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# The Role of Calgranulins in Urinary Tract Infection

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## 1. Introduction

Calgranulins A (S100A8), B (S100A9) and C (S100A12) are members of the superfamily of EF-hand calcium binding proteins. The term calprotectin specifically refers to the Calgranulin A and B heterodimer that is formed by a non-covalent interaction. A distinguishing feature of calgranulins is their involvement in innate immunity and inflammation. As secreted proteins, calgranulins can directly inhibit microbial growth in the extracellular milieu, presumably through their ability to chelate zinc. Cells that actively express calgranulins are more resistant to bacterial adherence and invasion. Calgranulins also support optimal functioning of the cells that comprise the innate immune system. For example, granulocytes depend on the intracellular calgranulin A/B complex to adequately participate in cell adhesion, cytokinesis, cytokine secretion, and activation of the respiratory burst. Calgranulin A/B can down-regulate immune responses through inhibition of immunoglobulin production by lymphocytes and induction of myeloid derived suppressor cells. In addition, calgranulin induces apoptosis in cells that stimulate the inflammatory cascade. Calgranulin A expression may also be important in promoting the induction of a regulatory macrophage phenotype that down-regulates inflammation and facilitates a healing response.

Under normal conditions, calgranulins support an optimal immune response, leading to resolution of infection and inflammation. When normal expression and/or secretion of calgranulins are perturbed, inflammation is exacerbated with adverse implications for host tissues. As an example, dysregulation in the expression or secretion of calgranulins can have detrimental effects by promoting chronic active inflammation that leads to collateral tissue damage. Excessive amounts of calgranulin A/B in extracellular compartments of tissues correlates with a variety of inflammatory disorders. Expression and release of calgranulin A/B in the extracellular milieu can be triggered by IL-1α. Extracellular calgranulin A/B can then bind to and activate toll like receptor 4 (TLR 4), which in turn initiates further expression of pro-inflammatory mediators such as IL-1, IL-6, and IL-8. The interaction of these cytokines and chemokines with calgranulin A can create an autocrine / paracine mediated pro-inflammatory feedback loop that does not necessarily resolve infection. In the urinary tract, over expression of calgranulin A is not specific for infection. Over expression of calgranulins has been reported in a variety of inflammatory disorders of

the urinary tract, including interstitial cystitis, interstitial nephritis, glomerulonephritis and neoplasia. This suggests that while calgranulin A may be a useful biomarker for chronic active inflammation, in general it may not be important in the defence against bacterial pathogens. Recent studies in rodent models of urinary tract infection (UTI) suggest that calgranulins are not required for innate defence against urogenital pathogens. Moreover, the exaggerated expression of these proteins during infection may be contributing to complicated urinary tract diseases such as struvite urolithiasis.

This review will focus on the role of calgranulin A and B in innate immune responses with particular attention to diseases of the urinary tract. The interaction of calgranulins with various host cell signal transduction pathways important in innate immunity and inflammation of the urinary tract will be reviewed. Further, potential consequences of aberrant signalling that may contribute to perturbations of regulation of calgranulins will also be discussed.

## 2. Classification and general function of calgranulins

Calgranulins are members of the superfamily of EF-hand calcium binding proteins that are involved in innate immune and pro-inflammatory processes. In the literature, these proteins have been given a variety of names including S100A8, myeloid related protein 8 (MRP), and L1 light chain for calgranulin A. Calgranulin B is also known as MRP-14 and S100A9. Calgranulin C may be referred to as S100A12 and EN-RAGE (Nacken et al., 2003). The name calprotectin specifically refers to the Calgranulin A and B heterodimer that is formed by a non-covalent interaction. Calgranulin C does not appear to form a heterodimer complex with either Calgranulin A or B (Foell et al., 2007).

Calgranulin C appears to be exclusively expressed in granulocytes (Vogl et al., 1999). However, calgranulins A and B are constitutively expressed in a variety of cells including granulocytes, monocytes, dendritic cells, epithelial cells, and keratinocytes (Foell et al., 2007; Frosch et al., 2004; Nacken et al., 2003). In healthy individuals, low concentrations of calprotectin can be detected in plasma, saliva, cerebrospinal fluid, urine and feces (Johne et al., 1997). Typically calgranulin gene expression is tightly regulated. However, during inflammation, expression is induced in cells such as macrophages and fibroblasts that do not normally produce calgranulins (Foell et al., 2007; Frosch et al.., 2004; Hsu et al., 2005; Nacken et al., 2003). Further, cells that constitutively express calgranulins are induced to secrete calprotectin into the extracellular milieu when stimulated (Kido et al., 2003). Therefore, calprotectin is considered a biomarker of inflammatory conditions such as rheumatoid arthritis, Crohn's disease, and chorioamnionitis (Kostakis et al., 2010; Perere et al., 2010).

