Neonatal sepsis [50] and Atherosclerosis [51]. Calprotectin is detectable in CSF during neuroinflammation and is used to predict disease severity in multiple sclerosis [52]. Urinary calprotectin can be used to differentiate prerenal and intrinsic acute kidney injury [53]. Calprotectin present in tissues helps in immunohistochemical studies of pathological conditions like Alzheimer’s disease [36], allograft rejection [54] and various types of cancers. Calprotectin was found in synovial fluid of rheumatoid arthritis and osteoarthritis patients [55]. Zinc reversible growth inhibitory property of calprotectin was studied in empyema fluid by Sohnle *et al* [41]. Some of the diseases which involve the potential role of calprotectin in their pathophysiology are given in Table 2.

Table 2. *Pathological conditions associated with calprotectin*

Lung Cancer [56, 57]	Psoriasis [112]	Sepsis [97, 98]
Colorectal cancer [58, 59, 60]	Acute Kawasaki disease [80]	Celiac disease [99]
Multiple sclerosis [61]	Acute myocardial infarction [81]	Cerebral ischemia [100]
Cystic fibrosis [1, 62]	Autoimmune diseases [82]	Obesity [101]
Rheumatoid arthritis [63, 64]	Cryopyrin-associated periodic syndrome [83]	Obstructive sleep apnea [102]
Juvenile Rheumatoid Arthritis [65]	Systemic lupus erythromatosus [84, 85]	Nephropathy [103]
Acute kidney injury [66]	Schizophrenia [86]	Gastroenteritis [104]
Sjogren syndrome [67, 68]	Atherosclerosis [87, 88]	Chronic rhinosinusitis [105]
Dermatomyositis [69]	Periodontitis [89]	Osteoarthritis [106]
Transplant rejection 70]	Pancreatitis [90]	HIV [107]
Inflammatory bowel disease [71, 72]	Cardiovascular disease [91]	Cirrhosis [92]
Ulcerative colitis [73]	Hepatic encephalopathy[92]	Psoriatic arthritis [108]
Crohn’s disease [74, 75]	Necrotizing enterocolitis [93, 94]	Systemic sclerosis [109]
Appendicitis [76, 77]	Adult onset still’s disease [95]	Alzheimer’s Disease [36]
Ankylosing spondylitis [78]	Henoch-Schönlein purpura nephritis [96]	

Calprotectin exhibited potential biostatic activity against few bacteria and fungi like *C.albicans* [41], *E.coli* [26], *K.pneumoniae* [26], *S.epidermidis* [9, 26], *S.aureus* [26] through chelation of zinc ions [41]. Some of the physiological roles of calprotectin are summarized in Table 3. High concentration of calprotectin in local inflammatory sites causes delay in tissue repair and lethal effects on surrounding tissues [31]. Johne *et al* explained comprehensively the concentration of calprotectin in different

body fluids in various clinical settings [6]. Neutrophils and monocytes are the first cells that migrate to the inflammatory sites where they carry out defense mechanisms like phagocytosis, release of ROS, enzymes and antimicrobial peptides. Calprotectin triggers degradation of endothelial cell tight junction which induces the migration of neutrophils and monocytes to the site of inflammation [110].

Physiological Functions

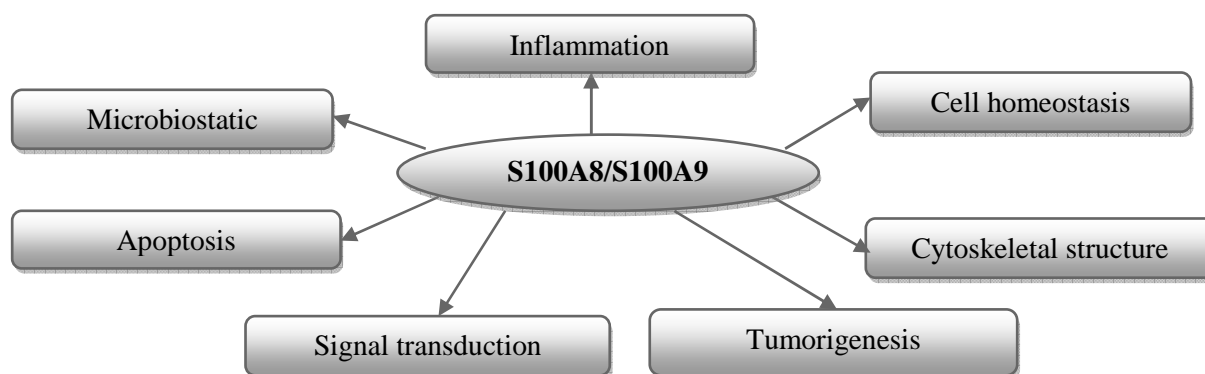


Figure. 1: Major Biological processes in which calprotectin is involved

Table 3. Calprotectin activities and associated functions.

