

Title: Cathelicidin AMP influence mast cell granule composition and release.**Summary****Atopic Dermatitis and Antimicrobial peptide expression**

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that is associated with skin infection or colonization of *Staphylococcus aureus* in 90% of patients (Leung and Bieber, 2003; Leung et al, 2004). Seventy to eighty percent have elevated serum IgE and allergic sensitization, whereas twenty to thirty percent of patients exhibit normal serum IgE levels and lack allergen-specific sensitization (Novak and Bieber, 2003). Recent studies have demonstrated that the Th2 cytokines, interleukin (IL)-4 and IL-13, downregulate anti-microbial peptide (AMP) expression in atopic eczema skin, and this may account for their propensity toward recurrent skin infections (Ong et al, 2002; Nomura et al, 2003). However, also the atopic dermatitis patients without increased IgE level suffer from itching and infections. **Interestingly both AD subtypes have a decreased level of AMP expression in lesional skin.** Two major classes of AMP— α -defensins (Harder et al, 1997) and cathelicidins (Gallo et al, 2002)—have been identified in mammalian tissue and have been shown to play an essential role in host defense against invading microbes (Nizet et al, 2001; Howell et al, 2004). Specifically, the antibacterial activities of human α -defensin (HBD)-2 and LL-37 against *S. aureus* have been previously described (Ong et al, 2002).

Hence, we hypothesize that mast cell AMPs deficiency in Atopic Dermatitis skin constitutes a breach to infections and a proclivity to inflammation.

Antimicrobial peptides are effectors of innate immunity with a broad spectrum of ability to kill bacteria, fungi and viruses. Caths also possess an immunomodulatory activity in vitro. It is an acquired knowledge that the level of antimicrobial peptide in patients with atopic dermatitis is decreased, especially in lesions where infections are more frequent and itching is severe. The data we collected over the last year in response to the reviewer comments on our proposal and as a follow up of our proposal served the purpose to answer the double question:

1-are mast cells important in atopic dermatitis pathogenesis?

2-Are mast cell antimicrobial peptide cathelicidin important in atopic dermatitis?

Our data indicates that mast cells have a role in eczema formation and as their presence reduces the edema formation; AMP deficiency is able to affect granule composition in mast cells, moreover this altered composition is also expressed on mast cells surface as receptor expression.

Mast cells in eczema development

Mast cells are of hematopoietic origin but typically complete their maturation in

peripheral connective tissues, especially those near epithelial surfaces. Mast cells express receptors that bind IgE antibodies with high affinity (FcεRI), and aggregation of these FcεRI by the reaction of cell-bound IgE with specific antigens induce mast cells to secrete a broad spectrum of biologically active preformed or lipid mediators, as well as many cytokines responsible for the skin inflammation and itching. Mast cells are widely thought to be essential for the expression of acute allergic reactions, but they are also important in late phase reactions and chronic allergic inflammation (Using mast cell knock-in mice to analyze the roles of mast cells in allergic responses in vivo. Tsai M, Grimaldeston MA, Yu M, Tam SY, Galli SJ.). Although it is clear that many cell types may be involved in the expression of late-phase reactions and chronic allergic inflammation, studies in genetically mast cell-deficient and congenic normal mice indicate that mast cells may be critical for the full expression of certain features of late-phase reactions and may also contribute importantly to clinically relevant aspects of chronic allergic inflammation. Moreover, the pattern of cytokines that can be produced by mast cell populations and the enhancement of such cytokine production in mast cells that have undergone IgE-dependent up-regulation of their surface expression of FcεRI, suggests that mast cells may contribute to allergic diseases (and host defense) by acting as immunoregulatory cells as well as by providing effector cell function.

Despite the recognized importance of MC in AD, no studies have been performed to evaluate the development of an atopic dermatitis model in absence of mast cells.

To answer this question we established an animal model: Mice deficient of mast cells (Kit^{wsash}/-) were repeatedly painted with DNFB at low concentration 0.01% once a week for 5 weeks. As a control C57BL6 litter-mates were used. It is known that repeated application of DNFB in mice is able to induce IgE production and eczematous lesions. The difference in the extent of the eczematous lesions and the interleukins expression in the lesions between the kit^{wsash} mice and the litter-mates will be an indicator of the importance of mast cells in AD lesion formation.

