# C3a and LPS mediated gene regulation in human mast cells examined by microarray and PCR

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# Contents


**1. Acknowledgements 3**

**2. Introduction 4**
2.1 Mast cells are important effector cells in $T_H2$ responses 4
2.2 Mast cells are important in the immune response to bacterial products 4
2.3 Complement is important in the development of asthma 4
2.4 Human mast cell-1 (HMC-1) 5
2.5 Cord blood derived mast cells (cbd-MC) 6
2.6 Aims of the study 6

**3. Materials and methods 7**
3.1 Antibodies 7
3.2 Cells and culture conditions 7
3.3 Cell culture for microarray analysis 7
3.4 Cell culture for CQ-RT-PCR analysis 8
3.5 C3aR expression examined by flow cytometry 8
3.6 Isolation of RNA, cDNA synthesis and CQ-RT-PCR analysis 9
3.7 Microarray analysis 14

**4. Results 17**
4.1 C3a and C3a/LPS stimulation of HMC-1 cells 17
4.2 C3a/LPS stimulation modifies cytokine mRNA expression in HMC-1 and cbd-MC 18
4.3 HMC-1 express the C3a receptor 24

**5. Discussion 25**
5.1 LPS induce $T_H2$ cytokines from mast cells 25
5.2 C3a induce airway hyper responsiveness 25
5.3 Regulation of $T_H2$ associated cytokines 25
5.4 Regulation of eicosanoid production 26
5.5 Mast cells as important regulators of airway remodeling in asthma 27

**6. Conclusion 27**

**7. References 28**

# 1. Acknowledgements

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# 2. Introduction

## *2.1 Mast cells are important effector cells in allergic $T_H2$ responses*

The mast cells are central effector and regulatory cells in IgE mediated allergic reactions and Th2-dominated immune responses (1). Binding of antigens to receptor bound IgE results in release of inflammatory mediators such as histamine, leukotriene (LT) $C_4$ and Prostaglandin (PG) $D_2$ (1), in addition to a variety of different ***$T_H2$*** cytokines, including IL-4, IL-5, IL-6, IL-10 and IL-13, which has been implemented in the pathogenesis of allergic reactions (2, 3, 4, 5). Asthmatic airway inflammation has been strongly linked to Th2 lymphocytes and their cytokines, particularly IL-4, IL-5 and IL-13, which regulate airway hyper responsiveness, eosinophil activation, mucus production and IgE secretion (6).

Mucosal mast cells lie adjacent to nerves, blood vessels and lymphatics, which highlights their pivotal importance in regulating allergic inflammatory processes. In allergic asthma, mast cells are predominantly activated by IgE receptor cross linking, and mediators from degranulating mast cells are critical to the pathology of the asthmatic lung. Mast cell proteases stimulate tissue remodeling, neuropeptide inactivation and enhanced mucus secretion, while histamine stimulates smooth muscle cell contraction, vasodilatation and increased venular permeability and further mucus secretion (7).

## *2.2 Mast cells are important in the immune response to bacterial products*

In addition to IgE-mediated allergic reactions, mast cells are important in the immune defense against Gram-negative bacteria through release of TNF-α (5, 8, 9) , and can perceive infectious agents either by complement-dependent mechanisms or through Toll-like receptors interacting with bacterial products (10). Endotoxin, or LPS, is a prominent pathogen-associated molecule embedded in the cell wall of Gram-negative bacteria. Mast cells respond to LPS by producing IL-1β, IL-5, IL-6, IL-13 and TNF-α without degranulation (5, 11-13).

## *2.3 Complement is important in the development of asthma*

C3a activate mast cells through their G-protein coupled receptor, C3aR, and this lead to a mobilization of intracellular calcium and thereby activation of the cell (6, 14, 15). The anaphylatoxin Complement C3a fragment and its receptor were recently shown to be important in the development of bronchial hyper reactivity, recruitment of airway eosinophils, IL-4 production, and IgE responses (13). Moreover, a deletion in the C3a receptor gene, resulting in no C3a receptor expression, ameliorated allergen induced bronchial contraction, mucus secretion and recruitment of inflammatory cells in a murine asthma model (16). Both mast cells and eosinophils express the C3a receptor; and the inability of the allergen induced C3a formation to activate these cells, may have inhibited the asthma reactions. Non-IgE dependent mast cell activation may actually be very important as IgE-deficient mice still developed antigen-induced airway hyper responsiveness (14, 17). IgE-mediated activation of mast cells normally induce secretion of preformed proteoglycan bound mediators such as, TNFα, IL-4, 5, 6, 10 and13, histamine, tryptase and chymase. In addition to these, new synthesis of arachidonic acid metabolites (leukotriene $C_4$ ($LTC_4$), leukotriene $B_4$ ($LTB_4$) and prostaglandin $D_2$ ($PGD_2$)) are stimulated. These substances are all important cytokines in the pathology of the asthmatic lung (2- 5, 7- 9).

Human asthmatics generate significant levels of C3a following intra-bronchial allergen deposition, but not saline. C3a was shown to be released to the bronchoalveolar lavage fluid (BAL) after allergen challenge in asthmatic lungs of 15 patients. The same study reports that the eosinophil and neutrophil infiltrate in BAL fluid correlates highly with the amount of C3a present (18). This indicates that the innate immune system and complement (C3a in particular) are involved in the pathogenesis of asthma.

## *2.4 Human mast cell-1 (HMC-1)*

The HMC-1 cell line was established from a patient with mast cell leukemia in 1987 by Dr. J. H. Butterfield and colleagues (19). HMC-1 has a unique chromosomal 10;16 translocation and bears many similarities to immature mast cells. HMC-1 grows in the absence of human plasma or leukocyte-conditioned medium, and is relatively stably arrested in a specific differentiation state. The cell line contain low levels of histamine, are stained metachromatically by toluidine blue, and contain chloroacetate esterase, aminocaproate esterase and tryptase activities. Heparin and chondroitin sulfate are found in approximately equal amounts. The cells lack T- and B-lymphocyte markers but express (similar to normal mast cells) the monocyte/macrophage marker CD68 and contain mRNA for the eosinophilic/basophilic-related differentiation marker: the Charcot-Leyden crystal. The cell does not, however, express the high affinity IgE receptors (19, 20).

There are two sublines of HMC-1, named HMC-$1^{560}$ and HMC-$1^{560,816}$, with different phenotypes and designated by the specific mutations in the c-kit proto-oncogene. Both sublines have a heterozygous T to G mutation at codon 560 in the juxtamembrane region of the c-kit gene causing an amino acid substitution of Gly-560 for Val. However, only HMC-$1^{560,816}$ cells have the V816 mutation found in mast cell neoplasms causing an Asp→Val substitution at the intracellular kinase domain. However, c-Kit is constitutively phosphorylated on tyrosine residues in both HMC-1 variants. This activation is associated with phosphatidylinositol 3´-kinase (PI 3-kinase) without constitutive phosphorylation of **Act** or extra cellular regulated protein kinase (ERK), which are signaling molecules normally activated when SCF bind c-Kit. This leads to the constant activation and growth of the mast cell lines. This documentation and characterization of the two HMC-1 sublines provides both information on the biological consequences of mutations in Kit and recognition of the availability of what in reality are two distinct cultured human mast cell lines (20- 23).

