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Induction by vitamin D_3 analog MC903

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Rollen hos mastceller och dess mediatorer i utvecklingen av atopisk dermatit i en musmodell

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SUMMARY

Atopic dermatitis (AD) is a complex, often lifelong allergic disease affecting around 10 % of both dogs and humans. The hallmark symptom is severe pruritus, causing a lowered quality of life. Mast cells (MCs) are known to play an important part of the immunopathogenesis, promoting a faulty T helper cell type 2 (Th-2) response which follows by a production of specific immunoglobulin E (IgE) antibodies towards environmental allergens (Ag). To further investigate the role of MCs and its mediators in the progression of AD, a low-calcemic vitamin D_3 analog (MC903) was used to induce AD-like symptoms locally on the ears of two different knock-out (KO) mouse strains. The first strain was $W^{sh-/-}$ mice deficient in MCs. The second strain was mice deficient in mouse mast cell protease 4 (mMCP-$4^{-/-}$), a homolog to the human and canine MC chymase (MCC).

The hypothesis was to observe an increase in inflammatory parameters following MC903-treatment, such as migration of inflammatory cells, higher amount of inflammatory mediators and clinically observed ear thickening. Thereby, MC903 would be proven to induce a functioning AD animal model. The results showed that daily topical MC903 application on the left ear induced changes mimicking AD macroscopically, microscopically and biochemically, including a Th-2 response represented by associated cytokines. No changes were seen in the vehicle-treated ears (treated with ethanol) used as an internal control.

The epithelial cell-derived thymic stromal lymphopoietin (TSLP) appeared to be an important cytokine in promoting the skin inflammation since the amount of TSLP was markedly high locally in the MC903-treated ears. In MC-deficient mice, TSLP-levels were significantly lower, thus implicating a central role of MCs in this model. Although the presence of MCs caused higher levels of both TSLP and interleukin 33 (IL-33) after MC903-treatment, the clinical appearance in $W^{sh+/-}$ mice was milder compared to mice lacking MCs. This indicates that MCs do promote the AD-like skin inflammation caused by MC903, but at the same time prohibit the inflammation from being too excessive.

The results from a small *in vitro* study performed as a part of this study, proved that MC903 also activates MCs directly to degranulate. The MCC homolog mMCP-4 did on the contrary only contribute to the lowering of IL-33 levels, most likely through cleavage of the cytokine by mMCP-4, therefore no apparent role of MCC could be observed. In the cleavage study of TSLP, a more rapid cleavage of TSLP was mediated by tryptase than chymase. Thus, as a continuation of this study, the investigation of the role of tryptase in the MC903-induced AD-model would be of high interest.

In conclusion, MC903 application induced an AD-like local inflammation, thus showing that the method provides a functioning AD-model. MCs contribute to disease progression through cytokine production, but have at the same time a limiting role in the course of inflammation.

SAMMANFATTNING

Atopisk dermatit (AD) är en komplex och ofta livslång allergisk sjukdom som drabbar ca 10 % av alla människor och hundar. Svår klåda kännetecknar sjukdomen, vilket medför en försämrad livskvalitet för drabbade individer. Det är känt att mastceller (MC) är viktiga i immunopatogenesen, då de bidrar till att skapa ett onormalt immunsvar med typ 2 T-hjälparceller (Th-2). Dessa är förknippade med produktionen av immunoglobulin E (IgE) antikroppar mot allergen i omgivningen.

I detta försök användes en vitamin D_3-analog (MC903) för att inducera AD-liknande symtom lokalt på öronen, tillhörande två olika knock-out (KO) musstammar. Detta gjordes för att ytterligare undersöka hur MC och dess mediatorer kan bidra i sjukdomsutvecklingen av AD. Den ena KO musstammen ($W^{sh-/-}$) saknar MC och den andra KO musstammen saknar mastcellsproteas 4 ($mMCP\text{-}4^{-/-}$), en mushomolog till mastcellsproteaset kymas (MCC) hos människa och hund.

Hypotesen i försöket var att lokalbehandling med MC903 skulle öka de inflammationsparametrar som valts att studera i studien (klinisk sjukdomsutveckling, migration av inflammatoriska celler, mängd av inflammatoriska mediatorer), vilket skulle bevisa att MC903 skapar en funktionell AD-modell. Resultatet i studien visade att en upprepad applikation av MC903 på ena örat inducerade förändringar som liknar dem som ses vid naturligt förekommande AD, både makroskopiskt och mikroskopiskt samt biokemiskt. Inga förändringar sågs i det andra örat som behandlades med vehikel (etanol) och användes som en intern kontroll.

TSLP (thymic stromal lymphopoietin), ett cytokin som främst produceras av epitelceller, bedömdes i försöket ha en viktig funktion i utvecklingen av den AD-liknande hudinflammationen då de MC903-behandlade öronen innehöll mycket stora mängder TSLP. Hos mössen som saknade MC var TSLP-nivåerna signifikant lägre, vilket tyder på en central roll för MC i denna modell. Även om MC903-behandlingen inducerade högre cytokinnivåer (bl.a. TSLP, interleukin 33 (IL-33)), var den kliniska bilden mildare hos $W^{sh+/-}$ möss jämfört med $W^{sh-/-}$ möss (som saknar MC). Detta indikerar en drivande roll för MC i den AD-liknande hudinflammationen som uppstår efter applikation av MC903, samtidigt som MC har en begränsande roll för inflammationen. En liten *in vitro* studie som utfördes under detta projekt, bevisade att MC903 även kan aktivera MC direkt till degranulering.

Ingen tydlig roll för mMCP-4 i denna musmodell kunde påvisas, då det enbart minskade nivåerna av IL-33, troligen via klyvning av cytokinet. Klyvningsstudien av TSLP visade en snabbare nedbrytning av TSLP med tryptas än med kymas. Som en fortsättning på denna studie vore det därför intressant att närmre undersöka rollen av tryptas i den MC903-inducerade AD-modellen.

Sammanfattningsvis gav en upprepad applikation av MC903 uppkomst till en AD-liknande, lokal hudinflammation vilket visar att metoden fungerar som en AD-modell. MC bidrar i sjukdomsutvecklingen genom att höja cytokin-nivåerna, men förhindrar samtidigt en överdriven inflammation.

CONTENT

INTRODUCTION

Atopic dermatitis is a lifelong disease with high prevalence (approximately 10 %) in both children and dogs, caused by an inflammatory response against common environmental Ag (Marsella & Girolomoni, 2009). Pruritus is the hallmark symptom of human as well as canine AD, which causes a lowered life quality of affected humans and dogs, as well as dog owners (Linek & Favrot, 2010). The pruritus is followed by typical lesions such as erythematous macules and flaky skin. These early lesions, with similar distribution in humans and dogs (Marsella & Girolomoni, 2009), aggravates into chronic lesions with hyperpigmentation and lichenification due to self-trauma. The MC has for a long period of time been considered an important part of driving the immune system towards a faulty Th type 2 reaction against Ag, but exactly in what way is still unknown. Although extensive scientific research has been done to examine the immunologic background of AD, there are without doubt discoveries left to be unveiled.

The purpose of this project was to establish a mouse model mimicking AD, induced by a low-calcemic vitamin D_3-analog MC903 (as described by Li *et al*. in 2006). MCs from humans, dogs and mice share many morphological and functional characteristics despite different origin, why the use of mouse models to widen the understanding of MC-dependent immunologic disorders (such as AD) is highly relevant. Therefore, a KO strain of mice lacking MCs (W^{sh}) was used to investigate the role of MCs in this mouse model mimicking both human and canine AD (Grimbaldeston *et al*., 2005). The predominantly expressed type of MCC in mice is mMCP-4 (Pejler *et al*., 2007) which share expression pattern with the human and canine MCC, as well as close to identical cleavage specificity and similar tissue distribution as the human MCC (Hellman & Thorpe, 2013). To further investigate the role of MCC, a KO mice lacking mMCP-4 was also used in this study.

The questions aimed to be answered were if MC903-induced AD is MC-dependent and how the inflammation differs when the tissue is free from MCs or mMCP-4 in KO mice strains, compared to when present in the tissue. The hypothesis, based on previously performed studies, was that MC903-treatment would increase the inflammatory parameters analyzed (*e.g.* clinical scoring, migration of inflammatory cells, inflammatory mediators), thus confirming that the model is mimicking AD. Significant differences between the KO and WT mice were expected since MCs are known to contribute in the immunopathogenesis of AD, thereby proving the importance of MCs in modulating the inflammation.

LITERATURE REVIEW

Mast cells

Characteristics

MCs have a characteristic morphologic appearance, whether of human, canine or murine origin. Their prominent feature is a large amount of cytoplasmic granules (secretory lysosomes), which are pronouncedly colored by toluidine blue (TB) (Garcia *et al*., 1998). The nucleus, which could be covered by granules, is round, especially after degranulation. Canine MCs varies between 7.5 and 12 μm in diameter (Garcia *et al*., 1998), whereas human MCs varies between 5 –10 μm (Voehringer, 2013). MCs originate from hematopoetic stem cells in the bone marrow and emigrate as precursor cells into the peripheral tissues were they mature and terminally differentiate. Their life span varies from weeks to months, according to Voehringer in a recent review article (2013).

Localization

MCs are present in most tissues but more prominent in tissues in contact with the surrounding environment, *i.e.* the skin, intestine and mucous membranes (Tsai *et al*., 2011; Voehringer, 2013). Moreover, they tend to assemble close to nerves, lymphatic vessels and blood vessels. This strategic placement gives MCs the capability to influence other inflammatory cells and to attract them into tissue migration via a large range of inflammatory mediators whenever necessary. Thus, MCs are able to act directly against environmental allergens and have a key role in driving the immune response toward a faulty reaction against non-harmful Ag, such as house dust mites or pollen, which causes the allergic reaction that AD is a symptom of. This refers to both human and canine MCs (Liu *et al*., 2011; DeMora *et al*., 2006).

Mediators

MCs carries preformed mediators in secretory granules which can be released upon several stimuli and used to create an instant immune reaction, for example when allergens cross-link two IgE antibodies connected to the high affinity receptors, FcεRI, on the surface of a MC (Voehringer, 2013). These granula mainly contain histamine, proteases (*e.g.* MCC, tryptase), cytokines (*e.g*. ILs, tumor necrosis factors (TNFs)) and proteoglycans (heparin) (Metcalfe *et al*., 1997). MCs do also have the capacity to produce new mediators from lipids in their cell membrane via arachidonic acid, such as prostaglandins and thromboxanes via cyclooxygenase and leukotrienes via lipoxygenase. Last but not least, MCs can up-regulate their cytokine expression after a longer time of antigen-binding or other stimuli. Cytokines produced by MCs are mostly pro-inflammatory, for example IL-4 that stimulates B-cells into producing IgE and TNF that has a positive effect on the migration and activation of other immunological cells (Tsai *et al*., 2011). However, MCs are also able to limit the inflammation through IL-10, for example by decimating the production of pro-inflammatory mediators from T-cells and keratinocytes.

Activation

Although the most known pathway of MC activation is through antigen cross-linking of two IgE bound to FcεRI high affinity receptor, there are many more ways to activate these immune cells. In fact, MCs have a very important function as sentinel cells in the innate immune response and not

just in initiating the acquired immune response (Metz *et al*., 2008). The placement of MCs in epithelial tissues enables notable contribution to the defense against pathogens, together with the expression of pattern recognition receptors (PRRs) on MC surface, creating an early response against both extra- and intracellular pathogens and their products. Activation of MCs through these PRRs induces the release of cytokines and antimicrobial peptides from not only MCs, but from other immune cells such as dendritic cells (DCs) as well (St. John & Abraham, 2013). MCs do also recognize and become activated by mediators that are upregulated during bacterial infections, such as complement factors (Metz *et al*., 2008). Furthermore, the MCs innate immune response against pathogens includes a direct defense towards bacterial endotoxins, which seem to be degraded by MC proteases.

Associations with atopic dermatitis

Through their strategic placement in the skin and wide range of inflammatory mediators excreted, MCs are able to make substantial effects on the local environment, through regulation of vascular permeability, recruitment of other inflammatory cells, alteration of the functioning of inflammatory cells as well as epithelial cells, professional antigen presentation and induction of a cytokine-driven Th-2 polarization (Liu *et al*., 2011). Comparative *in vitro* studies by DeMora *et al*. (1996) have shown that MCs derived from atopic dogs are more active than MCs from normal dogs. These changes include significantly higher amounts of histamine in the skin MCs, as well as higher spontaneous secretion of histamine from MCs isolated from atopic dogs.

Atopic dermatitis

Background

Atopic dermatitis is a skin inflammation characterized as a hypersensitivity reaction type 1, which implies an exaggerated Th-2 response and subsequent production of IgE antibodies against non-harmful Ag in the surrounding environment (Galli *et al*., 2008). According to Halliwell in 2006, the definition of canine AD is:

"*A genetically-predisposed and pruritic allergic skin disease with characteristic clinical features. It is associated most commonly with IgE antibodies to environmental allergens*".

As described above, heritable factors contribute to the development of AD. This is also true for human AD, were a large birth cohort study by Dold *et al*. (1992) showed a higher risk for a child to develop symptoms of AD with both parents suffering from the same disease (odds ratio (OR) 3.4), compared to the development of asthma (OR 1.5) or allergic rhinitis (OR 1.4) if both parents share those diseases.

One of the currently known inheritable trait of atopic individuals is alterations in the protective skin barrier; atopic dogs have consistently more unorganized components such as intercellular lipids and corneocytes in the *stratum corneum* and *granulosum*, before Ag challenge and in non-lesional skin, compared to non-atopic individuals (Marsella *et al*., 2010). These changes are visible with electron microscopy and the impaired skin barrier is likely to facilitate the contact between Ag and immunologically active cells. In humans, a null mutation in the gene coding for filaggrin, a protein

with important function in the skin barrier through the differentiation of epidermal cells, is associated with a significantly higher risk for AD-development (Palmer *et al*., 2006). In dogs, a genome-wide analysis showed a strong association between AD and two single nucleotide polymorphisms (SNP) in the gene coding for Plakophilin 2 which affects the function of desmosomes as adhesion proteins, hence important in the skin barrier (Tengvall *et al*., 2013). For the complete development of atopy, the immune cells have to display certain changes in function as well. In skin specimens from canine AD-patients, MCs are proven to be more reactive after activation by antigen cross-linking of IgE to FcεRI, in terms of histamine release (DeMora *et al*., 1996).

Beyond these congenital predisposing abnormalities, there are also environmental factors that contribute to the evolvement of AD. In fact, heritage and environmental factors have in a breeding group of guiding dogs for the blind been shown to be of almost equal importance (Shaw *et al*., 2004). One environmental risk factor is early exposure to Ag, in this case house dust mites, *Dermatophagoides farinae*, especially in combination with an inherited predisposition for high IgE-production (Schiessl *et al*., 2003). Another risk factor, shown in a study with 1500 pruritic dogs, is indoor living condition (Favrot, 2010) which also enables contact with the house dust mite Ag which is the most common cause of AD in Swedish dogs (Öhlén, 1992).

Ultimately, for the AD to establish in a patient, scratching with following self-damage on the skin is required. The cause of pruritus has for a long period of time been investigated, and there are most likely multiple factors involved, including TSLP, IL-25, IL-33 (Wilson *et al*., 2013), IL-2 and IL-31 (Kabashima, 2013). Pruritus and its mediators are further reviewed in later paragraphs.