Calprotectin deficiency has never been reported in humans, suggesting that this protein is essential for life. The need for calgranulin A was confirmed with a null mutation in mice, which resulted in early resorption of mouse embryos (Passey et al., 1999a). Null mutation of the S100A9 gene (calgranulin B) was not lethal in the mouse (Hobb et al., 2003). Interestingly, although the S100A9 -/- mouse expresses S100A8 (calgranulin A) mRNA, the protein is not detected in peripheral tissues.. Consequently, this mouse strain does not produce calprotectin, making it convenient for use in studying the role of calprotectin in various disease processes. Although the calgranulin genes are conserved among higher vertebrates, there are structural and functional differences among the various species (Nacken et al., 2003). For example, in rodents, calgranulin A is chemotactic for granulocytes but this is not the case in humans (Foell et al., 2007). Calgranulin C, which is not present in

rodents, is the potent granulocyte chemoattractant in humans (Yang et al., 2001) and is highly expressed in activated tissue macrophages localized within sites of inflammation (Perera et al., 2010). Calgranulin C also serves as a ligand for RAGE (Perera et al., 2010). Although, rodents possess RAGE, there is no evidence that either calgranulin A, B, or calprotectin act as a ligand for this receptor. To date, there are no published studies demonstrating a role for calgranulin C in urinary tract infection. Therefore, this review will focus on calgranulins A and B.

### 2.1 The contribution of calgranulins to defense against infection

The innate immune system comprises the first attack against invading microorganisms. Both intracellular and extracellular calgranulins play an integral role in modulating leukocyte activation, trafficking, and amplification of immune responses during infection. Intracellular calgranulins regulate cell adhesion, migration, phagocytosis, and bacterial killing through direct interactions with the cell cytoskeleton and plasma membrane components. Calprotectin and calgranulin B facilitate phagocyte transmigration by coordinating microtubule dynamics (Vogl, et al., 2004). Specifically, the calprotectin complex induces polymerization of microtubules that can be disrupted by phosphorylation of calgranulin B by p38 mitogen-activated protein kinase. With disruption of the calprotectin complex the microtubule depolymerizes. A similar mechanism in squamous epithelial cells increases their resistance to intracellular invasion by mucosal pathogens such as *Porphyromonas gingivalis, Listeria monocytogenes,* and *Salmonella enterica* serovar *Typhimurium* (Nisapakultorn et al., 2001a, 2001b; Zaia et al., 2009). In neutrophils, calgranulin B is a selective stimulator of MAC-1 mediated adhesion (Newton & Hogg, et al., 1995). Calgranulin B does not directly bind to MAC-1 but its stimulatory effects on β2 integrin / MAC-1 appear to be mediated through a G protein coupled receptor. Calgranulin A can inhibit MAC-1 affinity activation by dimerizing with calgranulin B. In addition to modulating leukocyte trafficking, calgranulins also enhance microbial killing through enhancing the generation of reactive oxygen species in phagocytes. In neutrophils, calgranulin A and the calprotectin complex activate NADPH oxidase by facilitating enzyme complex assembly at the plasma membrane (Kerkoff, et al., 2005). The calprotectin complex transfers arachidonic acid, an essential cofactor, to the enzyme complex while the calgranulin A subunit contributes to NADPH enzyme assembly by directly binding to the $p67^{phox}$ subunit (Kerkoff, et al., 2005). In macrophages, calgranulins contribute to the generation of nitric oxide mediated killing through induction of nitric oxide synthase gene expression (Pouliot et al., 2008).

Active secretion of calgranulins from neutrophils can be induced through engagement of toll-like receptor/CD14 and nuclear factor κβ (Kido et al., 2003) or through protein kinase C in monocytes (Nacken et al., 2003). Calgranulins can be found in mucosal body fluids that are normally colonized with bacteria, such as the oral cavity. Moreover, calprotectin concentrations increase in mucosal fluids in response to certain infections such as *Helicobacter pylori* (Leach et al., 2008). It has been suggested that the increased expression of calprotectin at these sites confers resistance to bacterial invasion or dissemination into deeper tissues (Hsu et al., 2009). Increased expression of calprotectin in humans with chronic sinusitis directly correlates with resistance to bacterial infection (Hsu et al., 2009). In our rodent model of ureaplasmal induced urinary tract infection, some animals with asymptomatic bladder infection and baseline levels of calprotectin developed ascending renal infections. However, animals with bladder infections accompanied by high amounts of calprotectin did not develop ascending renal infection (Reyes et al., 2008, 2009). Therefore, suggesting that calprotectin may be somewhat protective in the urinary tract.

Calprotectin microbicidal activity towards *Candida albicans, Staphylococcus aureus, Straphylococcus epidermis, Escherichia coli, and Klebisiella species* has been demonstrated *in vito* (Brandtzaeg, et al., 1995). Further, calprotectin has been shown to be effective in inhibiting the growth of *S. aureus* within liver abscesses of mice (Corbin et al., 2008). The mechanism of action by calprotectin appears to be chelation of magnesium, manganese, and zinc, which are required for growth by bacteria (Corbin et al., 2008, Hsu et al., 2009).