HMC-1 express mRNA for TNF-α, TGF-β, IL-1β, IL-3, IL-4; IL-8 and a low level of M-CSF, constitutively. They can also be induced to express several cytokines i.e. IL-1β, IL-3, IL-6, GM-CSF, TNF-β and PDGF-A (Platelet derived growth factor-A, which stimulate fibroblast proliferation and migration and has been implicated in a number of fibroproliferative disorders). Interleukin-4 (IL-4) is a well-characterized mast cells regulator of growth and function. HMC-1 expresses IL-4 receptor and IL-4 down-regulated IL-1β mRNA and protein expression, but had no effect on TNF-α mRNA expression. Increased transcripts for $T_H2$ cytokines are not detected after stimulation with IL-4, IL-2, IL-3, IL-6 and IL-8 (24). Thus, human mast cells have the capacity to express not only cytokines mediating immune response, but also cytokines affecting other cell types, e.g. fibroblasts and endothelial cells, involved in later steps of the inflammatory response (25, 26).

HMC-1 expresses both the high affinity C3aR1 receptor, and the low affinity C3aR2 receptor. Both are expressed at a high level (27). C3a function as a chemotaxin to HMC-1. This anaphylatoxin-mediated recruitment of mast cells might play an important role in hypersensitivity and inflammatory processes (28). Data also suggest that the HMC-1 high affinity C3aR receptor respond to C3a only, and not to C3a(desArg) (15).

HMC-1 express 5-lipoxygenase (5-LO) activity and produce 5-hydroxyeicosatetraenoic acid (5-HETE) as the major metabolite (29). Following this HMC-1 (and cord blood derived mast cells) is equipped with two enzymes that can catalyze the committed step in the biosynthesis of cysteinyl-leukotrienes (cys-LTs). The expression of the cognate receptor $CysLT_1$ suggests moreover, that these lipid mediators may be involved in autocrine signaling pathways regulating mast cell functions. These enzymes synthesize leukotriene $C_4$ ($LTC_4$). Both are GSH transferases termed $LTC_4$ synthase ($LTC_4S$, typically expressed in certain bone marrow-derived cells such as eosinophils and mast cells) and microsomal GSH transferase type 2 (mGST-II). The Cys-LTs $LTC_4$ and 11-trans-$LTC_4$ are increased in allergic inflammation and induce smooth muscle contracting, mucus secretion and was previously known as the slow reacting substance of anaphylataxis (30). HMC-1 cells contain the enzyme activities required to metabolize $LTC_4$

further into $LTD_4$ and later into $LTE_4$. The prostanoid profile includes $PGE_2$, $PGF_{2\alpha}$ and PGD2, but not prostacyclin. The cells are also able to produce substantial amounts of thromboxane (TX) $A_2$, quantities exceeding those of $LTC_4$, suggesting that mast cells may be an important source of thromboxane and pointing to a possible role of these cells in hemostasis and thrombosis (29).

## *2.5 Cord blood derived mast cells (cbd-MC)*

The cord blood-derived human mast cells were developed by Christine Dahl et al (31). Human cord blood progenitors were purified with anti-CD133 antibody, and then cultured in a serum-free medium supplemented with SCF and IL-6 for 8 weeks (32-37). The cells were thereafter cultured for 4 weeks in media supplemented with 10% fetal calf serum (FCS). The addition of FCS increased histamine and FcεRI expression, and released more histamine after anti-IgE stimulation than those that had grown in serum containing media only (31). All cultured human MCs produce chymase in a clonally regulated but cytokine-independent manner (38) and express tryptase in their cytoplasmic granules (39).

Cbd-MC constitutively express the immune modulating and fibroblast activating cytokine transforming growth factor $\beta_1$ (TFG-$\beta_1$) and release it continually, with no increase after IgE-receptor cross-linking. This in contrast to platelet-derived growth factor-β (PDGF-β) and basic fibroblast growth factor (bFGF) which is up-regulated after IgE-receptor cross-linking. Activated human mast cells produce fibrinogenic factors such as PAI-1, suggesting an important role for mast cell cytokines in fibrosis (40, 41).

Cbd-MC cells release histamine, TNF-α, sulfidoleukotrienes (LTs) and prostaglandin $D_2$ ($PGD_2$) after IgE receptor cross-linking (34, 42, 43). Preincubation with SCF, IL-4, IL-5 or IL-6 for 24 hours during sensitization with IgE enhances FcεRI -receptor mediated histamine release, whereas IL-3 showed a negligible effect (44). IL-4 may also induce IL-5 production in cbd-MC (45).

Cbd-MC produce and release IL-13 after FcεRI -receptor and calsium ionophore A23187 stimulation in the presence of SCF only (46). Whereas the $T_H2$ cytokine IL-4 stimulate the IgE-mediated mast cell production of macrophage inflammatory protein 1α (MIP-1α), IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF), the $T_H1$ cytokine IFN-γ reciprocally inhibits the production (47). IFN-γ may even stimulate histamine release from cbd-MC (48). Thus, both $T_H2$ and $T_H1$ cytokines may influence human mast cell functions in allergic reactions.

Peptidoglycan (PGN) from Staphylococcus aureus induce cbd-MCs to release TNF-α, IL-5, IL-10 and IL-13 through interactions with Toll-like receptor 2 (TLR2), while lipopolysaccharide (LPS) from Escherichia coli invokes the same response through TLR4. The TNF-α release induced by LPS required priming of cbd-MC by IL-4 and the presence of serum components, in particular CD14. Stimulation with PGN, but not LPS, induce release of histamine. Activation of either TLR2 or TLR4 pathway may lead to a pro-$T_H2$ immune response. These findings show that human MC differentially responds towards bacterial components with production of specific cytokines, and that their response depends on TLR pathways (5).

## *2.6 Aims of the study*

Our objective was to examine gene regulation following C3a and LPS stimulation of human mast cells. HMC-1 and cbd-MC cells were used in the studies to simulate normal human mast cells. We used microarray, which is a powerful tool to investigate differential gene expression of thousands of genes of a cell type, tissue, or organism. Microarray was used to detect changes in gene expression upon stimulation, then we used PCR analysis to confirm and differentiate these findings. The fact that mast cells respond to complement and bacterial products may indicate an important role in modulating other inflammatory processes, e.g. periodontal disease and muco-cutaneous contact stomatitis.