Clinical appearance

AD, in humans also known as eczema, is a complex disease that in most part shares the same features in both human and canine patients (see Table 1, adapted from Marsella & Girolomoni, 2009). In both species, pruritus is the primarily and most prominent component, which leads to worsening of the inflammation through itching and self-damage (Marsella & Girolomoni, 2009). After pruritus, lesions with erythem and flaky skin appear which in dogs quickly aggravates into alopecia, lichenification and hyperpigmentation of the skin, if the itching continues (Olivry *et al*., 2010). Affected areas are also similar between the species: humans have most of their lesions in flexural surfaces of the joints (for example inner sides of knees and elbows) and hands, and dogs are pruritic in surfaces in contact with the floor (for example axillar and inguinal regions, paws and ears) (Marsella & Girolomoni, 2009). Furthermore, the first clinical signs of AD occur early in life of both humans and dogs. However, canine AD is considered to be a lifelong disease in all cases (Marsella & Girolomoni, 2009), whereas human patients might outgrow their symptoms with increasing age. In a Swedish study from 2000, Gustafsson *et al*. observed that around 35 % of the children suffering from eczema at the starting point (4 – 35 months old) did not show any skin changes at the end of the study (5 – 7 years old).

Another difference between the clinical appearance in human and canine patients is that for humans, it is common to go through the so called atopic march, *i.e.* a development of the disease into allergic rhinitis/conjunctivitis and asthma (Spergel, 2010). Only a few percent of the atopic

dogs develop rhinitis or conjunctivitis and no dogs have been reported to develop asthma (see Table 1, adapted from Marsella & Girolomoni, 2009). Gustafsson *et al*. (2000) showed that 50 % of the 94 participating children diagnosed with eczema at 4-35 months of age developed allergic rhinitis and 47 % developed asthma before 7 years of age.

Table 1. *Similarities between human and canine atopic dermatitis*

	Human AD	Canine AD
Prevalence (%)	5 – 20 of children	10 – 15 in total
Genetical inheritage	Yes	Yes
Age of onset (years)	<1 – 5	1 – 3
Pruritus	Severe	Severe
Skin areas affected	Face, skin folds	Face, skin folds
Spongiotic dermatitis	Yes	Yes
High skin colonization of *Staphylococcus spp.*	Yes	Yes
Th-2 domination of immune response	Yes	Yes
IgE-specific response (%)	55 – 90	80
Atopic march	Yes	No
Rhinitis and conjunctivitis (%)	<5	35

Adapted with permission from Marsella & Girolomoni, 2009

Microscopically visible features of atopic dermatitis

Lesional skin from humans suffering with AD shows so called spongiotic changes when analyzed by histopathology and electron microscopy (Kamsteeg *et al*, 2010). This means an edema in the epidermis that widens the intercellular space, thus impairing the skin barrier. An experimental study by Kamsteeg *et al*. (2010) showed that atopic dogs had similar changes as humans, including a disorganized *stratum corneum* with lower density than in normal dogs. Ag challenge in normal dogs led to a mildly increased intercellular space whilst in atopic dogs, severe spongiotic changes were seen. Together with the spongiosis, hyperplasia of the epidermis was seen as a typical histological finding in lesional skin of dogs with AD, corresponding to the thickening of the skin seen macroscopically (Olivry *et al.*, 1997). In skin biopsies from ten human AD patients, epidermis was significantly increased (>50 %) in lesional skin compared to non-lesional skin, a change that was even more apparent in chronic AD compared to acute lesions (Gittler *et al*., 2012).

Moreover, it is typical to see a dermal infiltration of inflammatory lymphocytes and MCs that secrete cytokines associated with a Th-2 type cytokine profile (*e.g*. IL-4, IL-13). Interestingly, an *in vitro* study from 2011 by Kamsteeg *et al*. showed that skin equivalents grown from human keratinocytes develop typical spongiotic AD-lesions when cultured with IL-4 and IL-13. In the same study, these cytokines were also shown to increase fragmentation of deoxyribonucleic acid

(DNA) which according to the authors implies that the higher frequency of keratinocyte apoptosis seen in acute AD-lesions could be induced by Th-2 cytokines such as IL-4 and IL-13.

Prevalence

Because of the complexity of AD and its appearance, prevalence studies have to be considered merely qualified estimations. These give percentage numbers from 3 to 15 of the total canine population (Hillier & Griffin, 2001). In Sweden, the prevalence according to calculations using national insurance companies' data bases was 3 % between year 1995 and 2002 (Holm, 2007). In humans, a lifetime prevalence study in USA showed that up to 17 % of school children suffered from AD (Laughter *et al*., 2000) and the disease is also a major problem in developing countries according to a global prevalence study by Odhiambo *et al*. (2009).

Immunopathogenesis including the role of mast cells

Basic immunology

As previously mentioned, atopy is categorized as a type 1 hypersensitivity reaction associated with IgE antibodies directed towards Ag (Galli *et al*., 2008), which implies the involvement of Ag-presenting cells (APC), *e.g.* DCs. These migrate to the regional lymph nodes where they present antigen to naïve T-lymphocytes, leading to the formation of T-h cells. MCs are also able to present antigens and thereafter drive the immune response toward a Th-2 cell type associated with humoral immunity and IgE production, through secretion of IL-4 (Metcalfe *et al*., 1997). IL-4 is a cytokine which presence has been shown to be required for a Th-2 response *in vitro* (Voehringer, 2013) and is expressed by more cells in both lesional and non-lesional skin of human AD patients compared to normal controls (Hamid *et al*., 1994). In a study by Nuttall *et al*. from 2002, skin biopsies from atopic dogs were shown to express significantly higher levels of IL-4 mRNA compared to healthy controls. Schlotter *et al*. did a similar study in 2011, showing a higher expression of the Th-2 cytokine IL-13 in the skin of atopic dogs compared to the controls, as well as a lowered expression of the Th1-associated cytokine IL-12. Another important cytokine is the epithelial cell-derived cytokine TSLP which has in an *in vitro* study been shown to activate and support survival of the professional APC, DC (Soumelis *et al*., 2002). This leads to the production of Th-2 associated cytokines such as IL-4 and IL-13 from naïve T-cells. The Th-2 cells stimulate B-cells to proliferate and produce antibodies (IgG, IgE) towards the Ag, which lead to sensitization of MCs by Ag-specific IgE binding to the high affinity receptor FcεRI. MC activation occurs through cross-binding of Ag with two IgE bound to FcεRI, which subsequently leads to the release of mediators which causes the allergic skin inflammation (Voeringer, 2013).

This IgE-associated immune response is an important part of the immunopathogenesis, however, explaining only one part of the complex background of AD. In fact, there are many AD-patients who do not carry any traceable amounts of Ag-specific IgE. According to Marsella & Girolomoni (2009), this refers to 20 % of atopic dogs and 10 – 45 % of atopic humans. Consequently, the immunological background of AD involves not only the adaptive, but also the innate immune system. For example, epithelial cell-derived cytokines like TSLP could directly activate innate immune cells such as MCs without the involvement of B- or T-cells, according to Wu *et al*., 2010.

TSLP and the related cytokine IL-33 do also have an important part in promoting the Th-2 associated cytokine cascade seen in AD-skin (Park *et al*., 2013; Iikura *et al*., 2007).

Early phase of atopic dermatitis

Experimental studies in atopic dogs showed a biphasic pattern with an early and a late reaction phase, and that MCs play an important role in both phases (Olivry et al., 2001). First of all, the Ag has to come in contact with immunologically active cells, which in both humans and dogs occurs via inhalation or by the epicutaneous route, the later though being the most important route in dogs (Marsella *et al*., 2006). The fact that AD in dogs is not as often associated with respiratory symptoms as in humans could be an explanation to why Ag in contact with the skin give the most potent inflammation in dogs, compared to air-born Ag (Marsella *et al*., 2006). Sensitized MCs, with Ag-specific IgE bound to FcεRI, that come in contact with the same Ag once again, are immediately activated (Galli *et al.*, 2008). This activation leads to MC degranulation with the release of a broad range of mediators that create an almost acute inflammation after Ag contact, thus creating the early phase of the allergic inflammation.

Late phase of atopic dermatitis

The acute inflammation caused by MC mediators lead to pruritus, which is the hallmark symptom of AD and is often present before any visible changes appear in the skin (Favrot *et al*., 2010). MCs contribute to pruritus through mediators that have the ability to provoke an itching sensation via nociceptive receptors on sensory neurons (Galli *et al.*, 2008). The MC mediator histamine is a well-known and important prurinogen, but the fact that anti-histamines only have a limited impact on the pruritus in both humans and dogs, supports the involvement of other mediators as well. Known pruritic mediators are for example nerve growth factor (NGF) which is produced by keratinocytes and supports further innervation of the skin, thus enabling the sensation of pruritus (Kabashima, 2013). The blood level of NGF is also related to disease severity of AD. In turn, nerve cells produce Substance P that stimulates dermal fibroblasts to release artemin, which leads to a lowering of the threshold towards a temperature-induced itch seen in human AD patients. Interestingly, Substance P has also got the ability to activate MCs (Kawakami *et al*., 2009) and can be degraded by the MC protease chymase (Caughey *et al*., 1988), thus indicating a role of MCs in balancing the pruritus. Moreover, MCs are able to produce both Substance P and NGF, thus giving them opportunity to affect the nervous system. Other possible prurinogens described further by Kabashima in a newly published review article (2013) is endothelin and acetylcholin from endothelial cells, IL-2 and IL-31 from T-cells and reactive oxygen from eosinophils. In a study from 2006, mRNA expression of IL-31 was significantly upregulated in skin specimens from humans with AD (Sonkoly *et al*., 2006). This result was most apparent in patients with severe pruritus. In skin specimens from humans with the non-pruritic skin disease psoriasis, no significant upregulation of IL-31 could be observed. Moreover, non-lesional skin from AD-patients had four times higher mRNA-levels compared to skin from healthy humans. This could, according to the authors, imply that IL-31 is one of the factors that lead to pruritus before a skin inflammation is visible (Sonkoly *et al.*, 2006).

The late reaction phase continues with migration of other immunologically active cells to the affected skin, such as granulocytes (*i.e.* neutrophils and eosinophils) and lymphocytes. MC

mediators contribute to this event, through alterations in blood flow which enables vascular adhesion and migration of neutrophils (Metcalfe *et al*., 1997), as well by attracting other immune cells to the area, for example via IL-8 and TNF-α (Galli *et al.*, 2008). A small study in dogs done by Olivry *et al*. (2001) showed a significantly higher rise in neutrophil counts in atopic dogs after experimental Ag skin injection (most apparent after 6-12 hours), compared to non-atopic dogs. T-lymphocyte and DC numbers peaked later than the neutrophils, at 24 hours after Ag challenge. The same author showed in a previous study that the cell infiltration in the skin of atopic dogs consisted of low numbers of neutrophils, which indicates that these cells might not be as important in naturally occurring AD as in experimental AD (Olivry *et al*., 2001). The exact function of neutrophils in AD is yet to discover. Not to be forgotten, MCs might also have a role in limiting the inflammation through mediators like IL-10 (Galli *et al*., 2008).

Chronic phase of atopic dermatitis

After pruritus has established in the AD-patient, changes in the skin develop. First, small papules, erythema and flaky skin are seen, lesions quickly worsening without treatment into explicit changes caused by scratching. These include lichenification and hyperpigmentation, as well as alopecia in dogs. Chronic lesions develop due to the self-induced trauma that sustains the skin inflammation via continuous release of inflammatory mediators, which further aggravate the pruritus in a vicious circle (Olivry *et al*., 2010). Analysis of biopsy specimens from humans suffering from AD has shown significant differences in the cytokine expression pattern between acute and chronic lesions. Acute lesions show a Th-2 immune response, dominated by IL-4, though IL-5, IL-10, IL-13 and IL-33 are also expressed (Hamid *et al*., 1994; Gittler *et al*., 2012). Low expression of Th-1 cytokines such as IFN_{γ} is seen. In chronic lesions, a decrease in IL-4 expression, an increase in IL-5 expression and an increase of the Th-1 cytokine IFN_{γ} is seen (Hamid *et al*., 1994; Gittler *et al*., 2012), thus indicating a less important role of MCs in chronic AD.

The disease also worsens as microbes often attach and grow in the defective skin. In fact, secondary infections infest 66 % of all atopic dogs according to Favrot *et al*. (2010). The most common microbe causing infection in canines, *Staphylococcus pseudintermedius*, has been shown to adhere with corneocytes (terminally differentiated keratinocytes) in significantly higher extent in both lesional and non-lesional skin of atopic dogs compared to normal dogs (McEwan *et al*., 2006). This colonization predisposes atopic dogs to pyoderma, and may be caused by the faulty Th-2 microenvironment seen in AD according to the authors. In humans suffering from AD, more than 90 % have skin colonization with *Staphylococcus aureus*, shown by Breuer *et al*. in 2002. Of all adult AD patients, 94 % were positive for the bacteria in swab tests from their skin. In a similar study, over 87 % of the investigated atopic dogs were colonized by *S. pseudintermedius*, compared to 37 % of normal dogs (Fazakerley *et al*. in 2009).

Associations between specific MC-derived mediators and atopic dermatitis

The following MC-mediators were chosen for further investigation in the experimental model of this study, due to their previously proven association with AD.

Thymic stromal lymphopoietin

TSLP plays a key role in the innate immunity; it is involved in creating a Th-2 associated cytokine expression and subsequently induce a Th-2 type of allergic inflammation, without the involvement of T- and B-cells according to Park *et al*. (2013). Instead, TSLP activates innate immune cells directly via the TSLP-receptor (TSLPR) on the cell surface of immune cells, including MCs (Allakhverdi *et al*., 2007) and DCs. The activated immune cells then start to produce cytokines associated with a type 2 Th immune response, for example IL-4, IL-5, IL-13 and TNF-α (Ziegler, 2012), cytokines that also are associated with AD. Furthermore, an *in vitro* study showed that TSLP was produced by human MCs incubated with IL-4 (Okayama *et al*., 2009), thus indicating that MCs may contribute to an allergic inflammation through production of TSLP. In the same study, TSLP was shown to be degraded by MC proteases, which could be a way for MCs to limit the inflammation. The increase in IL-4 seen in other mouse models mimicking atopy, was not seen in mice lacking TSLP in keratinocytes (Leyva-Castillo *et al*, 2013), suggesting that TSLP-expression is necessary for the Th-2 dominated immune response seen in patient with atopy.

Genetic studies have shown significant associations between SNP in the gene coding for the TSLPR and human AD (Gao *et al*., 2010) as well as in canine AD (Wood *et al*., 2010). In fact, the gene coding for TSLPR was the only gene with SNP in atopic dogs of all eight breeds examined in a meta-analysis study. High TSLP expression by epithelial cells derived from atopic humans was observed by Soumelis *et al*. in 2002. In atopic dogs, TSLP expression was significantly up-regulated in both inflammatory lesional and non-lesional skin (Klukowska-Rötzler *et al*., 2013). Keratinocytes produce TSLP in response to microbes, inflammation or trauma such as scratching, by which TSLP could have a part in aggravating the continuous inflammatory response seen in AD-patients according to Allakhverdi *et al*., 2007. A recent study by Wilson *et al*., (2013) showed that an injection of TSLP into the cheek of a mouse induced a pronounced itch, whilst a vehicle-injection (control substance) did not. This was true not only in WT mice, but also in mice lacking B- and T-cells (severe combined immunodeficient (SCID) and $Rag^{-/-}$ mice respectively) as well as in mice lacking MCs (W^{sh}). Therefore, the authors suggested that TSLP may induce itch through a direct activation of sensory neurons, without the involvement of inflammatory cells or their cytokines.

Functionally, human and murine TSLP show significant homology (Ziegler, 2012), implying TSLP being a useful parameter to assess in AD-mouse models. A recent study, using an AD mouse model where AD was induced by repeated applications with house dust mite extract and Staphylococcal enterotoxin B on the mouse skin, showed that MCs were required for a maximal skin inflammation and that TSLP contributes to the inflammation (Ando *et al*., 2013). This was concluded since KO mice lacking TSLPR ($TSLPR^{-/-}$) had significantly lower clinical scores (*e.g.* ear thickness) after induction of AD compared to WT-mice. Another AD-study in mice by Yoo *et al*. (2005) showed that transgenic mice over-expressing TSLP in the skin, spontaneously developed most of the features that are seen in AD-skin, *i.e.* epidermal thickening, dermal infiltration of leukocytes, local Th-2 associated cytokine production and presence of Th-2 cells in draining lymph nodes.