Extracellular calgranulins also augment immune defense mechanisms in a cytokine like manner. Calgranulin B enhances neutrophil microbial killing by stimulating degranulation of matrix metalloproteinase through p38 mitogen activated protein kinase (Simard et al., 2010). Calprotectin stimulates production of interleukin-8 in airway epithelial cells (Ahmad et al, 2003). Calprotectin binding to microvascular endothelial cells promotes chemokine secretions, up regulation of cell adhesion molecules, and disruption of the endothelial barrier (Viemman et al., 2005, Eue et al., 2000), facilitating leukocyte migration into sites of infection. Calprotectin may also contribute to bacterial clearance from uroepithelium through its ability to enhance activation of toll-like receptor (TLR) 4 (Ehrchen, et al., 2009). Song et al. identified an additional alternative pathway in uroepithelium in which activation of TLR 4 by uropathogenic type 1 fimbriated *E. coli* or *Klebsiella pneumoniae* results in rapid activation of adenylate cyclase 3. The increased intracellular cAMP causes rapid expression and secretion of interleukin-6 that precedes cytokine production through the classical NF-κβ mediated pathway (Song et al., 2007). This pathway may be critical for early defense against invading microbes. More importantly, cAMP blocks intracellular invasion of bacteria into uroepithelium and promotes expulsion of bacteria through inhibition of Rac-1 mediated mobilization of the cytoskeleton (Song et al., 2007). This is an intriguing concept, but it appears that calprotectin may not be contributing to this unique defense mechanism. Dessing et al., compared the ability of uropathogenic *E. coli* to establish acute ascending infection in wild type and calprotectin deficient mice (S100A9 -/-). In this study animals received an intraurethral inoculation of $10^8$ organisms and were examined at 24 and 48 hours post-inoculation. Despite significant increases in calprotectin in both the bladder and kidney tissues of wild type mice, they did not exhibit any difference in microbial load or lesion severity when compared S100A9 -/- mice. Therefore, suggesting that calprotectin is not critical for early clearance of bacteria from the urinary tract.

### 2.2 Regulation of immunity by calgranulins

Successful defense against infection depends on a well controlled inflammatory response that is able to remove the pathogen without extensive collateral damage of the surrounding host tissues. During an effective immune response the signal transduction pathways trigger production and /or release of pro-inflammatory mediators that potentiate removal of the pathogen, and simultaneously or sequentially activate anti-inflammatory factors and protect surrounding tissues from proteases and reactive oxygen species. Calgranulins participate in several of these processes most of which have been identified in macrophages (Lim et al. 2009; Passey et al., 1999b; Xu et al., 2001). For example, bacterial lipopolysaccaride, IFN-γ, TNF-α, and interleukin-10 all induce calgranulin gene expression in murine macrophages (Xu et al., 2001). Glucocorticoids also amplify calgranulin expression in macrophages that have been primed with lipopolysacarride (Hsu et al., 2005). Methylprednisolone treatment in rheumatoid arthritis patients significantly increases the number of calgranulin A and B expressing macrophages in synovium (Hsu et al., 2005).

Secreted calgranulins also exhibit anti-inflammatory properties in neutrophils. Sroussi et al., have shown that both calgranulin A and B can repel human neutrophils *in vitro*, and that

calgranulin A can inhibit the recruitment of neutrophils *in vivo* (Sroussi et al., 2006, 2007). Recently, they have demonstrated that calgranulin A and B also inhibit neutrophil oxidative metabolism (Sroussi et al., 2010). Although the mechanism of action has not been elucidated, adenosine metabolites are known to be involved.

Calgranulins that have undergone post-translational modifications can down-regulate inflammation or provide protection from granulocyte secreted products such as reactive oxygen species. Calgranulin A, in particular, is highly susceptible to oxidation by hydroxyapatite, hydrogen peroxide, and hypochlorite (Lim et al., 2009; Harrison et al, 1999). Calgranulins therefore act as sinks for reactive oxygen species, providing protection of the microenvironment from collateral damage. Both calgranulins A and B can be nitrosylated (addition of nitric oxide molecule) (Lim et al., 2008, 2009). Nitrosylated calgranulin A suppresses mast cell mediated activation, leukocyte adhesion, and extravasation into the microcirculation (Lim et al., 2008).

Calgranulins A and B modulate various aspects of adaptive immunity. Calgranulin A has been reported to inhibit immunoglobulin G production in lymphocytes (Brun et al., 1995). Calgranulin B may down regulate responsiveness to toll-like receptor mediated stimulation in dendritic cells (Averill et al., 2011). During murine myeloid differentiation, increased expression of calgranulin B in embryonic stem cells favors development into myeloid derived suppressor cells over dendritic cells (Lim et al. 2009). Myeloid derived suppressor cells promote immune tolerance during infection and inflammation by suppression of T cell activation and promotion of a T helper type 2 cytokine phenotype involving expression of interleukin-4 and interleukin-13 (Delano et al., 2007; Ezernitchi et al., 2006; Haile et al., 2008). Moreover, calprotectin secreted by myeloid derived suppressor cells prolongs their immunosuppressive effects through an autocrine positive feedback loop (Lim et al., 2009).