# 3. Materials and methods

## *3.1 Antibodies*

Recombinant human C3a was a gift from Dr. Jörg Zwirner at the University of Göttingen. Calsium Ionophore III A23187 ( Ionophore), Phorbol 12-Myristate 13-Acetate (PMA) and Lipopolysaccharide from E.coli 0111:B4 (LPS) were all obtained from Sigma-Aldrich (St. Louis, MO, US). Recombinant human Interleukin-4 (IL-4) and recombinant human Interferon-γ (IFN-γ) were both purchased from R&D systems (Minneapolis, MN, US). For the flow cytometric analysis C-kit antibody CD-117 1/2000 suspension (DAKO a/s, Denmark), C3aR from clone 76/415 mouse-$IgG_1$ 1/2000 suspension (kindly provided by Dr. Zwirner, Göttingen), Phytoerethrin (PE) goat anti-mouse $IgG_1$ RPE 1/200 suspension (Southern Biotechnologies Associated Inc, Birmingham, AL, US) and FITC goat anti-mouse $IgG_1(\gamma_1)$ (Molecular Probes, Oregon, US) were used.

## *3.2 Cells and culture conditions*

The human mast cell line, HMC-1 (kindly provided by J. H. Butterfield; 19), were grown in Iscove`s medium (PAA Laboratories GmbH, Austria) supplemented with 10% iron-supplemented bovine calf serum (Sigma), thioglycerol (Sigma) and penicillin/ streptomycin (GIBCO, Invitrogen corp.) at concentrations ranging from $2{,}5x10^5$ to $1x10^6$ cells pr ml. at 37°C in a humidified atmosphere with 5% $CO_2$ and passed twice a week. The cord blood derived mast cells, cbd-MC (kindly provided by C. Dahl; 31) were cultured from $10^5$ $CD133^+$ cells progenitor cells using a serum-free condition for weeks 0 through 8. After 8 weeks of culture $>8x10^6$ mast cells were always generated. This was then followed by a serum supplemented maturation phase for 4 weeks. This yielded a mast cell that had an increased amount of histamine pr cell (3.8 pg/cell), and expressed more FcεRIα. These were cultured in the same medium and conditions as the HMC-1 cells.

## *3.3 Cell culture for microarray analysis*

### 3.3.1 HMC-1 stimulated with C3a or Ionophore.

The aim of the study was to examine gene expression in HMC-1 following C3a stimulation. To obtain enough total RNA for microarray hybridization we needed between 40 and 80µg total RNA. There are approximately 5µg total RNA in$10^6$ HMC-1 cells. The cells were therefore grown at large scales using $20x10^6$ cells for each experiment and time point. For the microarray experiment 12x20ml cultures of HMC-1 cells were set up at a concentration of $1x10^6$ cells pr ml (20ml flasks; Nalge NUNC int., Denmark) and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. $20x10^6$ unstimulated HMC-1 cells were harvested at each time point (0, 2, 4, 8 and 16 hours as negative controls). $20x10^6$ Ionophore stimulated HMC-1 cells were harvested after 4 hours as a positive control, and 4x HMC-1 cells ($80x10^6$) stimulated with rhC3a (100ng/ml) and $20X10^6$ cells were harvested at each time points (2, 4, 8 and 16 hours). When harvested, the medium were transferred into 50ml falcon tubes (Nalge NUNC int., Denmark) and centrifuged at 800 rpm for 8 minutes at 4°C during which time the cells remaining adherent in the 20ml flask were lysed with 4ml TRI Reagent® (Molecular Research Center, Cincinnati, OH, US) and placed on ice. The supernatant was then removed from the falcon tube and the TRI Reagent® moved from the 20ml flask to the tube to lyse the cell pellet; the solution was then stored at -70°C prior to use.

### 3.3.2 HMC-1 stimulated with C3a, PMA, Ionophore and LPS

The aim of the study was examine gene expression in HMC-1 following C3a, PMA, Ionophore and LPS stimulation. 16x20ml cultures of HMC-1 cells (320 $x10^6$ cells) were set up at a concentration of $1x10^6$ cells pr ml (20ml flasks; Nalge NUNC int., Denmark) and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. Unstimulated HMC-1 cells ($80x10^6$) were harvested at each time point (2 and 8 hours) as negative controls. $2x20x10^6$ HMC-1 cells were stimulated with both PMA (50ng/ml) and Ionophore ($10^{-6}$M) and harvested after 2 and 8 hours as positive controls. HMC-1 cells ($20x10^6$ cells at each experiment/time point) were stimulated with either rhC3a (500ng/ml), LPS (2 µg /ml) or a combination of LPS (2 µg /ml) and rhC3a (500 ng/ml) and harvested after 2 and 8 hours. When harvested, the medium was transferred to a 50ml falcon tubes (Nalge NUNC int., Denmark) and centrifuged at 800 rpm for 8 minutes at 4°C during. The remaining adherent cells in the 20ml flask was then lysed with 4ml TRI Reagent® and placed on ice. The supernatant was then removed from the falcon tube and the TRI Reagent® moved over from the 20ml flask to the tube to lyse the cell pellet there; the solution was then stored at -70°C prior to use

## *3.4 Cell culture for CQ-RT-PCR analysis*

### 3.4.1 HMC-1 stimulated with C3a or Ionophore

The aim of the study was to examine mRNA expression in HMC-1 following C3a stimulation. Stimulation with ionophore induce calcium influx mimicking IgE-receptor cross linking and served as a positive control for cell activation. For the cq-RT-PCR experiment 16x1ml cultures of HMC-1 cells were set up at a concentration of $1x10^6$ cells pr ml (24 well trays; Nalge NUNC int., Denmark) and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. Of these, $4x10^6$ HMC-1 cells were harvested at time 0. HMC-1 cells ($12x10^6$ ) were stimulated with either recombinant C3a (100 ng/ml) or Ionophore ($1x10^{-6}$M), and harvested after 2, 4, 8, 12, 16 and 24 hours. The cells were centrifuged at 800 rpm for 5 minutes at 4°C, and the cell pellet was lysed with TRI Reagent® (200µl pr well) and stored at -70°C prior to use.

### 3.4.2 HMC-1 and cbd-MC stimulated with C3a, PMA, Ionophore and LPS

The aim of the study was to examine the mRNA expression in mast cells following stimulation with C3a, PMA, Ionophore and LPS. For the cq-RT-PCR experiment 11x2ml cultures of HMC-1 ($5x10^5$ cells pr ml) cells and cbd-MC ($5x10^5$ cells pr ml) were set up in parallel (24 well trays; Nalge NUNC int., Denmark) and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. $2x10^6$ HMC-1 cells and cbd-MC cells were harvested at time 2 and 4 as negative controls, $3x10^6$ HMC-1 cells and cbd-MC cells stimulated with Ionophore ($10^{-6}$M) and PMA (50 ng/ml) were harvested at time 2, 4 and 8 hours as positive controls, and $6x10^6$ HMC-1 cells and cbd-MC cells stimulated with either rhC3a (500ng/ml) or rhC3a (500ng/ml) and LPS (2 µg/ml) and harvested after 2, 4 and 8 hours. The cells were centrifuged at 800 rpm/min for 8 minutes at 4°C, and the cell pellet was lysed with 200µl TRI Reagent® pr well and stored at -70°C prior to use.