Interleukin 33

Similar to TSLP, IL-33 is produced by epithelial cells following inflammatory stimuli (Miller, 2011). The knowledge of IL-33 as an important inflammatory cytokine is currently expanding. IL-33 carries the ability to activate Th-2 associated inflammatory cells such as MCs (Iikura *et al*., 2007). *In vitro*, bone marrow-derived MCs were shown to express IL-33 mRNA when incubated with IgE (Hsu *et al*., 2010). MCs derived from peritoneal lavage were stained positive for IL-33 with immunohistochemistry (IHC). In the same study, IL-33 levels were significantly lower in the skin from MC-deficient mice (W/Wv) when compared to WT mice. Hueber *et al*. (2011) showed that intradermal IL-33 injections every other day for 16 days caused a skin inflammation with epidermal thickening as well as a local increase in MC and neutrofil numbers. In $W^{sh-/-}$ mice, the inflammatory response was delayed, thus indicating a role for MCs in the early phase of the IL-33 induced inflammation according to the authors.

A polymorphism in the gene coding for the IL-33 receptor (ST2) was shown in 2005 to be associated with AD in humans (Shimizu *et al*.). To further investigate this connection, Imai *et al*. (2013) used transgenic mice overexpressing IL-33 in the skin. This resulted in a spontaneously developed dermatitis mimicking AD, including the hallmark symptom pruritus. Skin sections showed infiltration of both MCs and eosinophils, including degranulated MCs. This activation of MCs by IL-33 was also apparent in the blood, since mice overexpressing IL-33 contained higher amounts of histamine and IgE in serum compared to WT mice.

Mast cell protease chymase

MCC is a chymotrypsin-like serine protease that is secreted through MC granules following activation of MCs (Badertscher *et al*., 2005). The most well known substance to be processed by the enzyme is Angiotensin 1, but MCC has a wide range of other substrates such as fibrinogen, components of the extracellular matrix (ECM), lipoproteins and several inflammatory substances, including Substance P (see a list with potential substrates in Pejler *et al*., 2007), associated with the pruritus that characterize AD (Kabashima, 2013). Experimental studies by Terakawa *et al*. (2008) using a mouse AD model induced by the chemical substance DNFB, showed that an oral chymase inhibitor (SUN13834) lowered skin inflammation and scratching behavior, thus indicating a role of MCC both in pruritus and clinical changes. In a small study by Badertscher *et al*. (2005), MCC was quantified using IHC in punch biopsy specimens from 19 humans suffering from AD. The results showed a significantly higher number of MCs containing MCC in lesional skin, compared to normal skin in the same individual and skin specimens from humans with psoriasis (both lesional and non-lesional). Moreover, non-lesional skin from humans with AD showed significantly more MCC than psoriatic skin. The authors finally discuss the possible involvement of MCC in the impairment of the skin barrier seen in AD, due to the ability of MCC to degrade ECM components, but if this assumption is correct is yet to discover. In a Swedish study from 2013 by Waern *et al*., also using mMCP-$4^{-/-}$ mice, MCC was shown to have a protective role in an induced airway inflammation model. Therefore, an effect by MCC in the present study was hypothesized.

MATERIAL AND METHODS

Laboratory work was performed at the Department of Biomedical Sciences and Veterinary Public Health, Section of Immunology, located at the Biomedical Centre (BMC) in Uppsala, Sweden.

Experimental animals

Two different types of KO mouse strains on C57Bl/6 genetic background were used in this study. The $W^{sh-/-}$ mouse strain with nearly a total loss of MCs (Grimbaldeston *et al*., 2005) and the mMCP-$4^{-/-}$ mouse strain that only lacks the MC protease chymase (Tchougounova *et al*., 2003). Mice were six to nine weeks old; both female and males were used. Both strains were fertile, appeared clinically healthy and had a normal life span. As controls, heterozygous and WT litter mates were used. All of the animals were kept in a controlled environment at BMC in individually ventilated cages, cared for by trained staff and fed with R3 Pellets (Lantmännen). The methods and use of described mouse strains in experimental AD was approved by the Ethical Committee for Use of Experimental Animals in Uppsala.

The number of animals used in this study is shown in Table 2. In the first experiment, the role of MCs in MC903-induced AD was investigated by comparing MC-deficient ($W^{sh-/-}$) mice with heterozygote MC-competent mice ($W^{sh+/-}$). As controls, five $W^{sh+/-}$ and five $W^{sh-/-}$ were used. In experiment 2 and 3, MC903-induced AD in mMCP-4-deficient, heterozygote and WT animals were scored in a blinded fashion, *i.e.* the genotype of the 36 treated mice and five control mice was determined by polymerase chain reaction (PCR) after the terminal endpoint of the experiment.

Table 2. *Number of animals used in the experiments*

	Genotypes Experiment 1		Genotypes Experiment 2 and 3		
	$W^{sh+/-}$	$W^{sh-/-}$	WT	mMCP-$4^{+/-}$	mMCP-$4^{-/-}$
Treated	7	7	6	19	11
Controls	5	5	5	-	-

Induction of atopic dermatitis on ears by MC903

An AD-like disease was induced by topical application of a low-calcemic vitamin D_3-analog, MC903. This chemical substance is also known as calcipotriol (Dovonex), used for treatment of psoriasis. Several studies have been performed to investigate the functions of MC903 and its potential in AD-treatment. Li *et al*. (2006) showed that skin application of MC903 up-regulates the expression of TSLP in keratinocytes, which is also seen in the skin of human and canine AD-patients (Soumelis *et al*., 2002; Klukowska-Rötzler *et al*., 2013). This together with the fact that the known side effects of calcipotriol-treatment (redness, dry skin, pruritus) mimics AD-symptoms, rose a hypothesis that long-term skin-treatment with MC903 could induce an AD-like phenotype. Li *et al*. (2006) proved that this was true in WT mice as well as in KO mice deficient in B- and T-cells ($RAG^{-/-}$), thus showing that the MC903-induced AD-phenotype was not dependent of either B- or

T-cells. Instead, the cellular infiltration in the MC903-treated skin consisted of eosinophils, DCs and MCs. Another study using MC903 to trigger AD-like symptoms showed that MC903-treatment induced T-cells to produce Th-2 associated cytokines (Elentner *et al*., 2009). Since cultured keratinocytes did not produce any other of the measured cytokines (e.g. IL-1, IL-2 and IL-4), the authors suggested that TSLP is the most important cytokine in mediating the function of MC903. In the same study, DCs were shown to be necessary for the development of an AD-like phenotype.

In the present study, 1 nmol MC903 in 20 µl 95 % ethanol were topically applied on the left ear, 10 µl on each side of the ear. On the right ear, the same amount of 95 % ethanol only was applied as an internal vehicle control. This procedure followed once a day for a total of 13 subsequent days. The development of AD-like changes was followed by photography, measuring of ear thickness using a digital engineer´s micrometer (Mitutoyo Corporation, Japan) and by clinical scoring: 0 = no symptoms, 1 = mild redness/swelling, 2 = redness/swelling, 3 = redness/swelling/bleeding.

Sampling of material from mice

Blood was obtained from the tail vein twice during the trial, at day 2 and day 6, in amounts up to 100 µl. To enhance peripheral blood flow, a heating lamp was placed above the cage for five minutes before blood sampling. At the end of trial, mice were sacrificed using carbon dioxide (CO_2). Thereafter, both ears, the spleen, the auricular lymph nodes, blood, and a piece of the tail were collected from all mice included in the three experiments plus 15 non-treated, age-matched controls with the same genetic background. In Figure 1, the anatomical location of the ear draining lymph nodes is shown (adapted from ICCVAM Immunotoxicology Working Group, 2001). All of the collected materials were used for different analyses in this study except for the spleens, which were frozen in -20˚ C and saved for future studies. The goal was to use materials from every individual KO mice in all analyses performed when possible, material from WT, heterozygous and control mice were selected either randomly or due to the amount of material available.

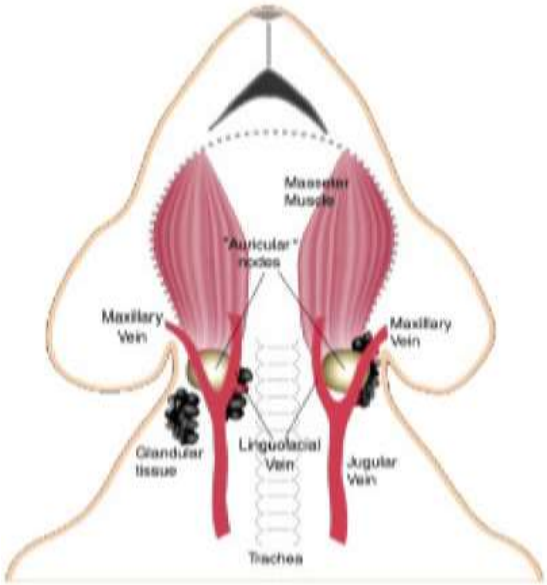

Figure 1. *Anatomical location of the auricular lymph nodes (adapted with permission from ICCVAM, 2001).*

Genotyping of WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice

Although being on the C57Bl/6 genetic background (with black coat color), the genotype in the W^{sh} mouse strain is apparent in the phenotype since homozygous mice are white, heterozygous mice are black and white (with a white sash around the waistline) and WT mice are black, this phenomenon is caused by the inversion mutation in the c-kit gene that do not only inhibit MCs but also affects melanocytes (Grimbaldeston *et al*., 2005). Therefore, only black WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ C57Bl/6 mice were genotyped.

Genotyping was performed using a KAPA Mouse Genotyping kit (KAPA Biosystems). A 2 mm^3 mouse tail fragment, combined with 10 µl extract buffer and 2 µl extract enzyme (kit appurtenant) in a small tube filled up with PCR-water to a final volume of 100 µl, were incubated for 10 minutes at 75˚C. Thereafter, the enzymes were inactivated during 5 minutes of incubation at 95˚C. After a quick vortexing of the tube, one minute centrifugation at 13' revolutions per minute (RPM) created the DNA-containing supernatant used for amplification. Reaction component, other than 1 µl supernatant, was 10 µl master mix (5 µl forward, reverse and neoprimers respectively, 6 µl MgCl and 29 µl PCR water). Cycling steps followed as instructed in kit, with denaturation, annealing, and extension in different temperatures and time points, to amplify DNA. Samples were thereafter loaded to a 1 % agarose gel and run for 30-60 minutes at 200 V to separate PCR products. Results were read as follows: upper band (700 base pairs) corresponds to WT genotype; lower band (550 base pairs) to KO genotype and both bands mean a heterozygous mouse.

Histopathology of ears

To estimate microscopically visible inflammatory parameters, histopathologic evaluation was performed on all mouse ears collected at the end of the trial. Cryostatic sectioning was done to make the procedure as efficient as possible because of the project's time limitation. Ears were cut into two halves; one half of the ears were soaked in 1 ml of 30 % sucrose for 48 h before they were embedded in medium (CryoMount), and quick frozen on dry ice. Subsequently, the embedded tissues were sectioned in a Cryostat at 8 µm slices and transferred onto microscope slides (Superfrost® plus, Thermo Scientific). The sectioned tissues were stained with hematoxylin-eosin (H&E) or TB to study cellular infiltration. Slides were digitally captured for presentation using NIS-Elements Microscope Imaging Software (Nikon) in a Nikon Eclipse 90i microscope.

Inflammatory cell counts

Lymph node cell counts

Each auricular lymph node (draining the ears) was mixed with 600 µl phosphate buffered saline (PBS) into a suspension. Lymph nodes from all of the treated mice in the trial plus one lymph node from each of the 15 control animals was analyzed. In the first experiment on W^{sh} animals, 100 µl lymph node cell suspensions, diluted 1:5 to 1:20 depending on cell density and containing 50' to 100' cells, were loaded into the funnel of the cytospin glass container. In the second and third experiments, 200 µl lymph node cell suspensions diluted 1:30 for vehicle side and 1:60 for MC903 side, were loaded into the funnel of the cytospin glass container. The container had a microscope slide and a punched filter paper mounted to it, with the funnel pointing towards the punched hole in

the filter paper on the glass slide. Glass containers were spun in a cytospin centrifuge for 5 minutes at 700 RPM, causing the fluid to be absorbed in the filter paper and the cells to attach on the microscope slide.

All slides were subsequently stained with May-Grünwald-Giemsa solution. Thereafter, manual cell counts were performed on the whole surface containing cells, using a light microscope (Nikon Eclipse E200) at 400x magnification. In the results, lymph nodes were divided into groups by genotype (WT and $^{+/-}$ or $^{-/-}$), and by treatment (MC903 or vehicle).

Cell counts in ear histological slides

Inflammatory cell counts in ears were made using light microscopy (Nikon Eclipse E200) at 400x magnification of the histological slides. Numbers of cells were manually counted in five to twelve fields in each slide; results presented as individual means in genotype groups. All slides were blinded from individual markings during cell counting. Neutrophils were counted in slides stained with H&E and MCs were counted in slides stained with TB.

Methods to analyze inflammatory mediators

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) is a quantitative biochemical analyzing method, using antibodies linked to enzymes to detect and sometimes quantify specific substances in liquids. In this study, quantitative analysis of TSLP and IL-33 were performed.

Thymic stromal lymphopoietin

As one of the inflammatory parameters in this study, TSLP was chosen because of its connection with human and canine AD, described earlier. To analyze quantities of TSLP in serum and ear tissue, a commercial ELISA kit was used. For preparation of protein suspension used in the ELISA, the other halves of the mouse ears were deep frozen with liquid nitrogen and crushed into a fine powder. The powder was solved in 500 µl PBS with 1 % Triton-X; divided into two eppendorf tubes in which one of them, proteinase inhibitor (PI) was added to prevent enzymatic digestion (Complete, Mini PI cocktail Roche). All tubes were held on ice for 2 hours; centrifuged for 10 minutes at 13’ RPM, thereafter both supernatants and pellet were frozen in -20˚C.

Ear suspension supernatants in duplicates and diluted 1:20 were used for measuring local TSLP-levels using mouse TSLP ELISA Ready-SET-Go! (eBioscience’s), according to instructions and with materials that followed the kit. Ear suspensions from five MC903-treated Wsh$^{+/-}$, six Wsh$^{-/-}$ mice and five non-treated controls in both genotypes were analyzed, as well as 14 WT/mMCP-4$^{+/-}$ mice and six mMCP-4$^{-/-}$ mice. The plates were read in a wavelength of 450 nm (A_{450}) to produce optical density (OD) values, subsequently converted into concentrations using a standard curve. With the same kit, TSLP-levels were also analyzed in serum (diluted 1:7) obtained at day 2, 6 and at the end of trial.

Interleukin 33

The concentration of the cytokine IL-33 was analyzed in ear suspensions because of its previously described association with AD as well as its potential as an in vivo substrate for chymase (Waern *et al*. 2013; Roy *et al*., 2013). Ear suspension supernatants in duplicates, diluted 1:20, from ten WT/mMCP-$4^{+/-}$ mice and ten mMCP-$4^{-/-}$ KO mice were analyzed using a DuoSet mouse IL-33 ELISA Development kit (R&D Systems). Manufacturer's protocol was followed, using supplied materials following the kit.

Cytokine array

A commercial kit, Mouse Cytokine Array Panel A (R&D Systems, Inc.), containing duplicate spots of capture antibodies directed against 40 different inflammatory cytokines and chemokines, was use to detect the mediators that are locally present after induction of AD with MC903.