After 7 days both groups of mice (8 mice for each group) developed

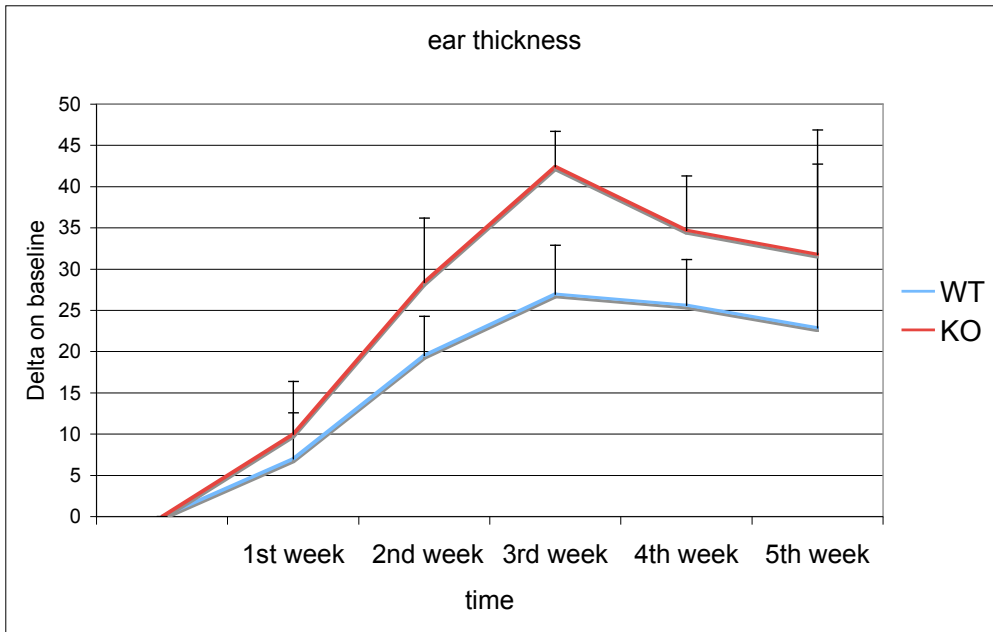


Figure 1: Mice deficient of mast cells (Kitwsah^{-/-}) were repeatedly painted on the ears with DNFB at low concentration 0.01% once a week for 5 weeks. As a control C57BL6 litter-mates were used. It is known that repeated application of DNFB in mice is able to induce IgE production and eczematous lesions. It is clear that the Kit wsah mice developed a higher edema, the difference was statistical significant for the first 3 weeks.

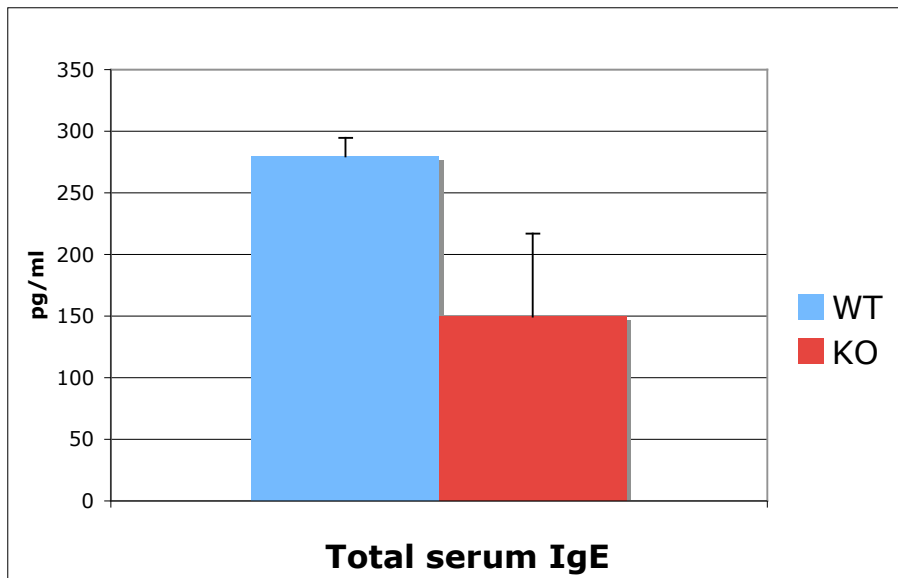


Figure 2: After 5 weeks the animals were sacrificed and both the blood serum and the tissue proteins extracted for ELISA analysis. This figure shows the level of IgE in the blood. The KO for mast cells developed less IgE

eczematous lesions at the site of application accompanied by itching. The measurement of infiltration was performed with a micro-caliper. After 5 weeks the animals were sacrificed and both the blood serum and the tissue proteins extracted for ELISA analysis.