## *3.5 C3aR expression examined by flow cytometry*

### 3.5.1 C3a-induced C3aR internalization in HMC-1 cells

This experiment was conducted to examine the levels of receptor expression on the cells and to examine whether C3a stimulation would induce C3a-receptor internalization. Three times $1,5x10^6$ (in a 24 well tray; Nalge NUNC int., Denmark) HMC-1 cells ($1x10^6$ cells/ml) were incubated at 37°C in a humidified atmosphere with 5% $CO_2$ with rhC3a at various concentrations (0 ng/ml, 100 ng/ml and 1000ng/ml) for 20 hours; then harvested on ice and labeled for flow cytometri. Cells ($5x10^4$) were incubated (30 min on a gyrating board) on ice with either mAb to c-Kit (clone 104D2, mouse IgG1, cons. 1/2000; DAKO, Denmark), or mAb to C3aR (clone 76/415, mouse

IgG1, cons. 1/2000), or BSA, in a 96 well tray (Greiner). The cells were then washed with 100µl washing buffer (PBS+1,5%BSA+azid) and centrifuged at 275G for 5 minutes at 4°C and then washed again with 200µl of buffer. The cell pellet was then resuspended in 50µl buffer before adding a combination of FITC-labeled goat anti mouse IgG1 (1:200, Southern Biotecnology, CA, US) and phytoerythrin (PE) –labeled goat anti mouse IgG1 (Southern biotechnology) for 30 min on ice. The cells were then resuspended in 200µl buffer after they had been washed for 5 minutes in 100µl buffer and centrifuged as described above. The cells were assessed in Becton/Dickinson FACSCalibur flow cytometer (Becton/Dickinson, Indianapolis, IN, US) using the Cell Quest Software (Becton/Dickinson).

### 3.5.2 C3aR expression in HMC-1 and cbd-MC after IL-4 or IFN-γ stimulation

The aim of the study was to examine if pre-stimulation with IL-4 or IFN-γ influence the C3a receptor expression on mast cells. Three times $1x10^6$ (in a 24 well tray; Nalge NUNC int., Denmark) HMC-1 cells and cbd-MC ($5x10^5$ cells/experiment) were incubated at 37°C in a humidified atmosphere with 5% $CO_2$ with either IL-4 (100ng/ml), IFNγ (15ng/ml) or nothing for 24 hrs. The cells were then harvested and prepared for flow cytometry as described above.

## *3.6 Isolation of RNA, cDNA synthesis and CQ-RT-PCR analysis*

### 3.6.1 Homogenization and Phase separation

The cells were lysed in 1.0ml TRI Reagent® per $5\times10^6$ cells by repetitive pipetting and stored for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, chloroform (0.2ml/ml TRI Reagent®) was added and the tube shaken vigorously for 15 seconds. The resulting mixture was stored at room temperature for 10 minutes and centrifuged at 12,000g for 10 minutes at 4ºC. The mixture then separated into a lower protein containing phenol-chloroform phase, a DNA-containing interphase and the RNA-containing colorless upper aqueous phase. The *Phase lock gel™Heavy 0.5ml* tubes (Eppendorf, Mansfield, TX, US) were used to facilitate phase separation when working with small volumes (less than $1\times10^6$ cells).

### 3.6.2 RNA precipitation, washing and solubilization

The aqueous phase was transferred to a fresh tube and added isopropanol for precipitation using 0.5 ml of isopropanol per 1 ml of TRI Reagent® initially. After mixing, the samples were stored in room temperature for 5 minutes followed by centrifugation at 12,000G for 8 minutes at 4º C. The RNA precipitate would then form a gel-like or white pellet at the bottom of the tube. The supernatant was then removed and the pellet was washed with 75% ethanol and centrifuged at 12,000G for 5 minutes at 4º C. This step was repeated using at least 1ml of 75 ethanol per 1ml TRI Reagent®. The ethanol was removed and the pellet air-dried for 2-5 minutes. The pellet was then dissolved by adding 50µl DEPC- $H_2O$ containing 40u of RNasin® (Promega, Madison, WI,US) per 1µl. Samples were then quantified and checked for contamination by looking at the 260/280 nm ratio. RNA aliquots were diluted with distilled water and spectrophotometry (*Perkin Elmer MRA2000, Rochester, NY, US*) were performed using a distilled water-RNasin® mix for calibration.

### 3.6.3 Reverse transcription

Reverse transcription (RT) was carried out on 50µl mixtures in 200µl PCR tubes (Perkin Elmer). The mixtures contained 0.5µg total RNA based on the spectrophotometric quantization, 2.5µl Oligo dt, 10µl of M-MLV-RT buffer (Promega), 5µl DTT (100mM), 1µl dNTP, 1,6µl of RNase inhibitor (RNA guard®, Promega) and 2µl of the reverse transcription enzyme M-MLV-RT (Promega). Finally DEPC $H_2O$ was added to the total reaction volume of 50µl. After 60 min. at 42ºC the reaction was terminated by heating for 10 min. at 81ºC, followed by immersion on ice.

### 3.6.4 PCR amplification

PCR reactions were carried out on 25µl mixtures in strips of 200µl MicroAmp® (Perkin Elmer) reaction tubes plugged with MicroAmp®Caps (Perkin Elmer). The mixtures contained cDNA, 2.5µl 10× PCR buffer, 62.5µM dNTP, and 1 unit of TAQ polymerase (In house made recombinant TAQ polymerase) and the following primers (100ng of each):

**Table.1 Sequence of the PCR-primers used for amplification**

| PRIMER | SENSE 5` | ANTISENSE 5` |
|---|---|---|
| β-actin | GGGTCAGAAGGATTCCTATG | GGTCTCAAACATGATCTGGG |
| IL-1β | GGATATGGAGCAAACAAGTGG | ATGTACCAGTTGGGGAACTG |
| IL-5 | CTTGGCACTGCTTTCTACTC | GCAGGTAGTCTAGGAATTGG |
| IL-10 | AAATTTGGTTCTAGGCCGGG | GAGTACAGGGGCATGATATC |
| IL-13 | GAGTGTGTTTGGTCACCGTTG | TACTCGTTGGCTGAGAGCTG |
| TNF-α | ACAAGCCTGTAGCCCATGTT | AAAGTAGACCTGCCCAGACT |
| C3aR | CAGACGGGACTCGTGGAGAC | GACAATGATGGAGGGGATGAG |
| $PGD_2S$ | CACAGATTTGGCTGGAAAC | CAGAGTTACCAATAAGCCATTC |
| $COX^2$ | ATTGTCATACGACTTGCAGTGAG | TTTACCTTTGACACCCAAG |

The mixtures were set up in a sterile environment on ice, followed by centrifugation at 3500rpm at 4ºC to prevent air bubbles and ensure complete mixing of the reagents.
Amplification (40 cycles) was performed on a GeneAmp®PCR System 9700 (PE Applied Biosystems, Rochester, NY, US) starting at 94ºC for 3 min, then 40 cycles with 20 sec at 94ºC , 30 sec at 57ºC and 45 sec at 72ºC, ending at 72ºC for 4 min before cooling down to 4ºC.