Ear suspensions from twelve chymase competent or - deficient mice were chosen, due to their high TSLP concentration as a marker of inflammation. Four mice had WT genotype, four mice mMCP-$4^{+/-}$ heterozygous genotype and four mice were of mMCP-4 KO genotype. Ear suspensions with PI, 50 µl from each mouse in one genotype, were pooled into three samples with 200 µl that were used on each membrane. These samples were analyzed using Array procedure was done according to instructions appurtenant in the kit, using the kit-specific buffers and reagents. The detection of bound cytokines was mediated with paired antibodies. The secondary antibody was biotin labeled and thereafter streptavidin/dye was added. The signal was read in the Odyssey system (Li-Cor). Results were calculated as mean pixel density of the two spots, representing one cytokine or chemokine in the two genotypes. Since negative background values were removed in calculations, all cytokines with a visible bar in the figure were also present in the ear.

Enzymatic assays

Enzymatic assays are used for investigation of a specific enzymes' activity on a substrate, measuring either the consumption of substrate or the amount of products generated during a specified period of time. In this study, the activity of the neutrophil enzyme elastase and the MC enzyme tryptase were measured in terms of changes in OD-values per hour, measured in a spectrophotometer. A cleavage assay was also performed to investigate whether the MC proteases tryptase and chymase are able to use TSLP as a substrate; samples were run in a gel to visualize cleavage products.

Tryptase activity

The activity of the MC protease tryptase was measured in duplicate ear suspension samples from 20 WT/mMCP-$4^{+/-}$ and 19 mMCP-$4^{-/-}$ mice, using a chromogenic substrate (S-2288). Per well, 10 µl of ear suspension supernatant was mixed with 90 µl H_2O in a 96-well plate which was read at 405 nm. Thereafter, 20 µl of the 1.8 mM enzyme substrate (S-2288) was added, and the plate was read every 15 minutes until 2 hours, after which one final over night reading was done. Results were calculated as differences in OD-values per hour (A_{405}).

Elastase activity

To measure the local activity of the neutrophil enzyme elastase in ears, as a second parameter of neutrophil activity complementing the neutrophil counts, ear suspension supernatants from 25 MC903-treated ears (whereof eleven KO mice) and twelve vehicle-treated mice (whereof four KO mice) were analyzed. 20 µl duplicate samples from each ear suspension were incubated in a 96-well plate at room temperature, together with 20 µl of enzyme substrate (Suc-Ala-Ala-Pro-Val-pNA) in a final concentration of 1 mM. To control the salt concentration, a balanced buffer containing 150 mM NaCl 0,05 %, 100 mM TrisHCl, 0,1 % BSA and Tween-20 (pH 8,5) was added to a final volume of 200 µl per well. Duplicates with only buffer, and buffer with substrate, were used as blanks. OD values (A_{405}) were determined every 15 minutes for 2 hours. One last reading was performed after incubation over night. Elastase activity was calculated as the difference in OD-values per hour.

TSLP cleavage by chymase and tryptase

Three samples were prepared in eppendorf tubes, to study if TSLP is cleaved by the MC proteases tryptase and/or chymase:

- 0.5 µl TSLP (1 µg/µl) in 4.5 µl PBS (negative control)
- 0.5 µl TSLP (1 µg/µl) and 0.25 µl human (H) chymase in 4.25 µl PBS
- 0.5 µl TSLP (1 µg/µl) and 0.25 µl H-tryptase in 4.25 µl PBS

Tubes were subsequently incubated in 37˚C for 40 minutes. To half of the samples, 5 µl 2x Sample buffer with 1 % 1 M dithiothreitol (DTT) was added. The sample buffer contains mainly sodium dodecyl sulfate (SDS), glycerol and bromophenol blue. SDS makes proteins negatively charged proportionally to their length, thus enabling separation of the proteins in gel electrophoresis. Glycerol gives the sample buffer higher density than the surrounding running buffer of the protein gel, enabling easy loading into the gel pockets. DTT breaks disulphide bonds. Thereafter, samples were boiled at 95˚ C for 6 minutes, quickly centrifuged and loaded onto a SDS-PAGE (polyacrylamide gel electrophoresis) 4 – 20 % gradient gel for separation of sample proteins. Gel was run at 200 V for 20 minutes, and visualization done through silver staining.

The other half of the samples were sent to SciLifeLab for proteomic analyzes, using mass spectrometry to determinate possible cleavage sites for chymase and tryptase in TSLP.

In vitro *stimulation of peritoneal derived mast cells*

Peritoneal cell mast cells (PCMCs) were derived as described by Malbec *et al*. in 2007. Two WT mice were sacrificed using CO_2 and peritoneal lavage with 4 ml PBS was performed to collect cells. These were subsequently cultured in media containing stem cell factor, with half of the media replaced once every third day. After three weeks, the PCMCs were transferred into duplicate wells containing media with either MC903 (1, 5 or 10 nmol) diluted in EtOH or only EtOH (5, 25 or 50 µl) with $1x10^6$ PCMCs per ml as a concentration. Incubation with calcium ionophore was used as a positive control. After incubation for 1 hour or overnight, the cell suspension was cytospun onto glass slides and stained with May-Grünwald-Giemsa's solution. Activation of MCs was calculated

and presented as percentage degranulated MCs of total cell number per field at 400x magnification, (Nikon Eclipse E200).

Statistical analysis

Statistical analyses were performed with Prism4 (GraphPad Software) using the Mann-Whitney test to compare rank values between different genotypes. This test was chosen because the parameters analyzed in the present biological systems do not follow normal distribution. The result of tryptase activity was analyzed according to the Unpaired T-test with Welsh correction because of the substantial individual variation. P-values <0.05 were considered significant. In the figures, P-values <0.001 were shown as ***, P <0.01 as **, and P <0.05 as *.

RESULTS

The results of this study are presented in three separate parts: first, a pilot study was performed on W^{sh} mice, followed by a similar study using MCC deficient and - competent mice. A separate cleavage study that did not involve any experimental animals was also performed.

Results in W^{sh}-mice

MC903 induce visible AD-like skin changes, most apparent in MC deficient mice

Treatment with MC903 created redness on the treated ear after 3 – 7 days. These changes developed into clearly visible thickening and dryness of the skin (see Figure 2). The lesions correspond well to those seen in canine and human patients with AD. After 13 days of treatment, all MC903-treated ears had at least a clinical score of 1, which corresponds to a mild redness and swelling. In vehicle-treated ears, no visible changes were seen (clinical scoring = 0), which proves that it was MC903 that caused these changes and not the ethanol-treatment.

Figure 2. *MC903-treated ear from a $Wsh^{-/-}$ mice at day 0 and 13.*

Statistical analysis showed that the scores were not only significantly higher in MC903-treated ears compared to vehicle-treated ears, but a significantly higher score was also recorded for the $W^{sh-/-}$ genotype lacking MCs, compared to MC903-treated MC-competent mice (see Figure 3 below). This result indicates a protective role of MCs in the clinical appearance of MC903-induced dermatitis.

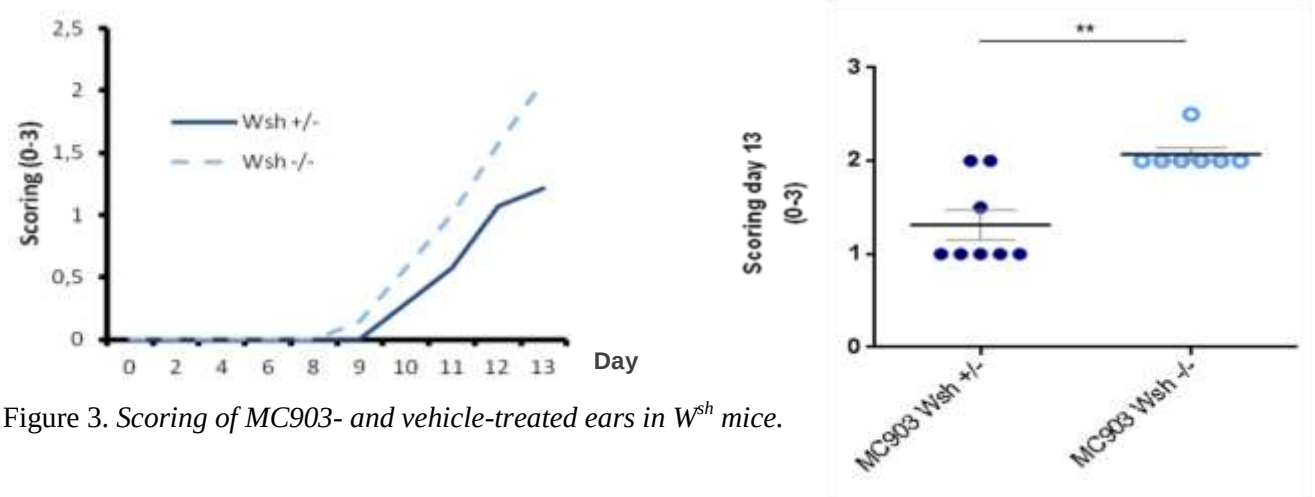

Figure 3. *Scoring of MC903- and vehicle-treated ears in W^{sh} mice.*

MC903-treated ears increase in thickness, most apparent in MC deficient mice

Ear thickness measured with an engineer's micrometer was increased in all MC903-treated ears. The first week, only a minor increase in ear thickness was seen, which developed during the last days of the trial into a considerable thickening. The highest increase in one MC903-treated ear from day 0 to day 13 was 0.57 mm (measured in a $W^{sh-/-}$ mice) and the lowest increase was 0.11 mm (measured in two $W^{sh+/-}$ mice). Compared to vehicle-treated mice, where the highest increase was 0.02 mm, did MC903 induce a significantly higher ear thickness (Figure 4). This proves that MC903-treatement induce thickening of the skin, a symptom that is also seen in atopic dermatitis. Statistical analysis between the two MC903-treated genotypes showed that ear thickness in MC deficient $W^{sh-/-}$ mice was significantly higher compared to MC competent $W^{sh+/-}$ mice (Figure 4), thus indicating a role of the MCs in limiting the ear thickening induced by MC903.

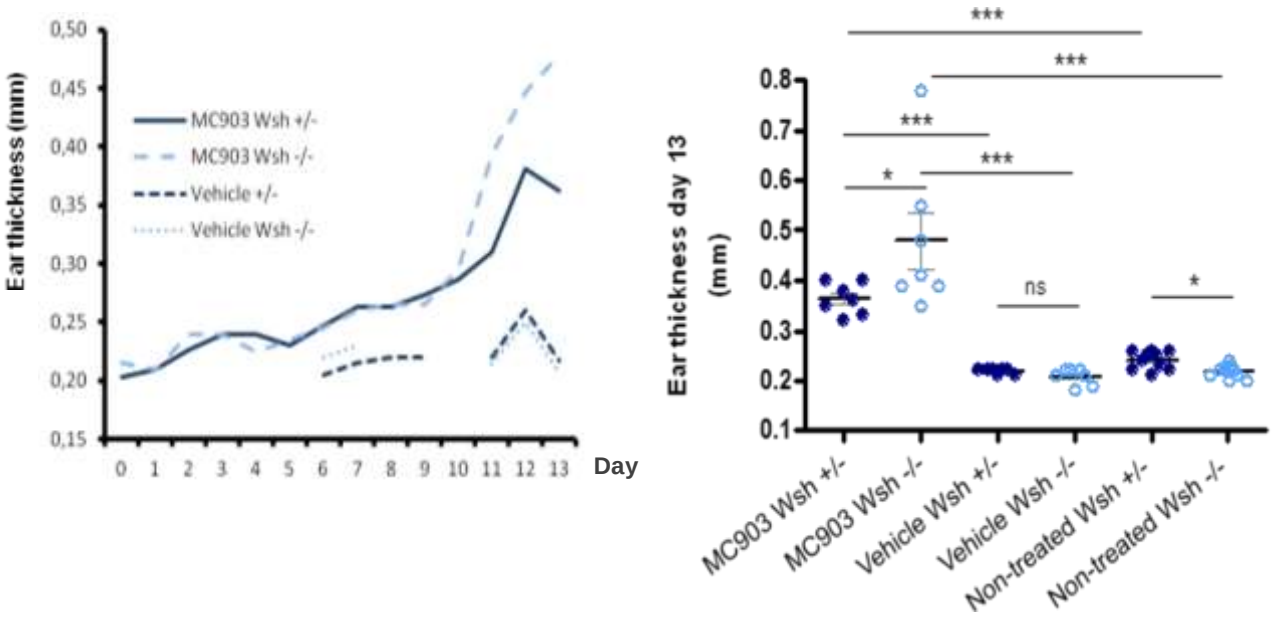

Figure 4. *Ear thickness in MC903-, vehicle- and non-treated ears in W^{sh} mice.*

Ear sections on histology slides stained in H&E (representative photographs seen in Figure 5 below), showed that the MC903-treated ears were substantially more thickened compared to the vehicle-treated ears, a result that corresponds well to the ear measurements done *in vivo*. MC903-treated ears from $W^{sh-/-}$ mice did also appear generally thicker than ears from $W^{sh+/-}$ mice, as seen macroscopically. The observation that the epidermis was thicker in ears from MC-deficient than in ears from MC-competent mice implicates that MCs might have a specific role in the epidermal thickening typically seen in lesional skin from humans and dogs suffering from AD. Histology slides stained in TB (Figure 5) show an almost complete depletion of MCs in $W^{sh-/-}$ mice and several degranulated MCs in $W^{sh+/-}$ mice.

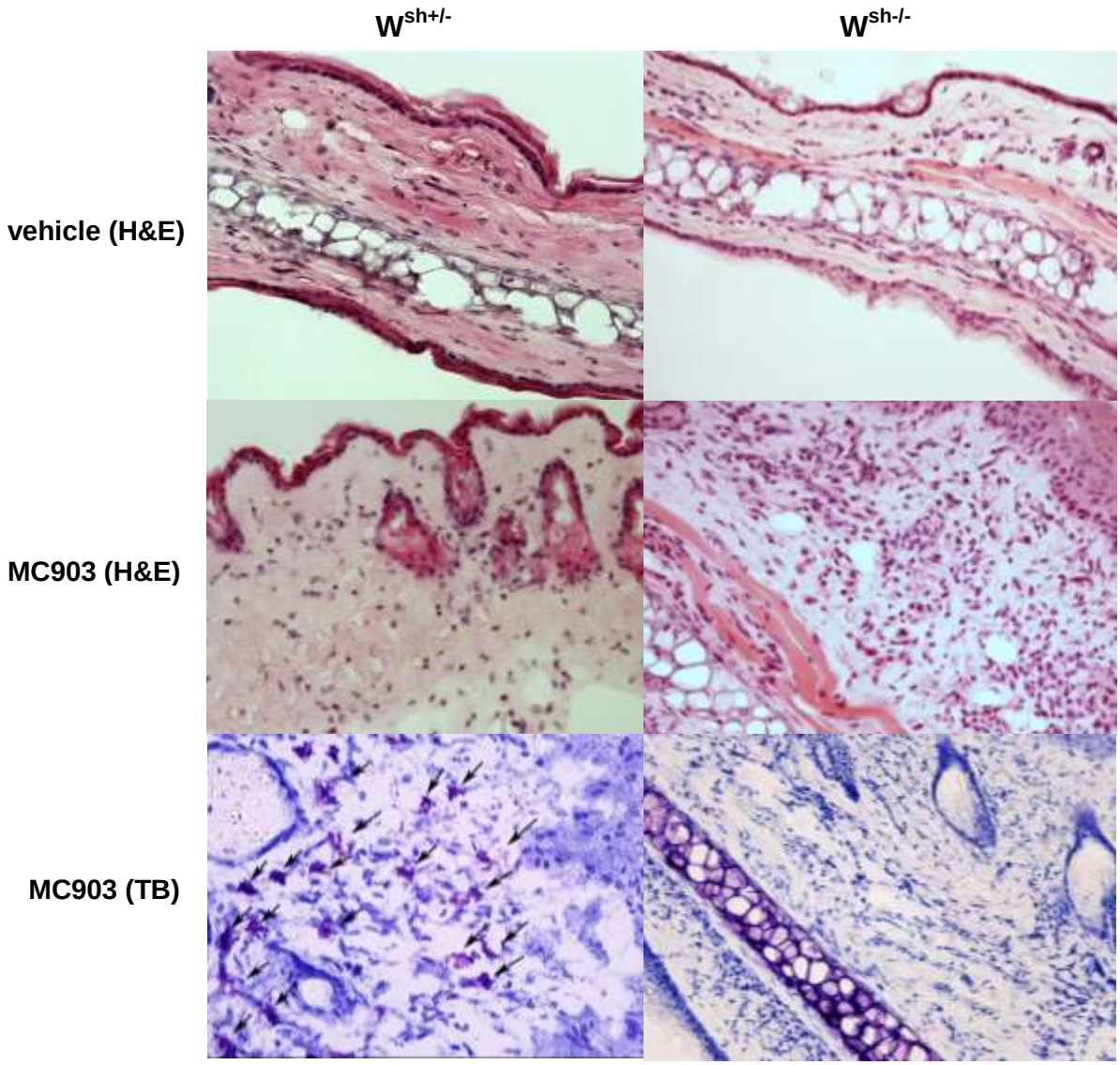

Figure 5. *Histologic sections from MC903- and vehicle-treated ears in W^{sh} mice, H&E stained in 200x and TB stained in 400x (arrows pointing to mast cells).*

More neutrophils in MC903-treated ears than in vehicle-treated ears in MC competent mice

Manual cell counts on histological slides showed a higher infiltration of neutrophils in MC903-treated ears compared to vehicle-treated ears. Statistical analysis showed a significant difference between MC903- and vehicle-treated $W^{sh+/-}$ mice (Figure 6). In $W^{sh-/-}$ mice, the difference between MC903- and vehicle-treated was non-significant. This could indicate that MCs contribute to the migration of neutrophils into the MC903-treated ear tissue. Inconclusively, no significant difference was seen between the two genotypes. This might be because of the high variability between individual mice in neutrophil migration and that some individuals reacted to vehicle-treatment with neutrophil migration. With one outlier removed, a vehicle-treated $W^{sh-/-}$ mice, the difference between MC903- and vehicle-treated $W^{sh-/-}$ mice is significant (p = *).