As shown in Fig.1, the absence of mast cells induces a bigger edema formation at the site of application of DNFB. However, the edema formation is not accompanied by an increase in IgE level, for which the presence of mast cells seems to be necessary (Fig.2). An analysis of the tissue protein at the site of lesion formation showed an increased level of IL-4 and a decreased level of IL-10 in the mast cell deficient mice. These data highlight the importance of mast cells in eczema formation. Despite a common belief that mast cells increase edema formation, our mast cells deficient mice presented with bigger lesions that can be justified by the lower expression of IL-10. Moreover, the mast cell deficient mice are skewed toward a TH2 response but they cannot mount an adequate IgE response. These information is important in defining the role of mast cells in an eczema model and later treatment of the disease. We now know that a mast cell

mediator is involved in IgE allergy response and that the edema formation is worse in the absence of mast cells.

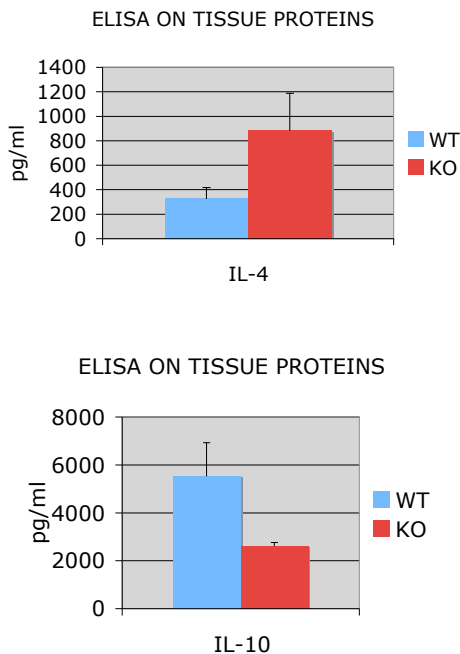


Figure 3: After the animals were sacrificed the tissue site of the reaction was analyzed. Analysis of the tissue protein at the site of lesion formation showed an increased level of IL-4 and a decreased level of IL-10 in the mast cell deficient mice.

Committed mast cell progenitors circulate in small numbers in the blood and are thought to migrate to tissues before undergoing the final stages of maturation, including the development of mature granules. MCs can be activated by very different factors leading to differential release of distinct mediators without degranulation. This process appears to involve the de novo synthesis of mediators with release through the secretory granules, a process distinct from those known in IgE dependent degranulation. How mast cells controls the granule formation and release is still unknown (Theoharides Immunological review 217:65; 2007)

We proposed the AMPs are able to affect MC granule composition and secretory granule release. The data we collected this year supports our hypothesis that AMPs deficiency, as in Atopic dermatitis, will change MCs granule composition and

stability making cells less responsive to infection and more prone to inflammation pathway. To pursue this investigation we derived mast cells from AMP deficient mice and from their wild type littermates.

We cultured the cells for 5 weeks to ensure differentiation. Cnlp^{-/-} (cath KO cells) were cultured in different conditions : in regular medium or medium that was supplemented with Cathelicidin at different concentrations, to verify if any difference in gene expression could have been corrected with cathelicidin supplementation. As you can see in figure 4, at 5 week differentiation, the expression of IL -10 is increased in the Cnlp^{-/-} (the same difference was present at 3 and 4 weeks-data not shown). The increase in IL-10 was correctable by the addition of Cathelicidin at intermediate concentrations. In a previous study dr Leung and coworkers noticed an increased level of IL-10 in the skin of AD patients (Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. Howell MD, Novak N, Bieber T, Pastore S, Girolomoni G, Boguniewicz M, Streib J, Wong C, Gallo RL, Leung DY. J Invest Dermatol. 2005 Oct;125(4):738-45. Erratum in: J Invest Dermatol. 2005 Dec;125(6):1320) In that study, they observed significantly decreased human beta-defensin (HBD)-2 gene expression in the skin of both Intrinsic atopic dermatitis (IAD) (p = 0.010) and Estrinsic atopic dermatitis (EAD) (p = 0.004), as compared with psoriasis patients. Conversely, IAD (p = 0.019) and EAD (p = 0.002) skin lesions exhibited elevated IL-10 gene expression when compared with psoriasis. Interestingly, neutralizing antibodies to IL-10 augmented the production of tumor necrosis factor-alpha and interferon-gamma by peripheral blood mononuclear cell from AD patients. Additionally, treatment of AD skin explants with anti-IL-10 augmented the expression of both HBD-2 and LL-37. Therefore, they hypothesized that the deficiency in AMP expression is an acquired rather than a constitutive defect. Our data, from mast cells also suggests that the intrinsic defect of Cathelicidin may lead to an increase in IL-10 and therefore increase susceptibility to infections.