### 3.6.5 Competitive PCR using an internal reference template

To do a quantitative analysis of our samples we used the competitive PCR method. (TaKaRa Biomedicals, Shiga, Japan). The principle of competitive PCR is to ad a known amount of a DNA fragment (competitor) to the sample. This competitor must contain sequences for the same primers used to amplify the target (our sample).When the target cDNA and the competitor (C) are amplified together; both templates will compete for the same set of primers. Because of this competition, the ratio of the amounts of the two amplified products reflects the ratio of the amounts of the target cDNA and competitor. Since the initial amount of competitor is known, the amount of the target cDNA can be estimated according to the target/C ratio
(Figure1 – 3)

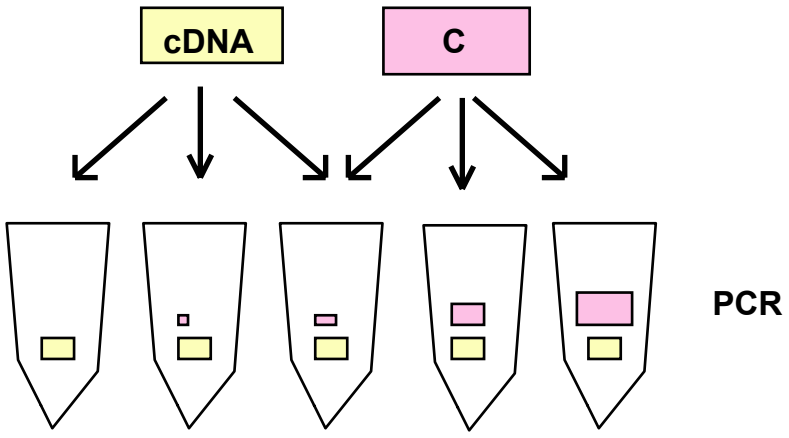



***Figure 1.***
***Equal amount of sample cDNA (yellow) is added together with increasing amount of the control (C) template (rose). The templates will compete during PCR reaction making it possible to estimate the amount of sample cDNA after gel electrophoresis.***

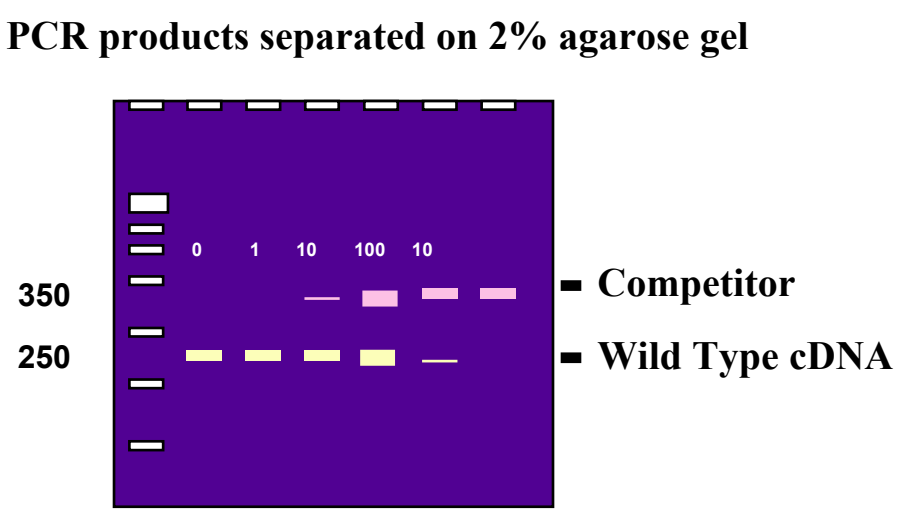
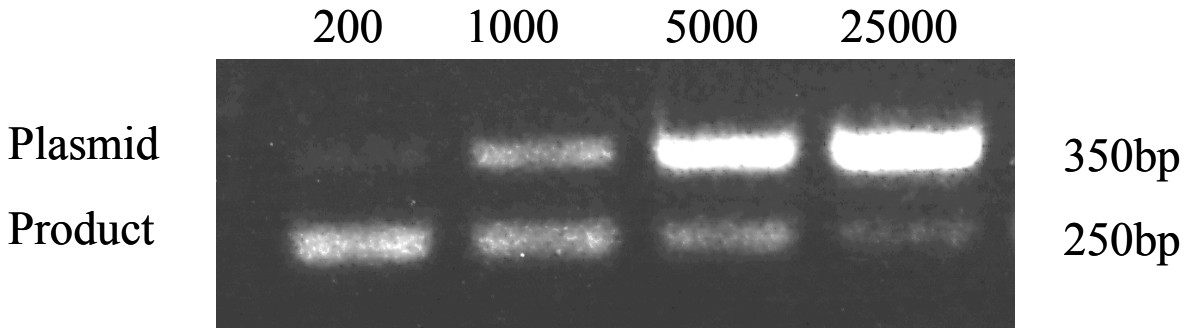



***Figure 2.***
***The size of the PCR product depend on the template being used. The sample contain same amount of cDNA for the given sequence when its short PCR product matches the longer competitor template (rose).***

We used the human β-actin gene as an *internal reference template*. This housekeeping gene is expressed at a constant level in most human cells and tissues and can therefore be used with any kind of sample derived from human cell lines (49).
As competitor we used a Plasmid, PQB2, synthesized by Pascal Legouux et al. (49). This is a heterologous competitor (different nucleotide sequence from the target cDNA except for the sequences of the primer annealing sites).PQB2 is an ideal competitor because it will be amplified by the same primers as our sample cDNA, the amplified product is distinguishable from the sample (different amplification length allows the two products to be separated by gel electrophoresis) and the competitor is purified and obtained at a known concentration.