### 2.3 Pro-inflammatory effects of calgranulins

Increases in extracellular calgranulin concentrations are associated with several inflammatory and auto-immune disorders including cystic fibrosis, chronic bronchitis, sepsis, pyelonephritis, oral candidiasis, periodontitis, *Helicobacter pyloris* infections, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, Sjogren's syndrome, and atherosclerosis (Foell et al., 2007; Johne et al., 1997; Perera et al., 2010). In the urinary tract calgranulins are found to be a major component of calcium oxalate, calcium phosphate, (Canales et al, 2008, 2010) and struvite uroliths (Bennett et al., 1994), all of which are associated with inflammation (Mushtaq et al., 2007; Reyes et al., 2009). In the case of calcium phosphate and calcium oxalate stones, renal injury with inflammation contributes to urolith formation (Khan, 2005). Struvite stones are sequela to severe inflammatory urinary tract infection caused by urease producing bacteria (Reyes et al., 2009). Due to their close association with inflammation, calgranulins are often used as diagnostic biomarkers for inflammatory disorders (Altwegg et al., 2007; Healy et al., 2006). There is a growing body of evidence that calgranulins play more than a passive role in at least some of these disorders. For example, Croce et al. showed that the inflammatory response to vascular injury is attenuated in calprotectin deficient animals. Further, deletion of calprotectin in atherosclerosis prone ApoE-/- mice reduces their susceptibility to the disease (Croce et al, 2009). Similar effects have been observed in CD40L over-expressing mice that spontaneously develop dermatitis, nephritis, auto-antibodies in serum, and auto-reactive $CD8^+$ T cells (Loser et al., 2010). In these animals, genetic deletion of S100A9 alleviates the progression of the disease.

One mechanism by which calprotectin amplifies inflammation is through direct activation of TLR 4. In the CD40L over-expressing mouse, calgranulin mediated activation of TLR 4

increases expression of interleukin-17, which activates autoreactive $CD8^+$ T cells (Loser et al., 2010). In experimentally induced sepsis, calprotectin enhancement of TLR 4 activation induces exaggerated secretion of TNF-α with endotoxic shock, which is not observed in S100A9 -/- mice. However, septic S100A9 -/- mice treated with recombinant calprotectin exhibit the same degree of endotoxic shock and mortality as their wild type counterparts. It is important to note that calprotectin mediated enhancement of TLR 4 pathway only occurs in systems that have already been immunologically activated. For example, in the case of the sepsis model, the initial activation of TLR 4 occurred by bacterial lipopolysaccaride. These results prompted us to re-evaluate our rat model of inflammation induced struvite urolithiasis secondary to experimental infection with *Ureaplasma parvum* (Reyes et al., 2008, 2009). In this model, expression of calprotectin is only observed in animals with inflammation secondary to urinary tract infection. Therefore, we wondered if activation of TLR 4 may also be a aspect in this disease. An important feature of *Ureaplasma parvum* is that this pathogen does not activate TLR 4, but does activate TLRs 1, 2, and 6 in macrophages (Shimizu et al., 2008). Moreover, ureaplasmas do not induce pro-inflammatory cytokine expression in uroepithelium as shown in Figure 1.

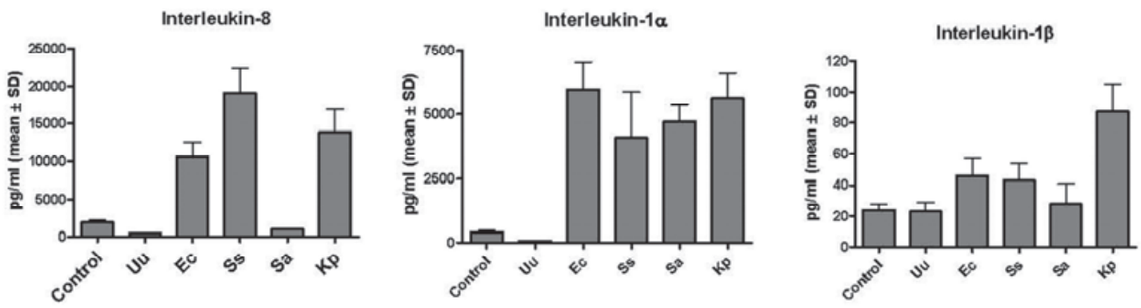


Fig. 1. Chemokine/cytokine profile of RT4 cell culture supernatants.

In an unpublished study of women with first time urinary tract infection, we identified the predominant pathogens present. *Escherichia coli* was isolated from 59.8% of 82 samples that had high numbers (>300,000 CFU/ml clean catch urine), and from 64.8% of 54 urines with medium microbial loads (100,000 to 300,000 CFU/ml clean catch urine). The next most common isolates present in high numbers were *Ureaplasma Spp* (20.7%), *Staphylococcus saprophyticus* (7.3%), and *Proteus mirabilis* (7.3%). *Escherichia fergusonii*, *Klebsiella pneumoniae*, and *Proteus vulgaris* each accounted for 1.2% of remaining urines with >300,000 CFU/ml. At CFU 100,000 to 300,000/ml urine, *S. saprophyticus* was isolated from 7.4% of urines. *K. pneumoniae, P. mirabilis, Staphylococcus aureus, S. epidermidis, S. haemolyticus, and Streptococcus agalactiae* were isolated from 3.7% of urines, with *Citrobacter koseri, Enterobacter aerogenes,* and *K. oxytoca* each accounting for 2% of urine samples. We determined the cytokine/chemokine profile of RT4 cells. Test pathogens were chosen on the basis of predominance in our study patients. RT4 cells were sham inoculated with sterile carrier (control) or, *U. urealyticum* (Uu), *E. coli* (Ec), *S. saprophyticus* (Ss), *S. agalactiae* (Sa), or *K. pneumoniae* (Kp) at a multiplicity of infection of 100. At 24 hours post-inoculation, cell culture supernatants were harvested and evaluated for the presence of interleukin-8, interleukin-1α, and interleukin-1β. Values in the graph represent the mean ± sd (n = 4) of 2 independent experiments. Chemokine and cytokine concentrations were measured with a multiplex antibody assay as previously described (Reyes, et al. 2006).