We next moved to analyze if the absence of Cathelicidin would also affect the expression of the enzyme composition and we analyzed perforin expression and granzyme expression. All of them were less abundant in the cnlp^{-/-} mice . Therefore, it is possible that the difference in expression of these enzymes will

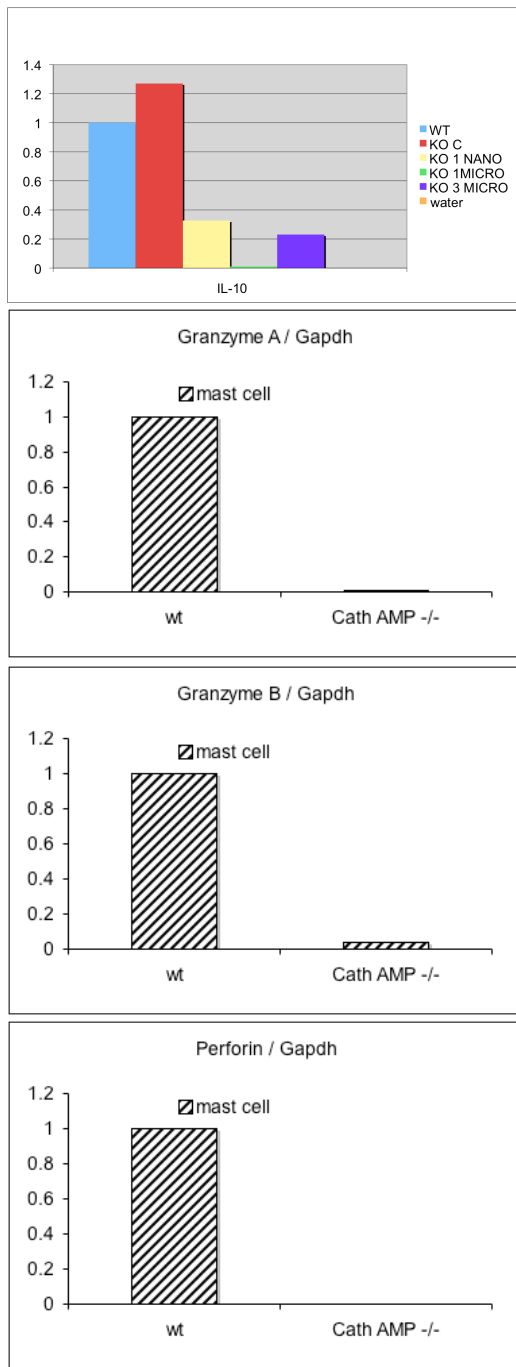


Figure 4: Mast cells derived from *cnlp*^{-/-} mice and wild type litter-mates were analyzed for IL-10 and enzyme expression. The cells deficient in Cathelicidin had a higher level of IL-10 but a lower level of perforin, granzyme A and granzyme B