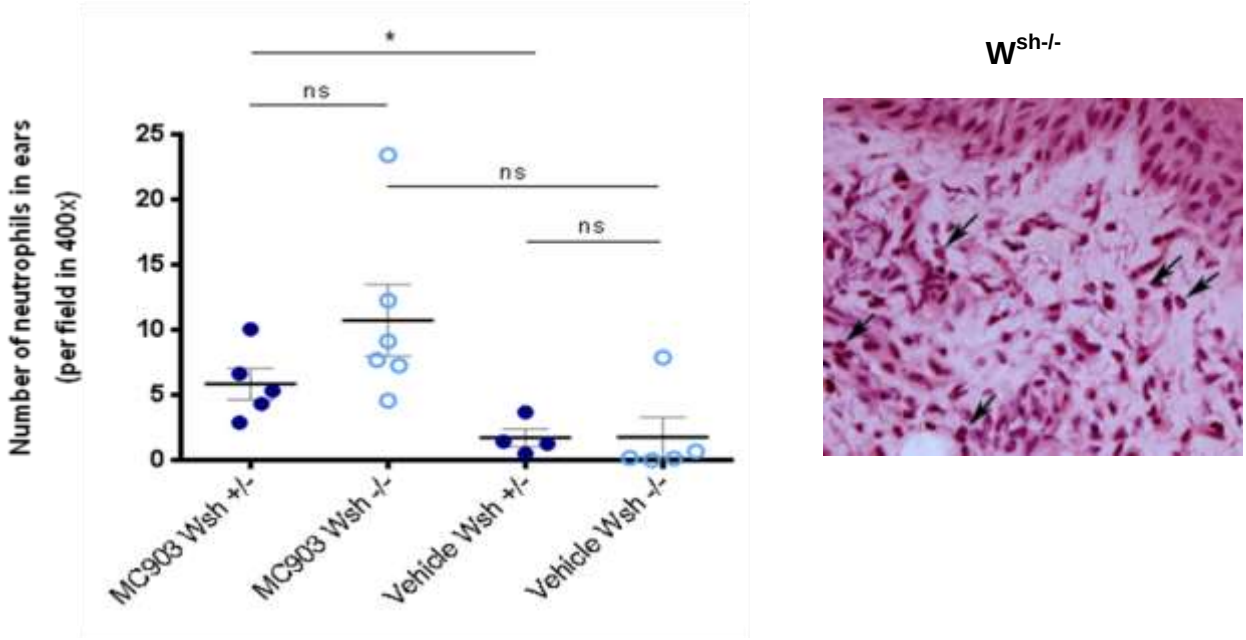

Figure 6. *Neutrophil numbers in MC903- and vehicle-treated ears in W^{sh} mice to the left, H&E stained ear from MC903-treated $W^{sh-/-}$ mouse to the right (arrows pointing to neutrophils).*

MC903 induce higher TSLP concentration in MC competent mice than in MC deficient mice

The ELISA kit for TSLP showed that MC903 induced increased concentrations of the AD-associated cytokine TSLP, in both serum and ear suspensions. Locally in the ears, MC903-treatment induced a 15 to 50-fold increase in TSLP compared to vehicle-treated ears, a statistically significant increase in both $W^{sh+/-}$ and $W^{sh-/-}$ mice. Not only was there statistically significance between MC903-treated and vehicle-treated ears, but the increase in TSLP concentration was also significantly higher in MC903-treated $W^{sh+/-}$ compared to MC903-treated $W^{sh-/-}$ mice. Data is shown in Figure 7.

At day 6, serum concentrations of TSLP were very low; no significant difference was seen between the two genotypes or compared to non-treated controls. At day 13, concentrations were two to four times higher than in the non-treated controls. Statistical analysis showed that $W^{sh+/-}$ mice had significantly higher amount of TSLP in their serum, compared to $W^{sh-/-}$ mice (Figure 7). These findings suggest that MCs have an important part in boosting the TSLP-production in this AD-model.

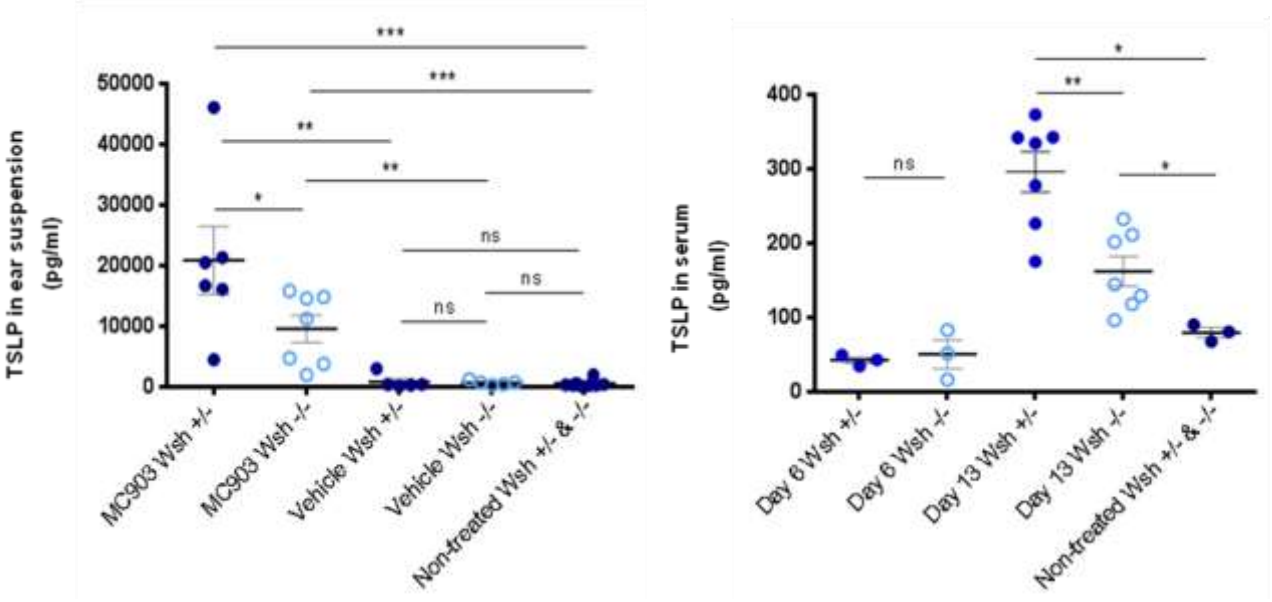

Figure 7. *TSLP in ear suspension and serum in W^{sh} mice.*

Higher amount of IL-33 is induced by MC903 in MC competent mice compared to MC deficient mice

The results from the ELISA-kit for IL-33 show that MC903-treated ears from $W^{sh+/-}$ mice contained significantly higher concentrations of IL-33 compared to MC903-treated ears from $W^{sh-/-}$ mice (see Figure 8). This supports the hypothesis that MCs is important in driving the production of IL-33.

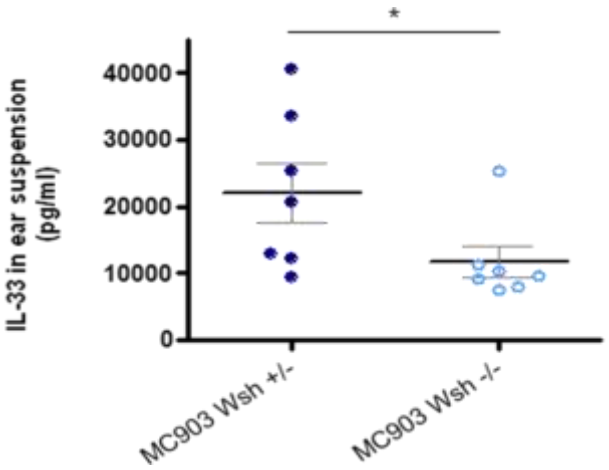

Figure 8. *IL-33 in ears from MC903-treated W^{sh} mice.*

Results in WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice

To investigate the role of MCC in this AD-model, the same kind of study was performed on WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice. Results from WT and mMCP-4$^{+/-}$ mice were presented together as one group since this did not affect the outcome of any of the statistical analysis performed.

Genotyping results shows mendelian randomization

Among the 41 mice genotyped, eleven mice were of WT genotype, eleven of KO genotype (mMCP-4$^{-/-}$) and the rest, 19 mice, of mMCP-4$^{+/-}$ genotype (Table 2). This result is close to a perfect mendelian randomization, which confirmed that the breeding is functioning as expected.

MC903 induce visible AD-like skin changes, in WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice

Ear-treatment of MC903 led to similar changes in WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice as in the W^{sh} mice: redness, flaky skin and ear thickening, seen in Figure 9 below.

Figure 9. *MC903-treated ears from one mMCP-4 mice at day 0 and 13.*

As in the group of W^{sh} mice, all MC903-treated ears had a clinical score of at least 1 at the end of trial whilst no visible changes were seen in vehicle-treated ears. Statistically, no significant differences were seen between the different genotypes, WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ (Figure 10). This shows that MC903 induce an AD-like phenotype without a critical involvement of the MC protease chymase.

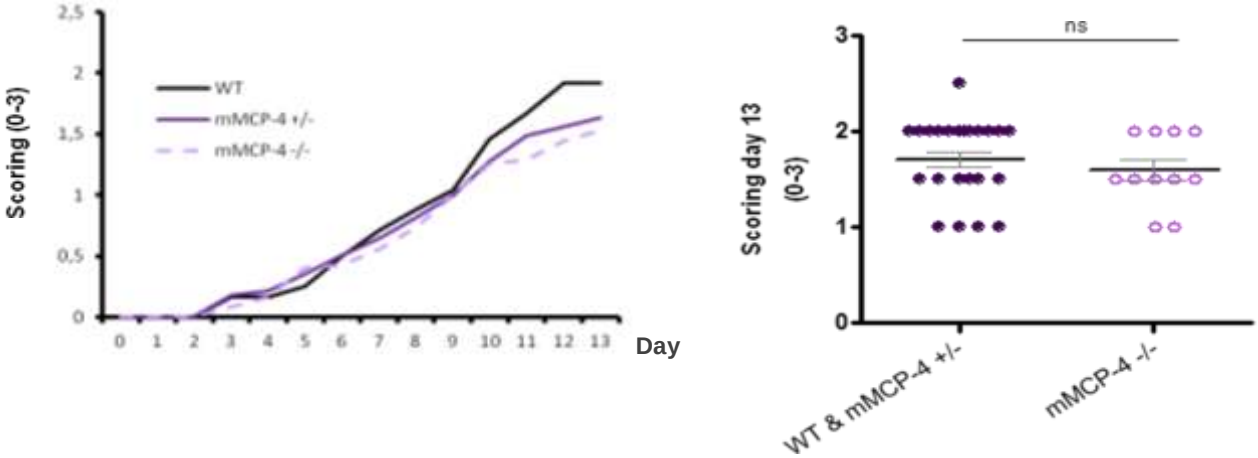

Figure 10. *Scoring of MC903-, vehicle- and non-treated ears in WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

MC903 induce an increase in ear thickness in WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice

Ear thickness was significantly increased by MC903-treatment in all genotypes, compared to both vehicle- and non-treatment. As seen in the W^{sh} mice, the increase in ear thickness in the WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice was mild until about one week into the trial, from where the ear thickness increased in a higher pace. No difference was seen between the two genotypes (Figure 11). This shows that the ear thickness induced by MC903 is apparent but is not influenced by MCC.

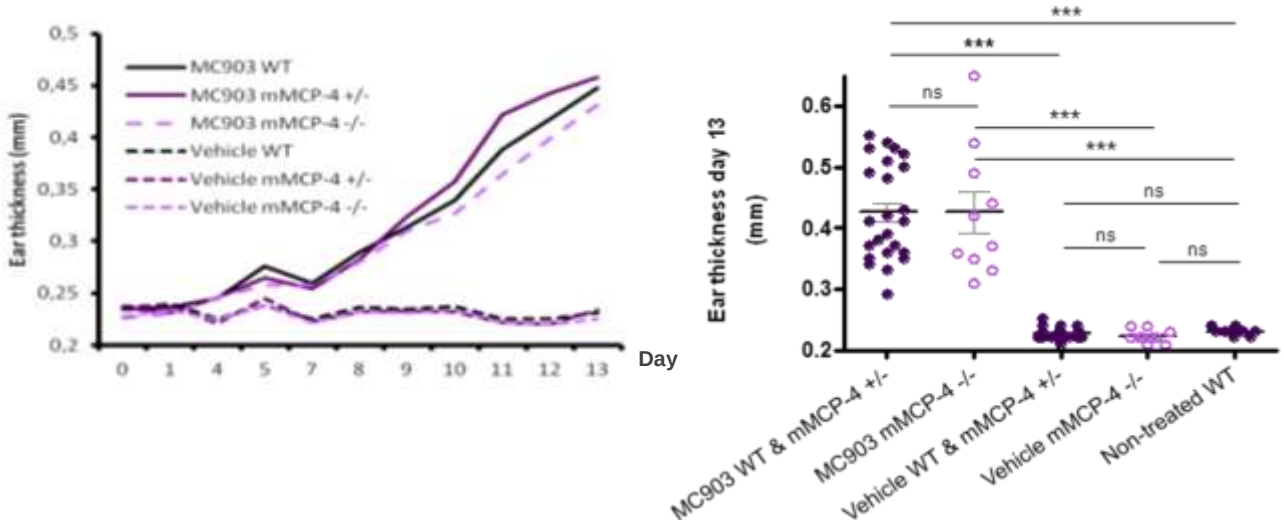

Figure 11. *Ear thickness in MC903-, vehicle- and non-treated ears in WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice.*

In ear sections on histology slides stained in H&E from mMCP-4 mice, seen in Figure 12 below, MC903-treated ears were substantially more thickened compared to the vehicle-treated ears, similar to the result seen in W^{sh} mice (Figure 5) and well corresponding to the ear measurements done *in vivo*. No apparent difference was seen between the genotypes.