Ureaplasmal infections are asymptomatic as long as their colonization is restricted to the urogenital mucosa. However, if these bacteria invade the deeper stromal layers, they induce an exaggerated host immune response that does not eliminate infection, but rather causes tremendous tissue damage at sites of colonization. In our rodent model of infection, invasion of bacteria into the submucosa triggered a smoldering inflammation within the submucosa that persisted. By two weeks post-inoculation, animals with ongoing submucosal infection developed struvite uroliths and an exaggerated pro-inflammatory cytokine profile in their urine. Since *U. parvum* is not capable of eliciting these responses in uroepithelium, we surmised that the initiator of uroepithelial cytokine production (see Figure 2) is a paracine factor originating from the inflamed submucosa. On the other hand, animals in which the microbe is found only on the mucosal layer exhibited asymptomatic infection. In these animals, bladder uroepithelium appears quiescent and urine cytokine concentrations are comparable to uninfected controls as shown in Figure 2. This is similar to what is observed in human RT4 cell study.

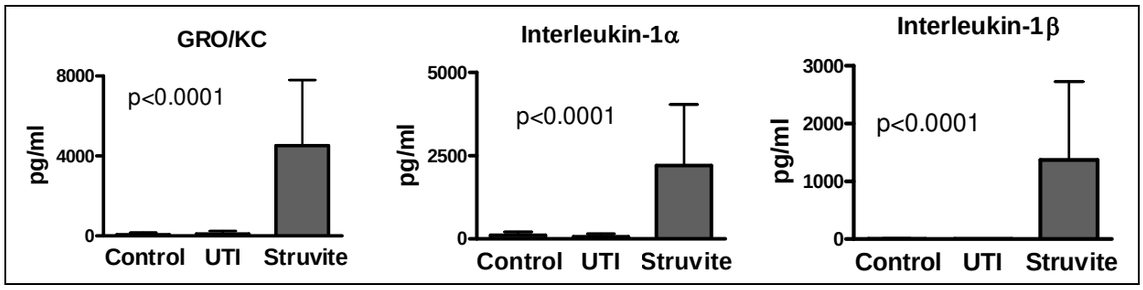


Fig. 2. Chemokine/cytokine profile in urine obtained from uninfected rats (Control), rats with asymptomatic urinary tract infection (UTI), or rats with struvite urolithiasis (Struvite). Values represent the mean ± sd of 5 biological replicates. Data was analyzed by ANOVA followed by Fishers PLSD test.

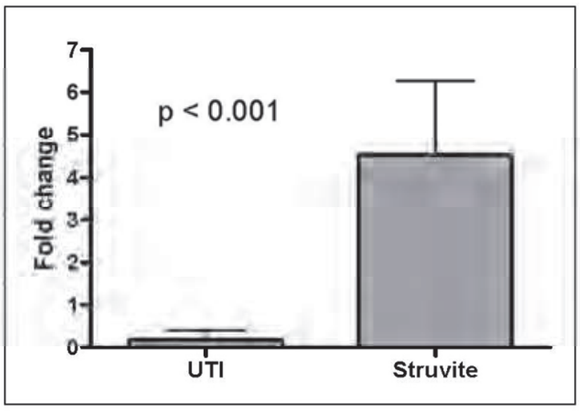


Fig. 3. Induction of calgranulin A in *U. parvum* infected rat bladder tissues. Calgranulin concentrations in rat bladder tissue homogenates from sham inoculated controls, animals with asymptomatic (UTI), and complicated (Struvite) urinary tract infection were measured by ELISA (Reyes et al., 2009). Absorbance values obtained by ELISA were normalized to total protein concentration of tissue homogenates. Values represent the mean ± sd fold change (infected/control) in the calgranulin concentration from 5 biological replicates. Data were analyzed by unpaired student's t test.

In our rodent model of *U. parvum* induced urinary tract infection, proteome analysis of bladder tissues revealed that concentrations of calgranulins A and B were seven fold higher in tissues from struvite positive animals (Reyes et al., 2009). Calgranulin A concentrations were confirmed by ELISA as shown in Figure 3. Moreover, the calgranulin A concentrations in bladder tissue homogenates directly correlated with urine pro-inflammatory cytokine concentrations, suggesting a link between calgranulin A, GRO/KC, IL-1α,and IL-1β.

We also evaluated the distribution of calprotectin within the bladder tissues of animals infected with *U. parvum* for 72 hours and 2 weeks post-inoculation. Calprotectin within uroepithelium was detected in bladder tissues obtained from struvite positive animals at 2 weeks post-inoculation (as shown in Figure 4). In these animals, calprotectin was also detected within endothelial cells that were surrounded by leukocyte infiltrates (Figure 4D). Despite the fact the uroepithelium of animals with asymptomatic urinary tract infection was colonized with *U. parvum*, these animals did not exhibit bladder inflammation or expression of calprotectin by uroepithelium (Figure 4B). We did not observe uroepithelial expression of calprotectin in the 72 hour group, even in animals with ongoing inflammation within the submucosa. Since calprotectin staining could be observed within neutrophils present in these tissues, we were confident that the lack of staining was not an artifact.