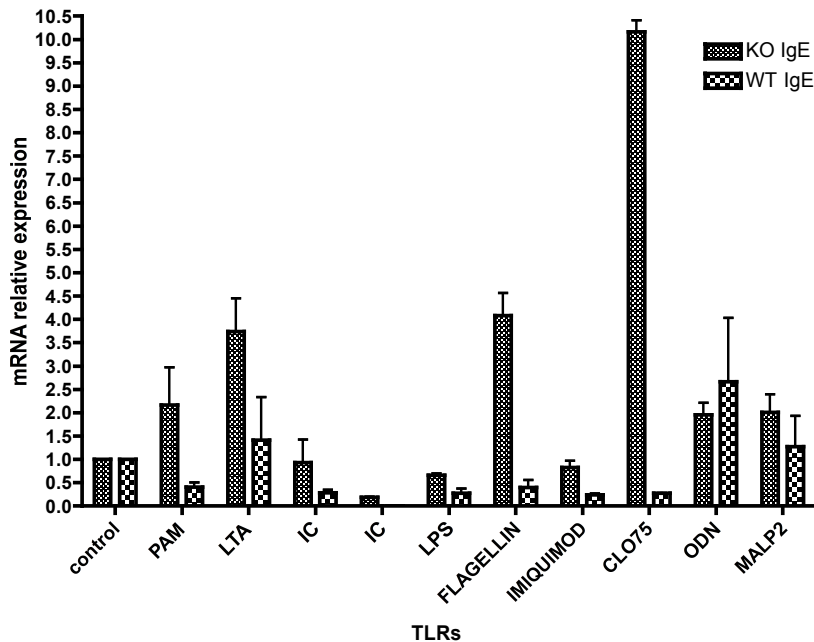
correspond to a difference in their capacity to fight infections. Mast cells can be activated by bacterial or viral antigens, cytokines, growth factors, and hormones, leading to differential release of distinct mediators without degranulation. This process appears to involve de novo synthesis of mediators, such as interleukin-6 and vascular endothelial growth factor, with release through secretory vesicles (50 nm), similar to those in synaptic transmission. Moreover, the signal transduction steps necessary for this process appear to be largely distinct from those known in Fc epsilon RI-dependent degranulation.

Two different mechanisms of granule release are known. One is non selective and is named de-granulation, a classical example of it is the IgE mediated mechanism in type 1 allergy. The second one is the differential release of mediators. A number of innate and endogenous molecules can trigger mast cell to release key mediators differentially or selectively. Toll Like receptors are critical in innate and acquired immunity. Rodent mast cells express TLR4, which binds LPS and induces the release of TNF alpha without degranulation, while peptidoglycan induces degranulation and histamine release. Activation by the innate system seems even more complex, as LPS produces TNF-alpha, IL-1, IL-6 and IL-13 but not IL-4 or IL-5 while TLR2 activation produces IL-4, IL-6 and IL-13. How these differential mast cell responses are controlled is still unresolved. But it is intuitive that a decrease or the absence of one granule component will be able to affect the composition of others.

Therefore we studied the expression of IgE RI on *cnlp*^{-/-} mast cells in comparison to WT during the stimulation with TLR ligands.

Cells from wt and *cnlp*^{-/-} have been treated with TLR ligands and changes in the mRNA expression for IgE RI have been calculated. As you can see in figure 5 MCs *cnlp*^{-/-} respond to pathogens producing much more IgE receptors.

This is a very important observation that remarks that the absence or deficiency of Cathelicidin in mast cells increase the expression of the IgE high affinity receptors. This explain how in the absence or deficiency of Cathelicidin antimicrobial peptide, patients with atopic dermatitis respond to infections with a dermatitis flare up.



Summary and comments

Mast cells in Atopic Dermatitis are surfacing as initiators and controllers of the innate immune system, modulators of the adaptive immune response, and regulators of tissue inflammation. Their activation appears to be sophisticated and highly regulated. Still, little is known about how mast cell granules are assembled and released and how their antibacterial property interferes with their pro-inflammatory functions. **The present study helped in answering some of these questions.**

- 1. We proved that mast cells are important in the pathogenesis of skin eczema, their absence increase the edema formation and this happens despite a lower IgE blood level. According to our data and the Dr. Galli group, the decrease in IL-10 expression is responsible for this**

phenomena. However, more experiments will be required to better define which mediator inside the mast cells is responsible for the edema formation in a chronic inflammation process.

2. We proved that the absence of Cathelicidin antimicrobial peptide inside mast cells alter cell granule composition. Not only the level of IL-10 is increased (as it happens in the AD patient skin) but also the IgE expression is completely subverted. Interestingly the *cnlp*^{-/-} cells have a lower mRNA IgE RI expressio at baseline but when challenged with bacterial products they react producing a large amount of IgE RI while the wt response is in downregulation of these receptors. This data give us a powerful explanation of how AD patients frequently present with an eczema flare up during an infection.

Further research is necessary to uncover the role of AMPs as key factors in mast cell control.