### 3.6.7 Gel electrophoresis, scanning and quantization

To visualize the competitive PCR results 10µl of each PCR mixture was separated on a 2% agarose-gel (Amesco®) in the presence of Ethidium Bromide (0,1%).
2µl of loading buffer (in house made) were added to the total volume (25µl) of each PCR mixture before separation. After a period of approximately 1 hour at 60V a good separation of the amplified products was achieved. (fig. 3)




| | | | |
|---|---|---|---|
| 1 534 | 24 728 | 83 457 | 128 236 |
| 46 640 | 34 170 | 27 273 | 9 089 |

***Figure 3.***
***Different amount of the control plasmids (200, 1000, 5000 or 25000) have been added to a fixed amount of samples cDNA. The PCR produce a 350 bp product from the plasmid compared to the 250bp product from the samples wild type b-actin. The differences in band size are visualized by gel electrophoresis and the band intensities are quantified by the use of TortalLab tm from phoretix (nonlinear Dynamics LtD). The bands pixelvolums are indicated at the bottom. The sample contain equal amount of b-actin when band intensities equal.***

Quantitative PCR was performed using 5µl of 1/50 diluted sample cDNA, and 5µl of the competitor at concentrations ranging from 40-5000 PQB2 per 1µl. PCR reactions were carried out on a total volume of 25µl as described above. A multiwavelenght fluoroimager, capable of optical density and fluorescence measurements (ProXPRESS™ProFinder by PerkinElmer), made the competitive PCR results available for computerized quantization. Using the built-in image analyzing software, taking into account background noise, a graph was plotted of *log(T /C)* against the initial amount of competitor ( T = amount of amplified target cDNA, C = amount of amplified competitor).
The amount of competitor corresponding to *log(T/C)=0* will then correspond to the amount of β-actin in our sample (fig.4)

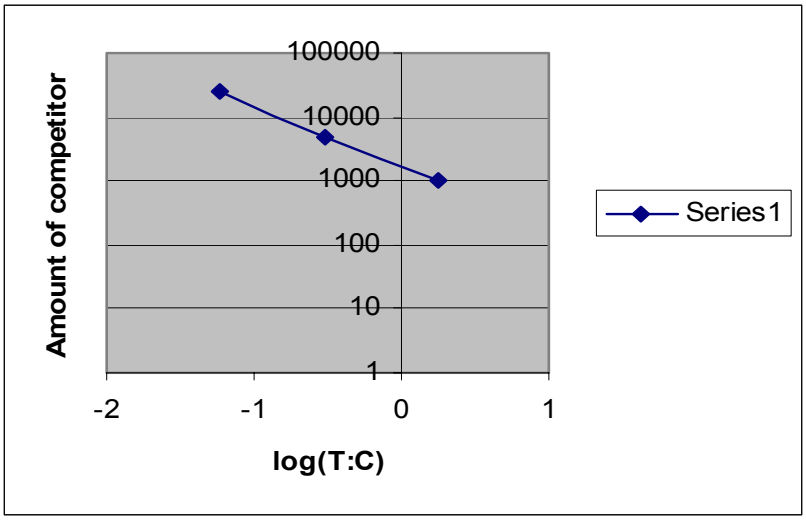



***Figure 4a: A graph was plotted of log(T/C) against the initial amount of competitor ( T = amount of amplified target cDNA, C = amount of amplified competitor). The amount of competitor corresponding to log(T:C)=0 corresponds to the amount of β-actin in sample***

Based on results obtained from the competitive PCR described above, correction of RNA amount among the samples was performed. Samples were diluted to $10^3$β-actin per 1µl. Using 10µl sample cDNA for the following PCR analyzes ($10^4$ copies of β-actin in each PCR mixture) we were able to compare the amount of amplified products from different samples, and relate the results to the expression of the housekeeping gene β-actin.

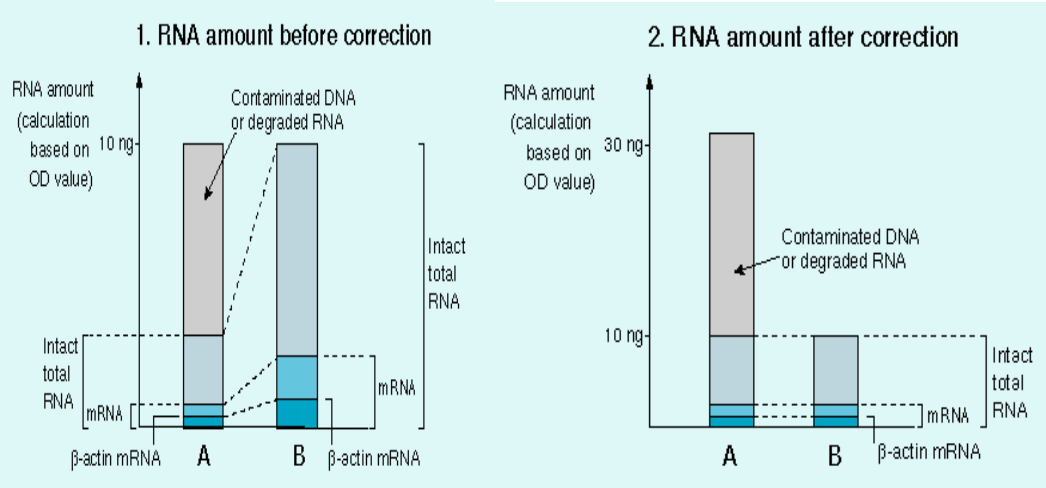



**Fig. 4b: Contaminated DNA or degraded RNA can potentially give false RNA amounts using optical density (OD) measurements. Following competitive PCR samples could be corrected allowing direct comparison of PCR results.**

## *3.7 Microarray analysis*

### 3.7.1 RNA Isolation, Homogenization, Phase separation and washing

The cells were lysed in TRI-Reagent®, using 4 ml TRI-Reagent® per $20\times10^6$ cells.

800 µl chloroform was added to the homogenate, shaken for 15 seconds and stored at room temperature for 10 minutes. Next, this was centrifuged at 1500g for 10 minutes at 20ºC. The aqueous phase was transferred to a new tube which was centrifuged at 1500g for 3 minutes at 20ºC.
2,4 ml 70% ethanol was added to 2,4 ml of the aqueous phase. 4 ml of this was applied to RNeasy midi column (Qiagen) and centrifuged at max speed for 5 minutes at 20ºC. The rest of ethanol/aqueous phase was applied to the column and centrifuged at max speed for 2 minutes at 20ºC. The RNA adsorbed to a membrane in the column, and the elute thrown.

*RNA wash*
2,5 ml RPE was added to the column and centrifuged at max speed for 2 minutes. Then, 2,5 ml RPE was added again and centrifuged at max speed for 6 minutes. Next, the column was transferred to a new tube, 150 µl RNase-free $H_2O$ added, and stored at room temperature for 1 minute. The tube was centrifuged at max speed for 3 minutes. The elute was transferred to an Eppendorf tube (elute 1). Another 150 µl RNase-free $H_2O$ was added to the column and stored at room temperature for 1 minute before centrifuging at max speed for 4 minutes. The elute was transferred to a new Eppendorf tube (elute 2). Elute 1 and elute 2 were then mixed and frozen. In the experiment of effect of C3a, PMA, Ionophore and LPS on gene expression in HMC-1, an additional precipitation was executed. To the elute, $^1/_{10}$ volume 8M LiCl was added and put on ice for 2 hours. Next, it was centrifuged at 13000 rpm for 15 minutes at 4ºC. Washing with 70% ethanol and centrifuged at 13000 rpm for 5 minutes at 4ºC. The RNA precipitant forms a pellet at the bottom of the tubes. They were air dried for 3 minutes before dissolved in DEPC-$H_2O$ and frozen at -70°C.