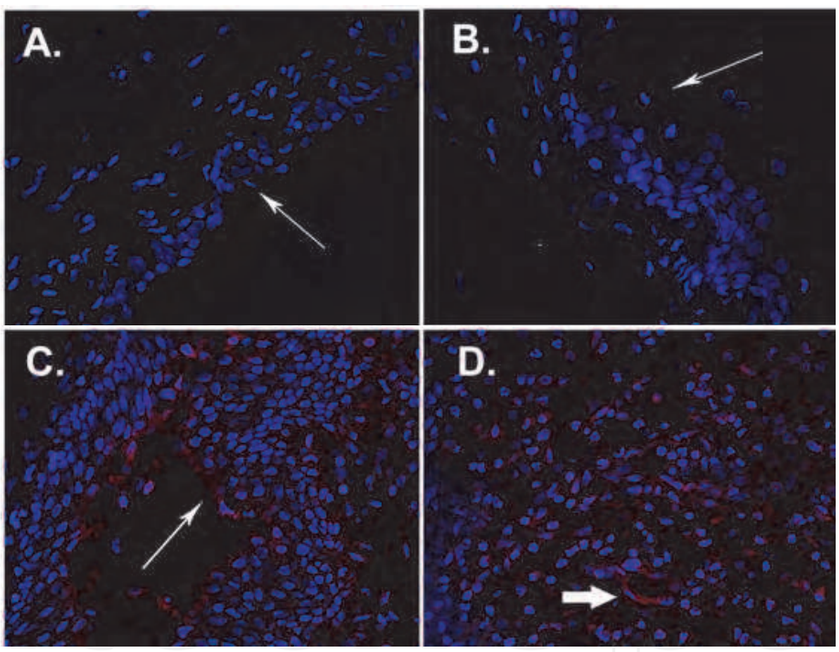


Fig. 4. Distribution of calprotectin in bladder tissues of Fisher rats. Images are 400x magnification of representative bladder tissue sections obtained from sham inoculated control (A), asymptomatic urinary tract infection (B), and struvite positive animals (C and D). Thin white arrows are placed within the lumen and point to the uroepithelial surface in panels A, B, and C. Panel D shows the distribution of calprotectin (red) detected within the submucosa that was infiltrated with, some of which are concentrated around the blood vessel (identified by the block arrow). Bladder tissue sections were processed as previously described (Reyes et al., 2009). Calprotectin was detected with monoclonal antibody (clone MAC 387, from Thermo Scientific, Fremont, CA., USA) followed by goat anti-rabbit conjugated to Alexa-660. Cell nuclei (blue) were labeled with DAPI stain. Images were captured with Olympus IX81 Spinning disk confocal microscope.

Danger-associated pattern molecules (DAMP) are endogenous ligands of toll like receptors. These molecules are often by-products of inflammation such as reactive oxygen species (Frantz et al., 2001) or tissue degradation products from the extracellular matrix (Okamura et al., 2001). Calprotectin is now considered a DAMP because of its ability to activate TLR4 (Ehrchen et al., 2009). Since we observed a correlation between uroepithelial calprotectin expression and pro-inflammatory cytokines in the urine of struvite positive animals, we measured the amount and distribution of TLR 4 in the bladder tissues of rats infected for 72 hours and 2 weeks post-inoculation.There were no appreciable differences in the amount of TLR 4 detected in bladder tissues from sham inoculated controls, or *U. parvum* infected animals at 72 hours post-inoculation. However, at 2 weeks post-inoculation, struvite positive animals exhibited more intense staining of TLR 4 in uroepithelium than uninfected controls or animals with asymptomatic urinary tract infection (see Figure 5).

Since *Ureaplasma* does not directly activate TLR 4, the increased expression observed in struvite positive animals is most likely mediated by DAMPS. Our current study cannot confirm if this activation was initiated by calprotectin or other DAMPS that were detected in our proteome studies such as heat shock protein 60 (Chen et al., 2007) and a variety of extracellular matrix proteins. Nevertheless, the lack of TLR 4 activation in inflamed tissues from the 72 hour time point, coupled with the direct association of uroepithelial calprotectin expression and TLR 4 expression suggests that calprotectin is contributing to an adverse inflammatory response in the struvite group.