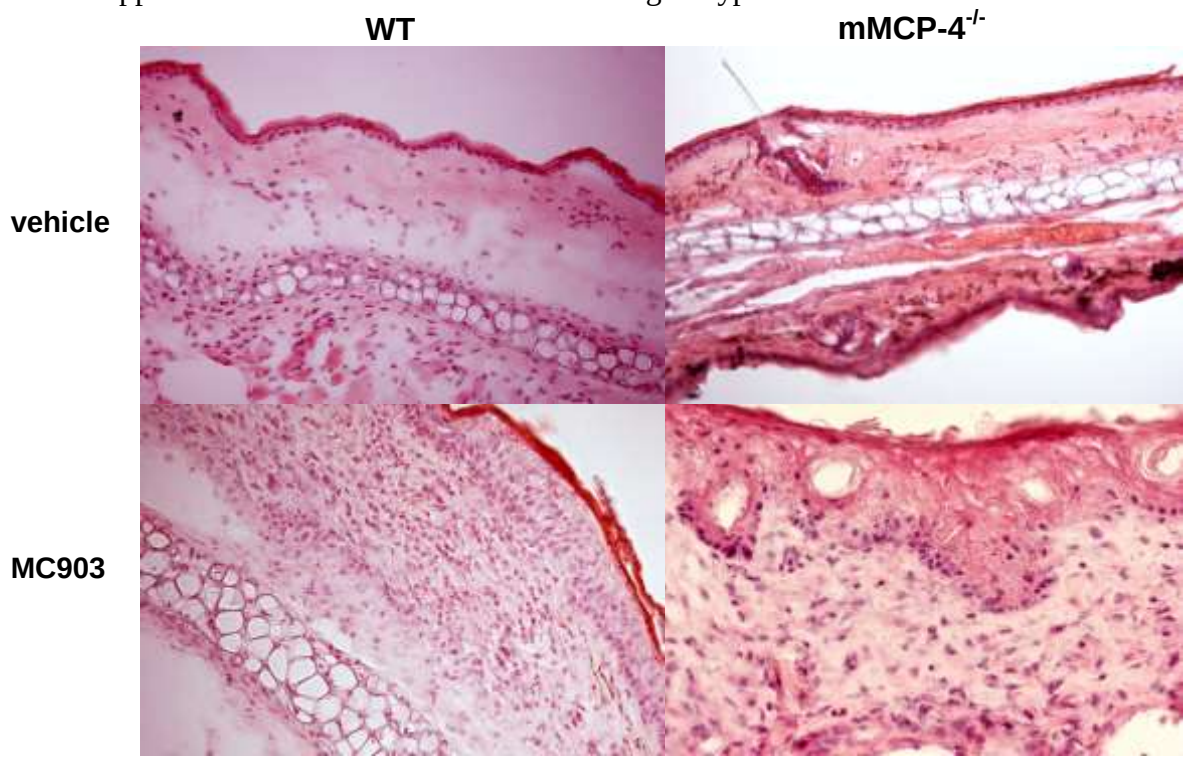

Figure 12. *Histologic sections in 200x from MC903- and vehicle-treated ears in WT and mMCP-$4^{-/-}$ mice.*

Topical application of MC903 induces MC migration to auricular draining lymph nodes

At the end of trial, the auricular lymph nodes were collected. Visually, the left sided lymph nodes (draining the MC903-treated ears) were clearly enlarged compared to the lymph nodes on the right side (draining the vehicle-treated ears). Manual cell counts in cytospun suspension from the auricular lymph nodes, showed no significant difference between the genotypes, whether MC903-treated or vehicle-treated (Figure 13), which indicates that the absence of chymase do not affect the migration of MCs in this model. However, in all genotypes (WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$) did MC903-treatment significantly increase the number of MCs in the ear draining lymph nodes, compared with vehicle-treatment (Figure 13) thus proving that this AD-model involves MCs.

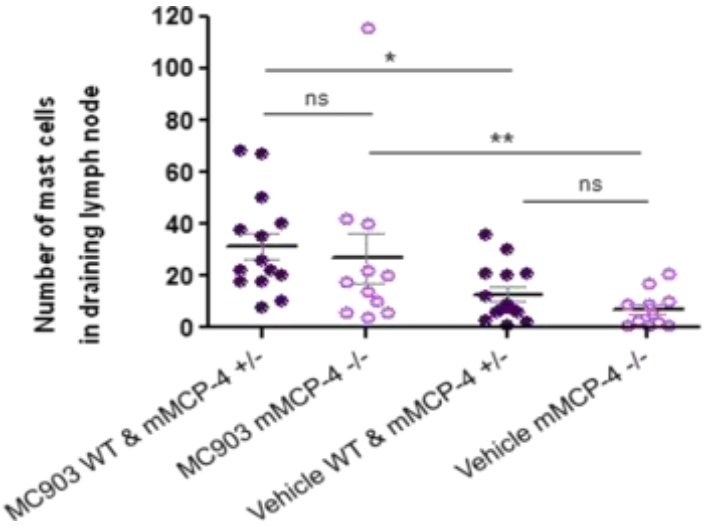

Figure 13. *MC count in draining lymph node of MC903- and vehicle-treated ears in WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice.*

MC903-treated ears have higher numbers of MCs than vehicle-treated ears

Significantly higher numbers of MCs was seen in MC903-treated ears compared to vehicle-treated ears (Figure 14), which shows that the inflammation started by MC903 involves MCs. The percentage of degranulated and thus activated MCs was also higher in MC903-treated ears (Figure 14), thus proving an activity of the MCs due to MC903-treatment. No significant difference was seen between the WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice which further add to the conclusion that MCC do not have an indispensible role in this MC903-induced AD-model.

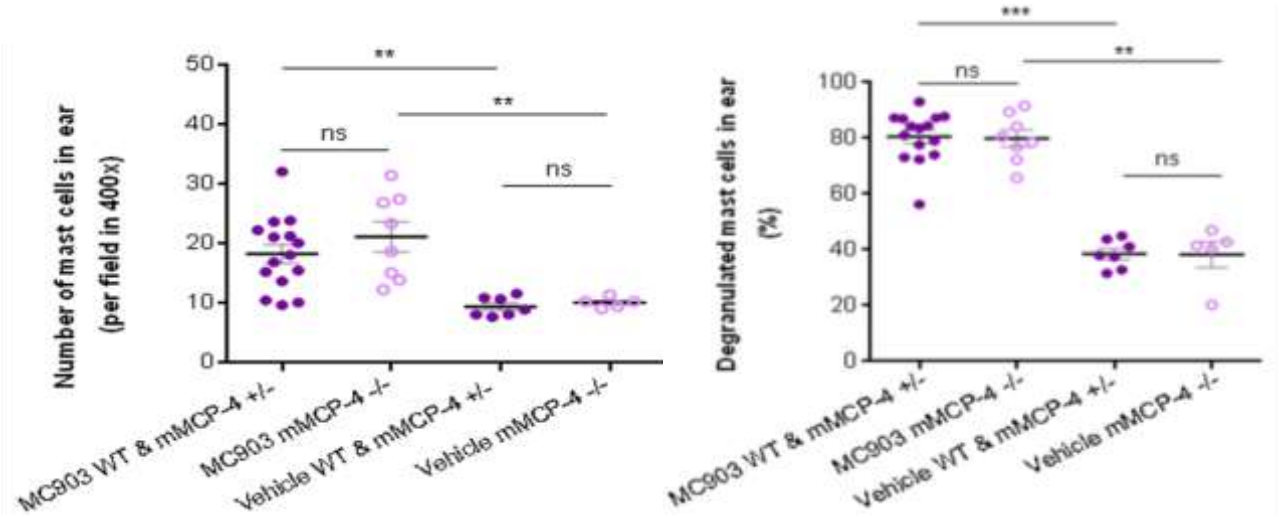

Figure 14. *MC count and degranulation in MC903- and vehicle-treated ears in WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

Tryptase activity is higher in MC903-treated ears than in vehicle-treated ears

A markedly high individual variance was observed in the activity of the MC protease tryptase; therefore, statistical analyze was performed using Unpaired T-test with Welch correction. This showed a significantly higher activity in both genotypes treated with MC903 compared to vehicle-treatment (Figure 15). This confirms MC activity in this AD-model and suggests that tryptase might have an important role in the inflammation. No significant difference was seen between the genotypes, an expected result as the lack of chymase should not affect the tryptase activity.

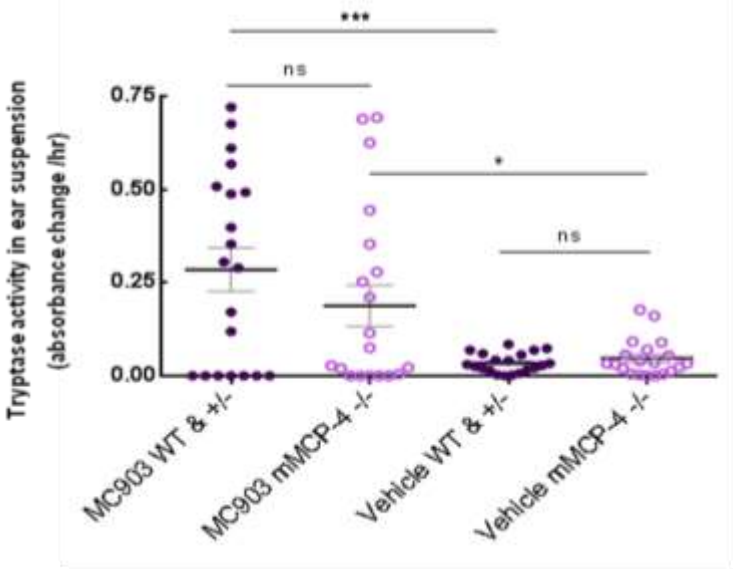

Figure 15. *Tryptase activity in MC903- and vehicle-treated ears in WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

More neutrophils and higher elastase activity in MC903-treated ears compared to vehicle-treated ears in MCC competent mice

Manual cell counts in ears sections stained with H&E, showed significantly higher numbers of neutrophils in MC903-treated WT, mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice compared to vehicle-treated ears from the corresponding genotype group (Figure 16). No significant difference was seen between the genotypes, thus proving that MC903 induce neutrophil skin migration but at the same time minimizing the likeliness of MCC being involved in the neutrophil migration.

The activity of the neutrophil enzyme elastase was significantly higher in MC903-treated WT and mMCP-$4^{+/-}$ ears compared to vehicle-treated ears from the same group (Figure 16), thus confirming that local neutrophil activity is increased as a response to MC903-treatment in MCC competent mice. Neutrophil activity was not significantly higher in MC903-treated mMCP-4 KO mice than in vehicle-treated mice of same genotype and no significant difference was seen between the two genotypes in neither MC903-treated nor vehicle-treated ears. There is however one outlier in the MC903-treated mMCP-$4^{-/-}$ genotype group, with much higher elastase activity than the other individuals in the same group. If this value is excluded, the difference between the two MC903-treated genotype groups is significant (*), thus giving a weak connection between MCC and neutrophil migration.

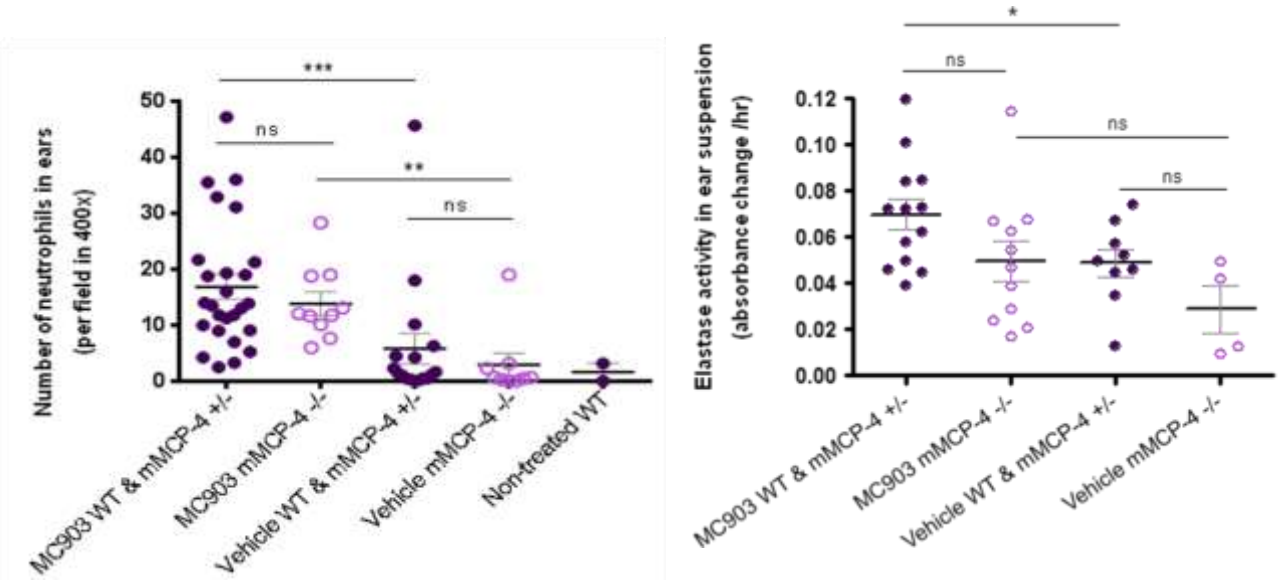

Figure 16. *Neutrophil numbers and elastase activity in MC903- and vehicle-treated ears in WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice.*

MC903 induce increased TSLP concentration in ear suspension and serum

In all of the genotypes analyzed (WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice), MC903-treated ears contained significantly more TSLP compared to vehicle-treated ears (Figure 17), a result that adds to the proof of TSLP being an important cytokine in driving the MC903-induced inflammation, mimicking AD. No difference separated mMCP-4 KO mice from the other genotypes; no effect of chymase on the TSLP-levels could therefore be detected.

Similar results were seen in serum. TSLP-levels were significantly higher in all MC903-treated mice on day 6 and 13, irrespectively of genotype, compared with serum-levels in non-treated mice (Figure 17). This result concludes that MC903 also induce systemically effects in the treated mice. On day 2, TSLP-levels were very low and mMCP-$4^{-/-}$ mice did not have significantly higher levels than non-treated mice. The highest levels of TSLP were detected on day 6, resulting in significant differences in both genotype groups compared to corresponding values seen in day 2 and 13. No significant differences were seen between the genotype groups, further adding to the conclusion that chymase is not needed for the MC903-induced increase in TSLP.