Activity	Associated Function	References
Protein Kinase Inhibitor	Myeloid cell maturation	Murao <i>et al.</i> [111]
Growth Inhibitory	Monomyelocytic cell differentiation, tumor cells, bone marrow cells	Murao <i>et al.</i> [112], Yui <i>et al.</i> [2], Yui <i>et al.</i> [113]
Immunoglobulin synthesis inhibitor	Immunoregulation	Brun <i>et al.</i> [114]
Resistance to infections (<i>in vitro</i>)	Barrier protection and innate immunity	Niaspakultom <i>et al.</i> [115]
Protease resistant	Post-translational regulation	Nacken <i>et al.</i> [116]
TLR4 agonist	Amplifies infection and inflammation promoting shock induced by endotoxin	Vogl <i>et al.</i> [117]
Cytokine like activity	Chemotaxis in inflammation and inflammation associated cancer	Gebhardt [118]
Elevated serum/plasma levels	Disease activity/severity	Benoit <i>et al.</i> [119]
Oxidant scavenging	Wound healing and prevention of excessive tissue damage	Lim <i>et al.</i> [120]
Binding of polyunsaturated fatty acids	Initiation and regulation of inflammation	Kerhoff <i>et al.</i> [29]
Inhibition of neutrophil oxidative metabolism	Oxidative stress disorders	Schwartz <i>et al.</i> [121]
Microtubule Polymerization		Vogl <i>et al.</i> [31], Therey <i>et al.</i> [122]
Cytoplasmic Ca ²⁺ sensor	Regulation of NOX2 activity in Phagocytosis	Schenten <i>et al.</i> [33]
Apoptotic / cytotoxic	Induces cell death in fibroblasts, lymphocytes and tumors	Yui <i>et al.</i> [2], Yui <i>et al.</i> [113], Mostafa <i>et al.</i> [123]
Inducer of NO production	Innate immune response	Pouliot <i>et al.</i> [124]
Induce expression of cell surface receptors (CD35, CD66b, CD18, CD11b)	Migration, adhesion and phagocytosis of neutrophils	Simard <i>et al.</i> [125]

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Calprotectin is referred as Damage-Associated Molecular Pattern (DAMPs) molecule due to its activities of maintaining cell homeostasis and its role as signaling molecule in signal transduction pathways like JNK, ERK1/2, p38, JAK/STAT [126, 127]. Like other S100 proteins, S100A9 and S100A8 act as ligand for RAGE receptor [128], which property was found significant in endotoxin induced cardiomyocyte dysfunction [129] and lung adenocarcinoma [130]. S100A8/S100A9 binds to TLR4 receptor and amplifies infection and inflammation [131]. Individually, S100A9 induces neutrophils degranulation which results in the release of secretory vesicles and specific/gelatinase granules [125]. S100A9 regulates B7 expression and reduces antigen presentation by dendritic cells [54]. S100A8 is involved in the translocation of

MyD88 from cytosol to TLR4 and activation of signal-transduction pathways like IRAK1, ERK, p38 MAPK, and PKC [131]. It also regulates NF- κ B pathway by the activation of TLR4-MD2 [117, 131].

Apoptosis inducing activity of calprotectin involves inhibition of antiapoptotic genes and activation of p53, bak, bax, caspase-3 and caspase-9. This reveals that apoptotic activity of calprotectin is via activation of mitochondrial apoptotic pathway [131]. Gene expression analysis showed that S100A8/S100A9 activates specific downstream genes such as Cxcl1, Ccl5 and Ccl7, Slc39a10, Lcn2, Zc3h12a, Enpp2 in tumorigenesis and promotes tumor growth, metastasis [132]. In pathological processes, calprotectin synthesis is induced by various factors as given in Fig 2.