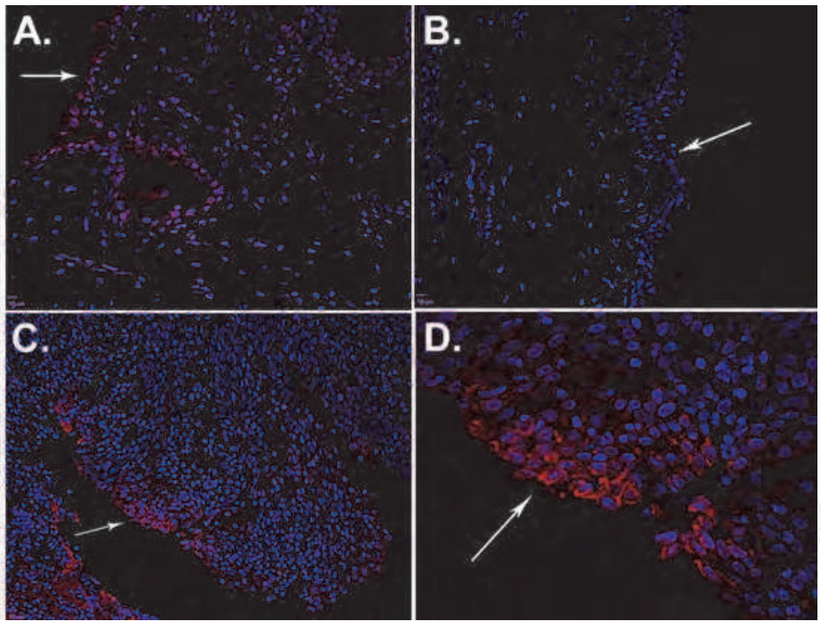


Fig. 5. Distribution of TLR 4 in rat bladder tissues of Fisher rats. Images in panels A, B, and C are 200x magnification of representative bladder tissue sections obtained from sham inoculated control (A), asymptomatic urinary tract infection (B), and struvite positive animals (C and D). Thin white arrows are placed within the lumen and point to the uroepithelial surface. Panel D is a 400x magnification of panel C. Tissues were processed as previously described (Reyes et al., 2009). TLR 4 (red) was detected with rabbit polyclonal antibody (Abbiotec, LLC, San Diego, CA) followed by goat anti-rabbit conjugated to Alexa-594. Cell nuclei (blue) were labeled with DAPI stain. Images were captured with Olympus IX81 Spinning disk confocal microscope.

### 2.4 Regulation of calgranulin gene expression in different cell populations

In addition to participating in immune defence, calgranulins are involved in embryogenesis, growth, and differentiation. Therefore, it is not surprising that the expression of these proteins is tightly regulated in a cell and tissue specific manner, which can change in response to environmental cues. For example, most mucosal epithelial cells have a basal level of calgranulin expression that changes in response to the appropriate stimulus (Ross & Hertzberg, 2001, Hsu et al., 2005, 2009). The appropriate stimulus for one tissue type is not necessarily the same for another. For example, calgranulin expression in oral squamous epithelial cells increases in response to interleukin-1α, but bacterial lipopolysaccaride does not induce an effect (Ross & Hertzberg, 2001). This may pertain to the fact that the oral cavity is heavily colonized with bacteria. On the other hand, bronchiolar epithelium increases its calgranulin expression in response to lipopolysaccaride mediated activation of TLR4 (Henke et al., 2006). Uroepithelium has a low basal expression of calgranulins A and B, which is significantly up regulated in cancer and inflammation (Yao et al., 2007; Tyagi et al., 2008). It is not known what specific cytokines or factors induce up regulation of calgranulin gene expression in these cells, but the studies performed by our laboratory and Dressing et al., imply that uroepithelium would respond to both interleukin-1α and toll like receptor activation.

Varieties of cell types that reside within bladder submucosa either repress or express calgranulins in response to changes within the tissue microenvironment. Cells pertinent to urinary tract infection are fibroblasts, dendritic cells, macrophages, and neutrophils (Cresswell, et al., 2001; Shafik, et al., 2004; Christmas & Botazzo, 1992; Shimizu et al., 2011). Fibroblasts do not participate in antimicrobial processes during urinary tract infection, but their response to mediators released by uroepithelial cells exposed to *E. coli* can influence their ability to migrate and remodel the extracellular matrix, which is particularly important in renal interstitial scarring (Kapoor et al., 2001). Furthermore, calgranulin expressing fibroblasts can be detected during the early stages of wound healing (Rahimi et al., 2005). Fibroblast growth factor-2 or interleukin-1β increases expression of calgranulins by fibroblasts. However, transforming growth factor-β, which promotes fibroblast migration in response to bacterial/urothelial products, can block calgranulin expression in these cells (Kapoor et al., 2001).

Dendritic cells are important in immune surveillance and can express calgranulins in varying intensity depending on their level of maturation (Kumar et al., 2003; Koga et al., 2008). Immature dendritic cells express higher amounts of calgranulins A and B than mature cells (Koga et al., 2008). Interleukin-10 promotes induction of tolerogenic dendritic cells, which then exhibit increased expression of calgranulins (Kumar et al., 2003). Further, calgranulin B may down regulate cytokine release in dendritic cells after toll-like receptor stimulation (Averill et al., 2011).

Macrophages represent differentiated monocytes that have migrated into tissues in response to pro-inflammatory signals or part of a homeostatic process. When differentiation is complete these cells cease to express calgranulins unless they become activated (Nacken et al., 2003). It is important to note that not all activated macrophages express calgranulins. Presently, three macrophage phenotypes with distinctly different functions have been identified (Benoit et al., 2008; Mosser & Edwards, 2008). The phenotype is determined by the type of stimulus the cell receives during the process of activation. For example, macrophages exposed to interferon-γ in combination with TNF-α, and induction of TLR

transforms them into the classically activated or microbicidal macrophage (Benoit et al., 2008; Mosser & Edwards, 2008). Classically activated macrophages are known for their enhanced microbial killing through induction of nitric oxide synthase, secretion of pro-inflammatory cytokines (TNF, interleukin-1β, interleukin-6, and interleukin-12) and secretion of chemokines (CCL2, CCL5, and CXCL8) (Benoit et al., 2008). Resident macrophages that are exposed to interleukin-4 and/or interleukin-13 transform into a wound healing phenotype. Instead of participating in pro-inflammatory events, these cells primarily secrete extracellular matrix proteins and contribute to tissue remodelling. The third activated phenotype is referred to as the regulatory macrophage because these cells express high amounts of co-regulatory molecules that bind to T cell receptors. In addition, regulatory macrophages secrete high amounts of interleukin-10, and these cells are primarily involved in down-regulation of the inflammatory response (Mosser & Edwards, 2008). Of the three phenotypes, only regulatory macrophages express calgranulins, in particular calgranulin A (Benoit et al., 2008; Hsu et al., 2009). Therefore, calgranulin A may be a useful molecular marker for the *in situ* identification of these cells.