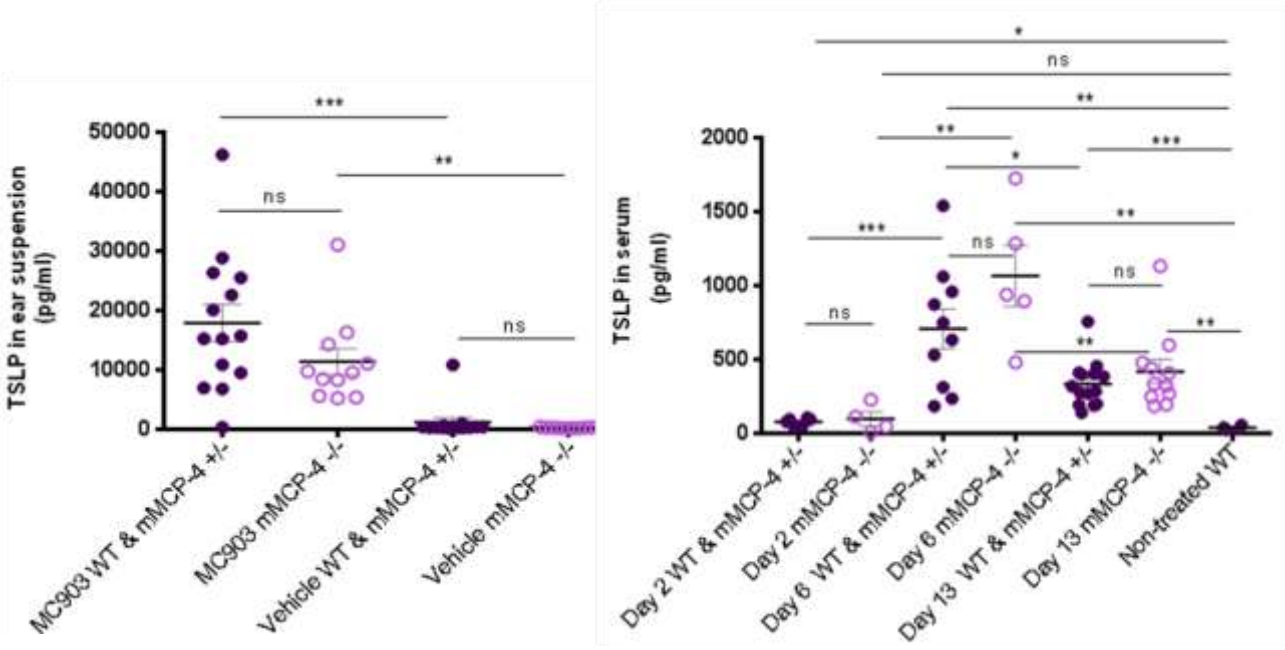

Figure 17. *TSLP in ear suspension and serum from WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

Higher amount of IL-33 is induced by MC903 in mice lacking chymase compared to WT mice

Significantly higher amounts of IL-33 were observed in the ears of mMCP-4$^{-/-}$ mice than in ears from WT and mMCP-4$^{+/-}$ mice (Figure 19), but with a lower concentration in MC-deficient mice., indicating that MCs either produce significant amount of IL-33 or promote the production of IL-33 in AD (Figure 8), the MC protease chymase seems to have a role in lowering the concentration of IL-33, probably through cleavage of the cytokine.

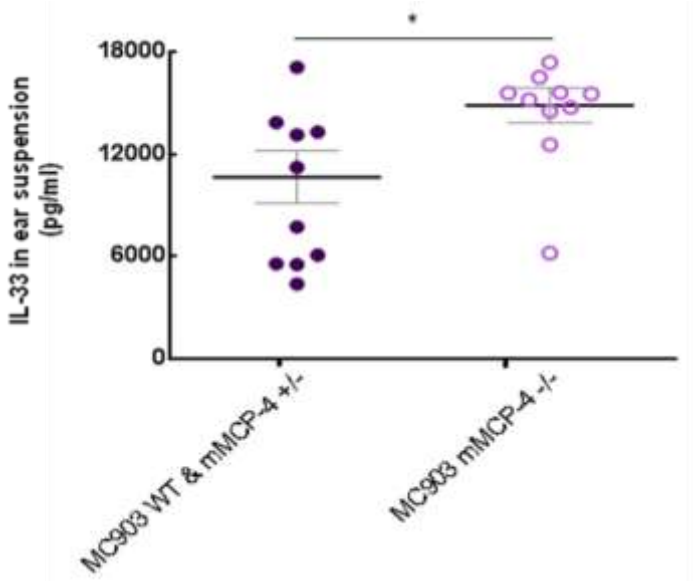

Figure 18. *IL-33 in MC903-treated ears from in WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

MC903 induce local production of a wide range of inflammatory cytokines

A qualitative cytokine array was performed, investigating which cytokines that were produced in ears following MC903-treatment. The result shows a massive production of inflammatory mediators, seen in Figure 19. Previously mentioned cytokines associated with Th-2 immune responses that were present in this AD-model are IL-4, IL-5, IL-6 and TNF-α, thus proving that the inflammation includes Th-2 cytokines; IL-13 is also present but not as obvious as the others. IL-12, an important Th-1 cytokine, is the only cytokine in this array that shows no activity, further implicating a predominant Th-2 milieu. One Th-1 associated cytokine, IFN-γ, is present, however with an indistinct result (low mean pixel density). The other cytokines present in the array will not be discussed in this study, because of the limited duration of the project. The information will instead be used in following research.

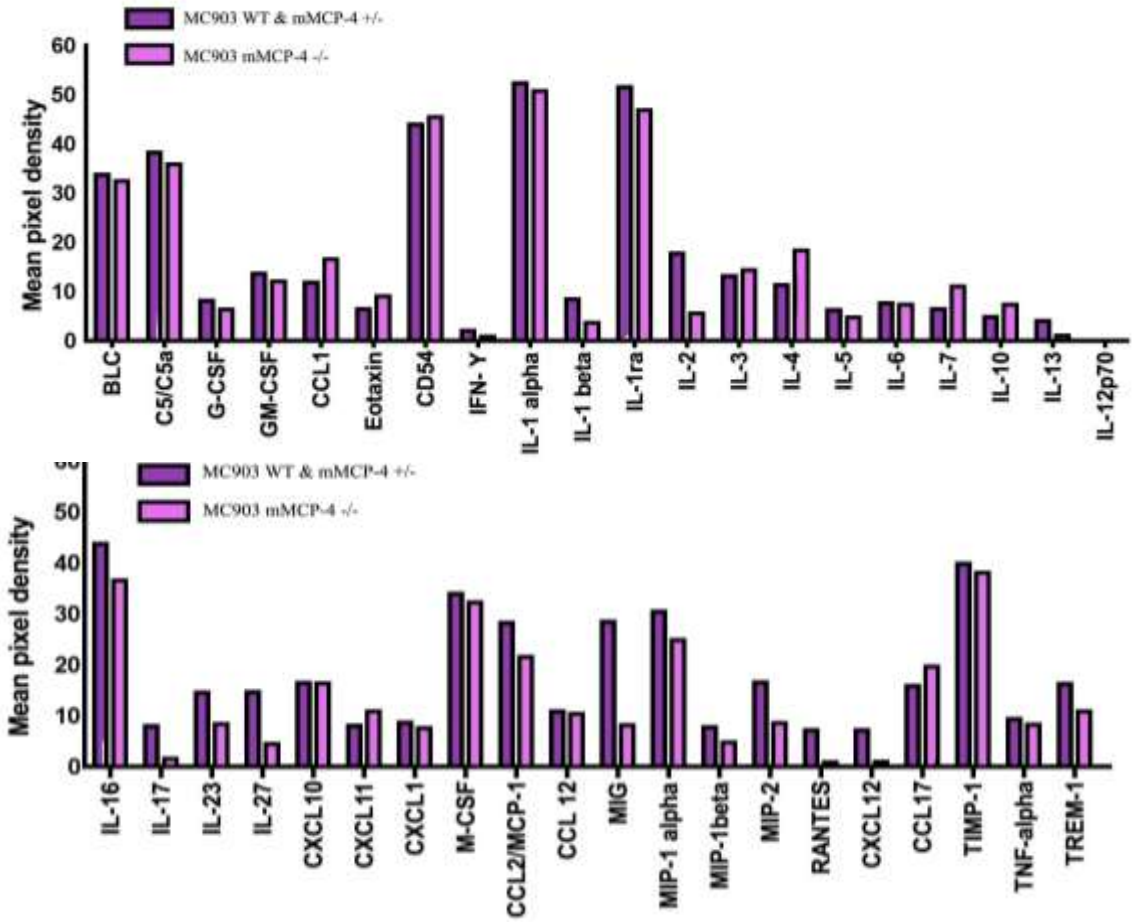

Figure 19.*Cytokines and chemokines in MC903-treated ears in WT/mMCP-4$^{+/-}$ and mMCP-4$^{-/-}$ mice.*

Results in cell culture

In vitro stimulation of peritoneal derived mast cells

MC903 cause degranulation of PCMCs in higher extent than vehicle

PCMCs incubated with MC903 did degranulate in significantly higher percentages compared to cells incubated with ethanol (EtOH), which was used as a vehicle in the *in vivo* part of present study (Figure 20). An average of 28 % of the PCMCs were degranulated after one hour of incubation and a mean of 50 % degranulated PCMCs were seen after overnight incubation, using the same amount of MC903 as in the *in vivo* study.

Higher amounts of MC903 (5 and 10 nmol) caused close to 100 % of the PCMCs to degranulate, but were also toxic as many of the cells died. PCMCs incubated with 95 % EtOH were degranulated in an average of 15 – 20 % regardless of amount (5, 25 or 50 µl) and time (1 hour or overnight), with one exception; PCMCs incubated with 50 µl EtOH overnight degranulated in a percentage close to 50, but this was still significantly lower than PCMCs incubated with high amount of MC903 overnight.

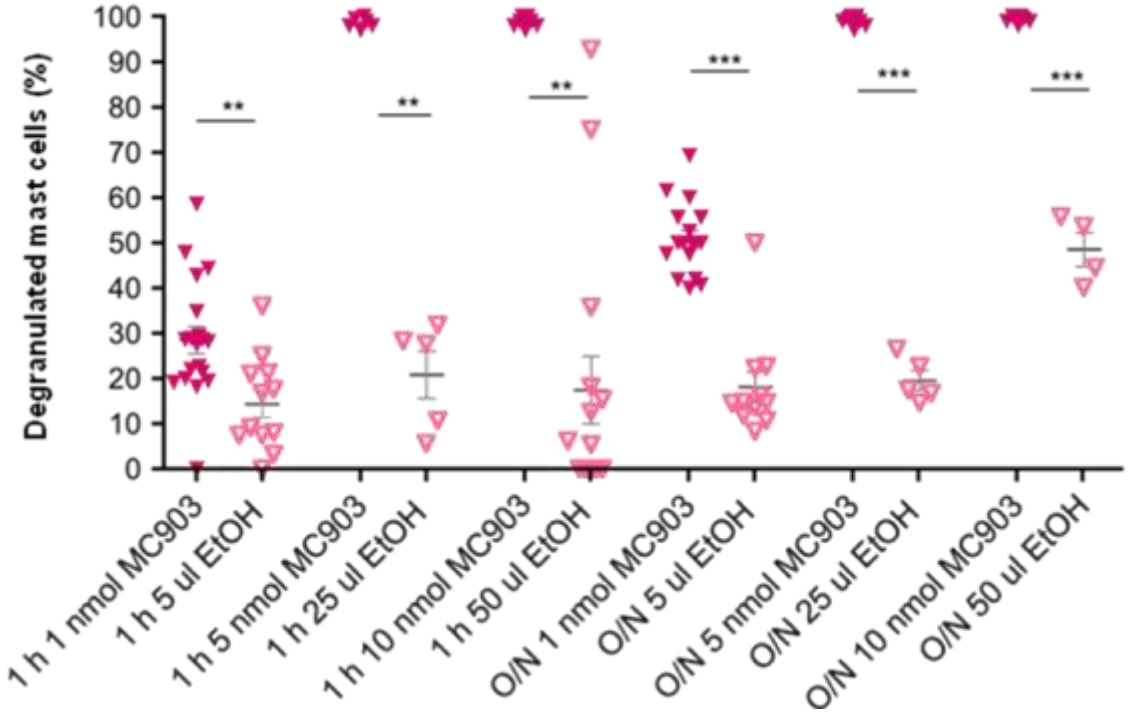

Figure 20. *Percentage of degranulated PCMCs incubated with MC903 and ethanol.*

Results of cleavage study

TSLP cleavage by chymase and tryptase

TSLP is cleaved to higher extent by tryptase than by chymase

The cleavage study, where TSLP was incubated for 40 minutes together with two different human MC proteases, showed that TSLP was cleaved at a higher extent by tryptase than by chymase. In Figure 21, cleavage is visualized by silver staining where 1 = molecular-weight size marker, 2 = TSLP + chymase, 3: TSLP + tryptase and 4 = negative control (only TSLP). As shown in Figure 21, the protein band in 2 is somewhat brighter than in 4, corresponding to a minor cleavage of TSLP by chymase. Number 3 shows a total cleavage of TSLP by tryptase. Thus, tryptase does degrade TSLP rapidly and completely, whilst chymase during a shorter period of time does minimal degradation of TSLP.

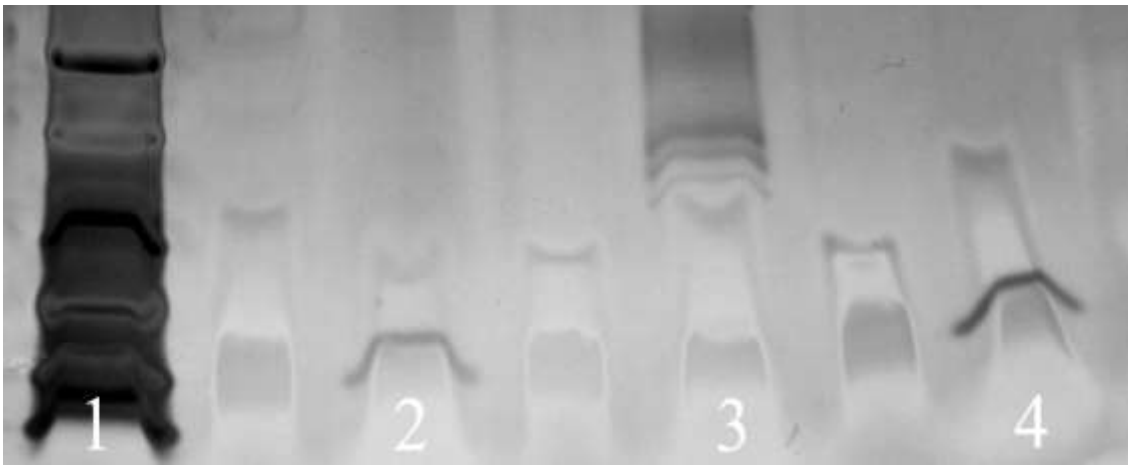

Figure 21. *Photography of a gel ran in the TSLP cleavage study with chymase and tryptase.*

These results are in agreement with those received from SciLifeLab performing proteomic analysis, showing that the MC protease tryptase had several potential target sites in TSLP, whilst chymase only had one (data not shown).

DISCUSSION

The aim of this study was to establish an AD mouse model, new to the laboratory. The use of a low-calcemic vitamin D_3 analog (MC903) on KO mice, deficient in MCs ($W^{sh-/-}$, Grimbaldeston *et al.*, 2005) and MCC (mMCP-$4^{-/-}$, Tchougounova *et al.*, 2003), to create an AD-like phenotype gave the opportunity to investigate the role of MCs and MC mediators in the clinical and biochemical progression. Analyzes performed during this study showed that MC903 induced a number of inflammatory changes in both W^{sh} and mMCP-4 (WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$) mice, similar to those seen in human and canine AD-patients thus confirming the use of MC903 to create a functioning mouse model.

There are some rather important aspects that separate this model from other AD-models. Tanaka *et al*. (2012) reviewed different AD animal models, induced either by an intravenous injection of IgE anti-haptens prior to antigen challenge, or by tape stripping prior to repeated allergen challenges. These methods have a more complicated performance, are more time consuming and do cause a substantially higher level of stress in the treated mice. Inducing dermatitis with one drop of MC903 on each side of the ear is a simple procedure that the mice get used to fairly quickly. No obvious protests are seen until only the last couple of days, when more apparent changes are visible. Therefore, the MC903-induced mouse model for AD is a non-expensive and simple method with minimal negative effects on the mice.