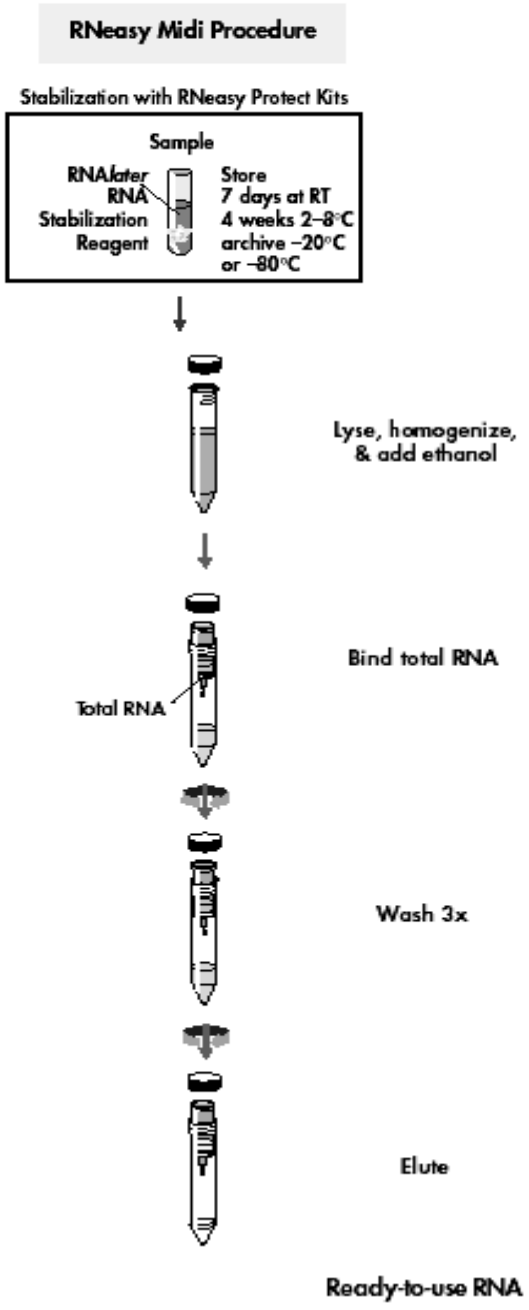



***Figure 5. Total mRNA isolation***

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### 3.7.2 Labeling by reverse transcription and hybridization

*Probe from total RNA*
4 µl 5×first strand buffer, 1 µl oligo dT primer (2 µg/µl $dT_{21}V$), 2 µl lowT dNTP (5 mM A, C, GTP, 2 mM dTTP), 2 µl Cy3-dUTP 1 mM (Amersham, Buckinghamshire, England) (test sample), 2 µl 100 mM DTT, 60 µg total RNA (test sample) and $H_2O$ up to 19 µl were mixed in tubes. The same was done with the control sample, except Cy3-dUTP 1 mM was exchanged with Cy5-dUTP (Amersham). Test samples and control samples from 2 hours and 8 hours were used.

The total RNA was concentrated by centrifuging under vacuum to concentration.
The tubes were well mixed and heated to 65ºC for 5 minutes and then cooled down to 42ºC for 2-3 minutes. 1 µl SuperscriptII (200u/µl Invitrogen) and 1 µl RNasin (Promega) were added to each tube and incubated at 42ºC for 30 minutes. Then 1 µl SuperscriptII was added and incubated at 42ºC for 40 minutes. At the end of the incubation 2,5 µl 0,5M EDTA was added and incubated at 65ºC for 1 minute. 5 µl NaOH was added and incubated at 65ºC for 15 minutes to hydrolyze RNA. Then 12,5 µl 1M tris was added immediately to neutralize the pH. The volume was brought to 70 µl by adding 35 µl TE-buffer.

Bio-Gel P-6 columns (Bio-Rad, Hemel Hempstead, UK) were prepared by re-suspending the gel and remove air bubbles, the tips were cut off, the caps opened and the fluid was allowed to be filtered through for 2 minutes and finally centrifuged at 3200 rpm for 2 minutes.
The samples were transferred to the Bio-Gel P-6 columns and centrifuged at 3200 rpm for 4 minutes. Then the correlating test sample and control sample were mixed, 200 µl TE-buffer added and transferred to microcon 100 (Millipore, Billerica, MA, US). This was centrifuged at 2300 rpm to ~20 µl. 400 µl TE-buffer and 10 µl cot DNA (1mg/ml Invitrogen) was added and centrifuged at 2300 rpm to <20 µl. Next, the columns were turned over in new tubes and centrifuged at 2300 rpm for 3 minutes.

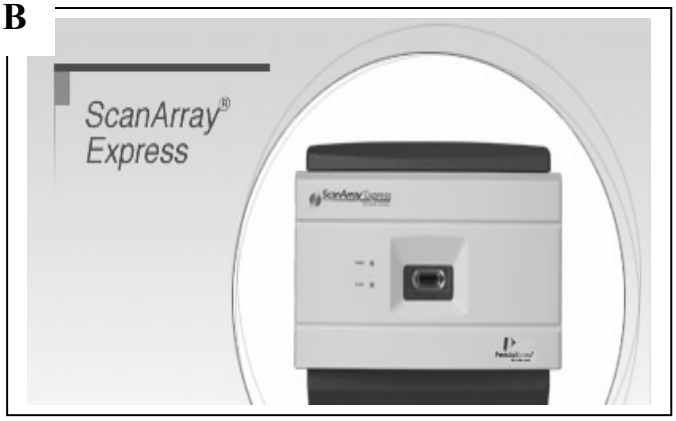
### 3.7.3 Hybridization
DEPC-$H_2O$ was added to the samples to a total volume of 24,75 µl. Then 50×Denhardt`s solution (SIGMA) 1,5 µl, Yeast tRNA (SIGMA) 1,5 µl, Poly dA (0,1 u/µl Amersham) 2,25 µl and 20×SSC 3,9 µl were added. This was incubated at 99ºC for 2 minutes. 0,9 µl 10% SDS (sterile-filtrated) was added and mixed. Next, it was centrifuged. The samples were applied gently against the edge of the lifterslips. Hybridization in 50 ml falcon tubes with two pieces of lens paper moisturized with 500 µl 2,3×SSC at 65ºC over night.
Washing: The lifterslips were loosened by holding the slides upside down in 0,5×SSC and 0,01% SDS. They were washed on a shaking table in coplin jar covered with aluminum foil. Washing for 15 minutes in 0,5×SSC and 0,01% SDS, 15 minutes in 0,06×SSC and 0,01% SDS and 15 minutes in 0,06×SSC. The slides were dried by centrifuging in falcon tubes at 2800 rpm for 2 minutes.