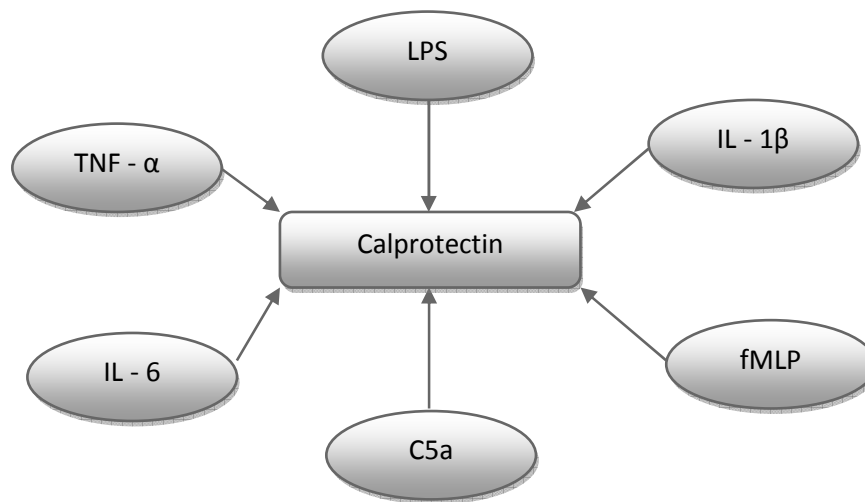


Figure 2. Inducers of calprotectin secretion (LPS: Lipopolysaccharide; IL-1 β : Interleukin-1 β ; fMLP: N-formyl-methionyl-leucyl-phenylalanine; C5a: complement component 5a receptor; IL-6: Interleukin-6; TNF- α : tumor necrosis factor- α).

Genetic Association with Diseases

Very few studies were conducted till date correlating genetic variations in calprotectin gene and diseases. Gene polymorphism studies revealed that two SNPs (rs3795391 and rs3806232) in S100A8 are associated with high risk of chronic periodontitis in males [133] and aggressive periodontitis [134, 135] in Chinese population. In North-eastern Thai population rs2916191 of S100A9 and rs3006488 of S100A8 are associated with kidney stone disease [136]. S100A9 with low CpG density shows less relationship between DNA methylation and gene expression in bladder cancer [26]. miRNA studies showed that, miR-196a serves as a potential marker of tumor progression during evolution of Barrett's esophagus to Esophageal adenocarcinoma with S100A9 as its target along with KRT5 and SPRR2C [137]. Mutation studies revealed that

substitution of cysteine 42 by alanine in S100A8 inhibited the enhancement of antifungal properties of S100A9, while substitution of Methionine 63 or 83 by alanine caused the total loss of antifungal effect of S100A9 [138].

Calprotectin Knock Down

Loss of MRP8 or MRP14 or MRP8/14 unveiled the secrets behind *in vivo* characteristics of calprotectin. MRP14-/- mice studied by Josie *et al* were fertile and showed no obvious phenotypic variation from wild type. Myeloid cell functions like phagocytosis, superoxide burst, and apoptosis were unaffected in MRP14-/- cells [97, 139, 140]. Table 4 summarizes the outcomes in various clinical conditions, when calprotectin or its subunits are knocked out.

Table 4. Phenotypic outcomes of calprotectin knock out in various clinical settings

Pathological Condition	Knocked out Protein	Expressed Phenotype	References
Cardiac allograft rejection	MRP8/14	Increased T-cell activation and exacerbate allograft rejection	Shimizu <i>et al</i> [54]
Alzheimer's Disease	MRP14	Decreased learning and memory impairment, Reduced number of amyloid plaques and pyknotic neurons	Ha <i>et al</i> [141]
Esophageal squamous cell carcinoma	MRP8/14	Weak differentiation of tumor cells	Kong <i>et al</i> [142]
Inflammation	MRP14	Reduced CD11b expression, impaired chemoattractant activity of IL-8, G to F actin shift in non-stimulated PMN cytoskeletal structure. Decreased neutrophil migration and release of cytokines. No effect on myeloid cell functions	Manitz <i>et al</i> [143] Averill <i>et al</i> [144] Josie <i>et al</i> [139]
Embryo implantation	MRP8	Rapid resorption of S100A8 null embryos	Passey <i>et al</i> [145]
Tumorigenesis	MRP14	Inhibition of tumor growth due to lack interaction of S100A9 with TLR4	Kallberg <i>et al</i> [146]
Resistant to bacterial invasion	MRP14	Increased colonization of bacterial cells in oral carcinoma and release of proinflammatory signals	Sorenson <i>et al</i> [140]
Wound healing	MRP14	Granulocytes with weakly polymerized tubulins, reduced recruitment of granulocytes into the granulation tissue	Vogl <i>et al</i> [32]
Endotoxin tolerance	MRP14	Protection against endotoxin induced lethality, diminished cytokine response, Reduced liver damage.	van Zoelen <i>et al</i> [97]

Conclusion

From the day of its discovery, Calprotectin is undergoing several controversies over its beneficial/harmful roles in biological process. But it is very obvious that calprotectin's adverse effects overwhelm its beneficial roles in diseased conditions. Studies had shown that inhibiting calprotectin can be a new therapeutic approach in suppressing the poor outcomes like cytotoxicity, liver damage, and tissue destruction during inflammation. Zinc acts as a potential neutralizer of calprotectin, reversing almost all of its properties. In summary, Calprotectin is a prominent player in various pathophysiological processes and

targeting calprotectin may help in preventing worse effects of inflammation and associated diseases. Genetic association studies may reveal the relation between genetic variation in S100A8/S100A9 and risk of diseases, which can be used as prognostic marker in future.

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