In most of the results produced in this study, MC903 was shown to induce changes mimicking a naturally occurring AD. Macroscopically, redness, dermal thickening and flaky skin was seen, which corresponds well to the early changes seen in humans and dogs suffering from AD (Marsella & Girolomoni, 2009). If pruritus, the most prominent symptom of human and canine AD, is evoked by MC903, was however not clarified in this study, since available methods used to measure scratching are too advanced and expensive for this kind of short term project. For example, Tanaka *et al*. (2012) describes a method with a high-speed camera connected to a computer program, but other methods are far more invasive, such as surgically insertion of a magnet into the hind paws of the mice done by Terakawa *et al*. in 2008. During the course of the MC903-treatment in this project, more scratching behavior was seen in the last period before the endpoint of the study. Without any measurement of this parameter, no conclusion concerning itch could be done. Since the study was ended before any mice showed severe changes, none of the later changes in AD, such as hyperpigmentation was seen. Surprisingly, mice lacking MCs reacted significantly higher to MC903-treatment in both scoring and ear thickening, thus implicating a protective role of MCs against clinical development. MCC cannot be accounted for this function, since no significant clinical difference was seen between the WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ genotypes. Knowing that MCs have the ability to limit inflammation, for example via the negative effects of IL-10 on the secretion of pro-inflammatory cytokines (Tsai *et al*., 2011), it would be interesting to further evaluate the levels of IL-10. To ensure that this limiting effect by MCs truly exists, at least two more separate experiments in W^{sh} mice would be necessary. Because of the short period of time, it was not possible to perform any more trials within this project, but continued studies on this subject are recommended.

Microscopically, epidermal thickening with inflammatory cell infiltration to a varying degree was seen in all MC903-treated ears of the present study ($Wsh^{+/-}$, $Wsh^{-/-}$, WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$), similar to the typical changes known to exist in naturally occurring AD (Olivry *et al.*, 1997). Histological examination did not show a spongiotic type of dermatitis, typically seen in skin from AD patients. This might be due to the fact that AD in humans and dogs is associated with inherited immunological traits creating a microenvironment with Th-2 character and impairing the skin barrier, leading to widened intracellular spaces in the stratum corneum called spongiosis (Kamsteeg *et al*. 2011). Therefore, mice without these genetic immunological traits will not exhibit those specific alterations, even though an AD-like phenotype is seen.

MC903-treatment did significantly increase the number of neutrophils in the ears of WT, mMCP-$4^{+/-}$, mMCP-$4^{-/-}$ and $W^{sh+/-}$ mice compared to vehicle-treated mice. In $W^{sh-/-}$ mice, the increase was however not significant. This result could be interpreted as a role of MCs in the migration of inflammatory cells in this AD-model, but since no significant difference in neutrophil numbers could be seen between $W^{sh+/-}$ and $W^{sh-/-}$ mice, no definitive conclusion could be made. MCs are known to alter homeostasis and enhancing adhesion of neutrophils to endothelium which enables migration into the skin (Liu *et al*., 2011). Elastase was measured as an additional parameter of neutrophil activity. It showed a similar result with significantly higher activity in MC903-treated WT and mMCP-$4^{+/-}$ mice compared to vehicle-treated ears, but no significance between treated and non-treated mMCP-$4^{-/-}$ mice. With one outlier excluded in the mMCP-$4^{-/-}$ genotype group, the difference between the MC903-treated WT/mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice was significant (*), thus supposing a connection between MCC and neutrophil migration. MCC is known to use Angiotensin I as a substrate why chymase also is proposed as having a role in generating Angiotensin II (Pejler *et al*., 2007). This component of the Angiotensin system has been shown to stimulate neutrophil migration by for example Elferink *et al*. (1997), hence making a role of MCs and MCC in the neutrophil migration explainable. Since these results were not all clear, a higher number of individuals should be analyzed for a more conclusive result.

MC numbers were significantly increased in lymph nodes draining MC903-treated ears, as was tryptase activity in all ears from WT, mMCP-$4^{+/-}$ and mMCP-$4^{-/-}$ mice, thus confirming that MC903 activates MCs and cause migration of the cells into the lymph nodes. MCs may contribute to the promotion of a Th-2 dominated environment in the lymph nodes, through production of Th-2 associated cytokines (Metcalfe *et al*., 1997). MCs might also present antigen to T-lymphocytes (although DCs are the most important APCs), which subsequently become Th cells of type 2 that are associated with humoral immunity and IgE production. The lack of chymase did not affect this studied parameter significantly, suggesting that MCC do not affect MC migration in this MC903-induced AD model. However, the potential involvement of MCC in MC migration could be further evaluated in the MC903-induced AD model with increased or lowered doses of MC903 in future projects.

Biochemical analyses also showed similar AD-like inflammatory changes due to MC903-treatment, for example the presence of Th-2 associated cytokines such as IL-4, IL-6 and TNF-α, as well as an increase of the AD-associated cytokines TSLP and IL-33. TSLP, a cytokine that functions as a

"*master switch for allergic inflammation*" according to Liu (2006), was shown to increase locally in the ears after repeated applications of MC903. In MC-competent mice, the increase was more than 65 times the levels of vehicle-treated ears and in MC-deficient mice, TSLP increased less than twelve times. The results indicate a role of MCs in driving the increase of TSLP. Although MCs are able to produce TSLP by themselves (Okayama *et al*., 2009), it is more likely that a significant part of their contribution to the TSLP-increase occurs indirectly, through production of mediators that enhance TSLP-expression in other cells, since the MCs are clearly outnumbered in the skin by epithelial cells and keratinocytes. For example, MCs are according to Tsai *et al*. (2011) able to promote the production of TSLP from epithelial cells through the cytokines IL-4, IL-13 and TNF-α. Since the TSLP-levels were similar in mice lacking MCC compared to MCC-competent mice, this protease does not seem to affect TSLP expression in MC903-induced AD.

The result of ELISA for IL-33 shows that MC903-treatment induces a local increase of the cytokine, with significantly higher levels in MC-competent mice. This is probably due to similar reasons as to why TSLP-levels are higher in mice with than without MCs, namely an indirect activation of IL-33 by epithelial cells through mediators produced by MCs. Since the numbers of MCs is comparatively low, it is less plausible that the loss of MC-produced cytokines would cause a significant difference. IL-33 was also increased in WT and mMCP-$4^{+/-}$ mice after MC903-treatment, although not significantly. These results are similar to those produced by Hueber *et al*. in 2011, where MC-deficient mice ($W^{sh-/-}$) had lower levels of IL-33. Interestingly, MCC-deficient mice had significantly higher levels of IL-33, which implies a limiting role of chymase, most likely due to cleavage of the cytokine. Recently, it was shown that IL-33 is in fact cleaved by chymase (Roy *et al*., 2013), thus confirming this hypothesis. Since the clinical parameters in the mice lacking mMCP-4 were not significantly different to mMCP-4 competent mice, the effect by chymase on IL-33 levels does not seem to be of major importance in this MC903-induced AD-model.

One question raised when using a KO mouse in research, is if there are other phenotypic changes caused by genetic alteration that could affect the clinical outcome. The mMCP-$4^{-/-}$ mice have a specific mutation in the gene coding for the MC protease 4 that share functional similarities to human chymase and no other functional or morphological changes in the MCs have been seen (Tchougounova *et al*., 2003). In this study, no visible differences were seen between the mMCP-4 competent mice compared to the mMCP-4 deficient mice. $W^{sh-/-}$ mice carries an inversion mutation in the white spotting (W) locus, also called the c-kit gene, which affects melanocytes and creates a major deficiency in MCs. Grimbaldeston *et al*. (2005) showed that $W^{sh-/-}$ mice have normal levels of B- and T-cells, granulocytes, macrophages and basophils in bone marrow; as well as splenic B-cells, granulocytes, macrophages, DCs and natural killer cells. In later research, W^{sh} mice have been shown to carry some other hematopoietic alterations, such as higher numbers of neutrophil granulocytes and thrombocytes, accompanied by an enlargened spleen (Nigrovic *et al*., 2008), the later phenomenon clearly apparent in this study as well. Since no significant difference in neutrophil numbers was seen between vehicle-treated ears from $W^{sh+/-}$ and ears from $W^{sh-/-}$ mice, the generally higher levels of neutrophils in $W^{sh-/-}$ mice does not seem to affect local neutrophil numbers and therefore should not affect the results seen in this study.

To strengthen the evidence that differences seen in experimental KO mouse studies is truly caused by the specific deficiency of cells/cell mediators, it is common to use a method in which the lacking component is reinserted. Changes between the genotypes should thereby no longer be detectable. This reconstitution is achieved either by injection of cultured MCs, for example from the bone marrow, or by transplantation of the whole bone marrow from WT mice (Grimbaldeston *et al.*, 2005). Although a reconstitution of MCs in the $W^{sh-/-}$ mice would be of interest to make sure that the changes seen in this AD-model are in fact MC-dependent, it was not an option due to the time limit of this project.

As always, it is important to recognize the weaknesses in these kinds of mouse models, since a mouse is neither a human nor a dog. The present differences between species make it difficult to extrapolate any conclusions directly to humans based on these results seen in mice, especially since there are tremendous amount of molecular biology that is still not fully understood. In spite of this, mouse models do contribute substantially to this essential area of research. As new discoveries in molecular biology are made, mice may serve as a potential source of relatively cheap *in vivo* models with short generation time and the possibility to study large litter sizes. Mice are small, thus acquiring considerable less space than dogs for example, easy to handle and have enough adaptability to thrive in a laboratory environment. More importantly, methods to alter their genotype and subsequently create mouse disease models are highly developed. Studies such as this one, inducing a disease-like phenotype in KO mice lacking specific cells or cell mediators due to genetic alteration, give a unique opportunity to thoroughly investigate the role of specific cells or mediators in a disease-model. New discoveries made in mice could subsequently be further investigated in humans, to conclude if similar connections are present.

To complement all of the analyses performed in the mouse model, a small *in vitro* experiment was also performed. Since the PCMCs used in the study did only originate from two individual mice, the result could not be completely signified. In spite of this, the result do imply that MC903 is able do directly activate MCs, since significantly more MCs did degranulate when incubated with MC903 in comparison to when incubated with ethanol. The fact that MC903 at higher doses were toxic to the MCs is not surprising, since mice treated with high doses of MC903 died in the previous study by Li *et al*. (2006).

Since no marked involvement of MCC was seen in this model, a new focus on tryptase could contribute to further understanding the role of MCs in AD. The result from the cleavage study performed, confirmed that the cleavage of TSLP by chymase was not as pronounced as the cleavage by tryptase. Tryptase was also shown to have several potential cleavage sites in TSLP, compared to only one by chymase. These results suggest that tryptase might be more important than chymase in the development of AD, thus giving an opportunity for continuous studies using MC903 to induce AD in KO mice lacking tryptase.

As a conclusion, it was shown that MC903 in a simple method induced an AD-like phenotype in both mouse strains used in this study, visible both macro- and microscopically. Without the impact of MCs, the ear thickness was highly increased, indicating a limiting role of MCs in the clinical development of the MC903-induced AD-like symptoms. This result needs further proof to be

conclusive, since only one trial with W^{sh} mice was performed. The other parameters were lowered without MCs, such as the cytokine levels of TSLP and IL-33 locally and TSLP systemically, creating a clear indication that the MC903-induced AD-model is MC-dependent. This result was expected since MCs have many important features in the immunopathogenesis of AD. A non-proved sign of lowered neutrophil migration was also seen, a result that also would need further research. MCC did exhibit one clear function in this study, namely to limit the local levels of IL-33, most likely through cleavage, as well as one less apparent function in the migration of neutrophils into to skin. TSLP, which seems to be a very important cytokine in the MC903-induced AD-model, was shown to be relatively unaffected by MCC.

As a continuation of this study, it would be interesting to shorten or lengthen the trial, to investigate whether MCs and MCC have different effect on the skin inflammation in earlier or later phases. Since the qualitative kind of cytokine array only answers the question *if* and not how *much* of the cytokine that is present, the next step was to perform specific analyzes on the cytokine of interest. Due to the short time limit of this project (three months), the results could not be followed with quantitative analysis. However, there will be continued research in this area, in where these results may be of importance.

CONCLUSIONS

In all of the mice included in this study, whether WT, MC-deficient or -competent, having or lacking MCC, MC903-treatment was shown to induce macroscopically visible and measurable changes locally in the ears. In histological examination, the treated ears had a clearly thickened epidermis with prominent inflammatory cell infiltration, thereby consisting of *e.g.* MCs, neutrophils and lymphocytes. These changes correspond to those seen in human and canine cases of AD. The typical spongiotic changes with epidermal edema were however not seen. This may probably be due to it being an inherited, primary feature of AD-patients and therefore not present in the mice used in this experimental study.

Biochemical analysis confirmed that MC903-treated ears contain AD-associated cytokines, both locally in the ears (TSLP, IL-33 measured by ELISA and IL-4, IL-13 measured by cytokine array) and systemically in serum (TSLP). These interleukines have also been shown to be produced by innate immune cells after TSLP-activation (Ziegler *et al.*, 2012), which further strengthen the idea that MC903 induce an AD-like phenotype via TSLP. A Th-2 dominated cytokine milieu was observed in the MC903-treated ears with *e.g.* IL-4, IL-6 and TNF-α, similar to naturally occurring AD. On the contrary, the Th1 cytokine IL-12 was the only cytokine not detected.

To summarize the vast number of results produced in this study, Table 3 shows the effect seen on the different genotypes following MC903-treatment, compared with the results from vehicle-treatment. In the left columns under the different genotypes, the number of arrows quantifies the increase of the different inflammatory parameters. One arrow corresponds to a low increase (↑), two means a moderate increase (↑↑) and three arrows correspond to a massive increase (↑↑↑), compared to vehicle-treatment. Statistical significance is shown inside parenthesis ($P < 0.001$ = ***, $P < 0.01$ = ** and $P < 0.05$ = *). The right columns under each genotype show statistical significance of the MC903-induced increase, compared to MC903-treated mice of opposite genotype.

Table 3. *Summary of results following MC903-treatment, compared to vehicle-treatment*

	$W^{sh+/-}$		$W^{sh-/-}$		WT & mMCP-$4^{+/-}$		mMCP-$4^{-/-}$	
	Treated vs. Control	P-value	Treated vs. Control	P-value	Treated vs. Control	P-value	Treated vs. Control	P-value
Clinical scoring	↑		↑↑	**	↑↑		↑	
Ear thickness	↑ (***)		↑↑ (***)	*	↑↑ (***)		↑↑ (**)	
MC numbers in LN	n.a.		n.a.		↑↑ (*)		↑↑ (**)	
MC numbers in ears	n.a.		n.a.		↑ (**)		↑ (**)	
Tryptase activity	n.a.		n.a.		↑↑ (***)		↑↑ (*)	
Neutrophil numbers in ears	↑ (*)		↑		↑↑ (***)		↑↑ (**)	
Elastase activity	n.a.		n.a.		↑ (*)		-	
TSLP in serum	↑↑ (*)	**	↑↑ (*)		↑↑		↑↑	
TSLP in ears	↑↑↑ (**)	*	↑↑ (**)		↑↑↑ (***)		↑↑ (**)	
IL-33 in ears	↑↑↑	*	↑↑		↑		↑↑	*

The results in Table 3 show that MC903-treatment induced significant increases in various means compared to vehicle-treatment, but that some of the significant changes were only seen in one of the genotypes, indicating some kind of effect on the AD-like development by MCs and MCC. For example, higher neutrophil counts accompanied by higher elastase activity were seen in mice having MCs and MCC but not in the KO mice, thus implying a contribution of both MCs and MCC to neutrophil migration. On the contrary, no significant differences were seen between the MC903-treated genotypes, making it somewhat difficult to draw a clear conclusion.

The involvement of MCs in this AD-model is hence only proved in three of the analyzed parameters: clinical development, TSLP-levels and IL-33 levels. MCC does only seem to affect the IL-33 levels, although some signs of involvement by MCC in the elastase activity are also seen. As a conclusion, MC-deficient mice have a higher clinical response to MC903, thus indicating a protective role of MCs in limiting the skin inflammation, but at the same time do they have lower levels of the cytokines TSLP and IL-33; both with previously mentioned connections to AD.

In the small *in vitro* study performed, incubation with MC903 was shown to induce activation through degranulation of PBMCs to a higher extent than incubation with ethanol, thus proving that MC903 also has a direct effect on MCs. This is likely to be one important cause of the significant changes in inflammatory response seen in MC-deficient mice compared to MC-competent mice *in vivo*.

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