A common feature of regulatory macrophages is that they develop from simultaneous exposure to two stimuli (Mosser & Edwards, 2008). The first signal has little to no stimulatory function. Weak agonists known to induce a regulatory phenotype include immunoglobulin complexes, prostaglandins, apoptotic cells, glucocorticoids, interleukin-10, or G-protein coupled receptor ligands. The second signal involves TLR activation. It has been postulated that calgranulin expression by regulatory macrophages contributes to their immunosuppressive function (Hsu et al., 2009). Support for this argument is that regulatory macrophages exclusively express calgranulin A, which can reduce the damaging effects of inflammation by acting as a scavenger for reactive oxygen species. Furthermore, secreted calgranulin A also repels or inhibits recruitment of neutrophils (Hsu et al., 2009; Sroussi et al., 2010; Xu et al., 2001). Although regulatory macrophages may be highly beneficial during sterile inflammation, their presence at sites of infection may be potentially detrimental. Regulatory macrophages can be exploited by bacterial, parasitic, and viral pathogens. For example, during invasion of macrophages, *Leishmania* species can utilize immunoglobulin complexes to promote the induction of a regulatory phenotype (Moser & Edwards, 2008). Regulatory macrophages may also be inadvertently assisting microbial persistence in the urogenital tract. For example, *Ureaplasmas* are chronic and persistent colonizers of the human urogenital tract, and infections are often asymptomatic (Reyes et al., 2008, 2009; Volgmann et al., 2005). However, these organisms are also associated with pro-inflammatory disorders including interstitial nephritis, urethritis, and overactive bladder (Latthe et al., 2008; Lee et al., 2010). As urease producers, *Ureaplasmas* promote development of struvite uroliths (Grenabo et al., 1988). During infection these microbes induce secretion of prostaglandin $E_2$ and interleukin-10 in host cells (Aaltonen et al., 2007; Estrada-Gutierrez et al., 2010; Moser et al., 2009), which are recognized promoters of the regulatory macrophage phenotype (Moser & Edwards, 2008). Although this connection has yet to be demonstrated with this organism, it is an intriguing notion that *Ureaplasmas* may be benefiting from such a mechanism.

## 3. Conclusion

The biological function of calgranulins is complex, diverse and paradoxical. Therefore, extrapolations from studies outside of the context of urinary tract infection must be made

with caution. To date, there are only two published reports that refer to calgranulins during urinary tract infection. Based on these reports, we can only conclude that the antimicrobial properties of calgranulins are minimally effective in the urinary tract. More than likely, the pro-inflammatory properties of calgranulins contribute to complicated inflammatory diseases such as struvite urolithiasis, pyelonephritis, and possibly interstitial cystitis. The positive correlation between increased calgranulin expression and inflammation in tissues, support the argument that these proteins may serve as biomarkers for chronic complicated urinary tract infections. However, additional studies are required in order to determine which clinical settings would benefit from monitoring calgranulin concentrations during urinary tract infections. Of more immediate benefit may be the use of calgranulins as molecular markers in basic science research. For example, monitoring these proteins during maturation and activation of different cell types that comprise innate defense may provide new insights into mechanisms of immune dysregulation induced by various microbial pathogens of the urinary tract.

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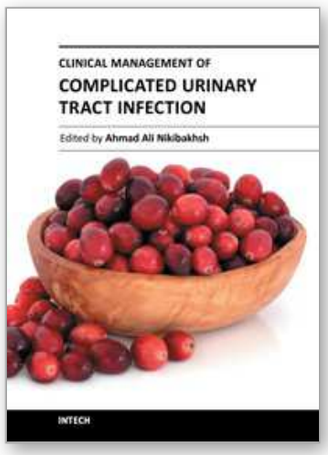


**Clinical Management of Complicated Urinary Tract Infection**
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Complicated urinary tract infections (cUTIs) are a major cause of hospital admissions and are associated with significant morbidity and health care costs. Knowledge of baseline risk of urinary tract infection can help clinicians make informed diagnostic and therapeutic decisions. Prevalence rates of UTI vary by age, gender, race, and other predisposing risk factors. In this regard, this book provides comprehensive information on etiology, epidemiology, immunology, pathology, pathogenic mechanisms, symptomatology, investigation and management of urinary tract infection. Chapters cover common problems in urinary tract infection and put emphasis on the importance of making a correct clinical decision and choosing the appropriate therapeutic approach. Topics are organized to address all of the major complicated conditions frequently seen in urinary tract infection. The authors have paid particular attention to urological problems like the outcome of patients with vesicoureteric reflux, the factors affecting renal scarring, obstructive uropathy, voiding dysfunction and catheter associated problems. This book will be indispensable for all professionals involved in the medical care of patients with urinary tract infection.



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