### 3.7.4 Computer analysis of Microarray-slides
The array-slides were scanned using either the GeneTAC UC4 Microarray Analyzer (Genomic Solutions, Ann Arbor, MI, US) or the ScanArray®Express Microarray Scanner. The lasers were set to a level in which the slides were scanned just below saturation for both Cy3 and Cy5. The analyzing tool used for gene identification was GeneTAC Integrator 3.3. Further analysis was done by using Spotfire® Decisionsite™.
QuantArray® Microarray Analysis Software and Microsoft Access were also used for gene identification and analysis.

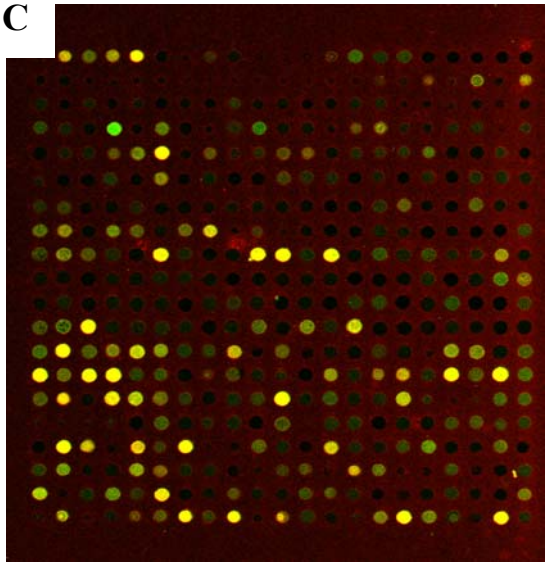
**A**

*The GeneTAC UC-4 is a compact, easy-to-use micro-array analyzer combining simplicity with performance.*

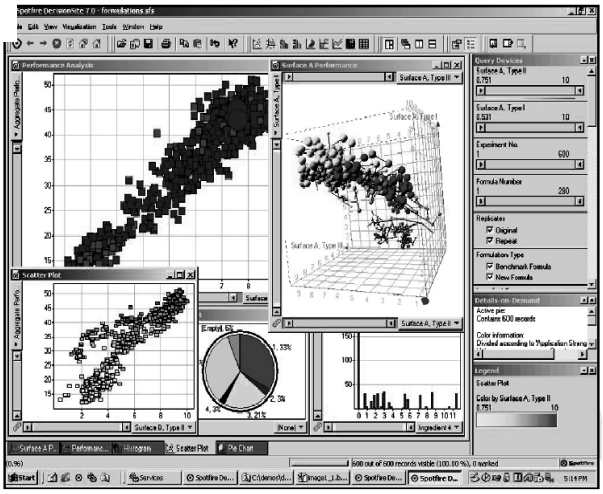
**B**




**C**



**D**



Spotfire®Decisionsite™

***Figure 6.: A shows the GeneTAC UC-4 scanner used for the first scans. Later, the ScanArray® Express (B) was used. C shows part of the scanned microarray, which was analyzed using Spotfire® Decisionsite™ (D) software.***

# 4. Results

## *4.1 C3a and C3a/LPS stimulation of HMC-1 cells*

| A | B | C | D |
|---|---|---|---|
| BIRC3 baculoviral IAP repeat-containing 3 | 35 | 602 | 17 |
| DEAF1 deformed epidermal autoregulatory factor 1 (Drosophila) | 56 | 561 | 10 |
| GEM GTP-binding protein overexpressed in skeletal muscle | 26 | 256 | 10 |
| SEMA5A | 42 | 414 | 10 |
| Interleukin 1- beta | 43 | 333 | 8 |
| MAOA monoamine oxidase A | 268 | 1286 | 5 |
| Mannosidase- alpha type II | 52 | 249 | 5 |
| NGB GTP-binding protein | 50 | 240 | 5 |
| TRAF1 TNF receptor-associated factor 1 | 144 | 664 | 5 |
| TCF12 transcription factor 12 (HTF4- helix-loop-helix transcription factors 4) | 90 | 414 | 5 |
| Homo sapiens protein kinase C-alpha mRNA- partial 3' UTR | 45 | 201 | 4 |
| PAI-I | 97 | 417 | 4 |
| Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- alpha | 123 | 508 | 4 |
| BIRC3 baculoviral IAP repeat-containing 3 | 55 | 211 | 4 |
| Homo sapiens- clone MGC:5618- mRNA- complete cds | 169 | 620 | 4 |
| MAN2A1 mannosidase- alpha- class 2A- member 1 | 143 | 517 | 4 |
| Thrombospondin 1 | 101 | 357 | 4 |
| ESTs- Moderately similar to unknown [H.sapiens] | 152 | 536 | 4 |
| CREM | 124 | 418 | 3 |
| IL1B interleukin 1- beta | 144 | 479 | 3 |
| PRCC papillary renal cell carcinoma | 142 | 446 | 3 |
| FLJ13164 hypothetical protein FLJ13164 | 64 | 201 | 3 |
| FLJ21016 hypothetical protein FLJ21016 | 80 | 249 | 3 |
| GTF2F1 general transcription factor IIF- polypeptide 1 | 66 | 205 | 3 |
| Small inducible cytokine A4 | 395 | 1212 | 3 |
| Homo sapiens cDNA: FLJ22380 fis- clone HRC07453 | 73 | 219 | 3 |
| SCYA7 small inducible cytokine A7 (monocyte chemotactic protein 3) | 88 | 265 | 3 |
| DDX5 DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase- 68kD) | 329 | 982 | 3 |
| SCYA4 small inducible cytokine A4 (homologous to mouse Mip-1b) | 613 | 1820 | 3 |
| STATI2 STAT induced STAT inhibitor-2 | 112 | 333 | 3 |
| FLJ20037 hypothetical protein FLJ20037 | 68 | 201 | 3 |
| CCNA2 cyclin A2 | 75 | 221 | 3 |
| GBE1 glucan (1-4-alpha-)- branching enzyme 1 | 118 | 338 | 3 |
| IFI16 interferon- gamma-inducible protein 16 | 162 | 463 | 3 |
| UBE3A ubiquitin protein ligase E3A | 247 | 706 | 3 |
| PDCD2 programmed cell death 2 | 85 | 242 | 3 |
| KIAA0332 KIAA0332 protein | 108 | 303 | 3 |
| FLJ13195 hypothetical protein FLJ13195 similar to stromal antigen 3 | 90 | 251 | 3 |
| BIRC2 baculoviral IAP repeat-containing 2 | 154 | 429 | 3 |
| Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A) | 243 | 672 | 3 |

***Table 2. Results from microarray analysis following C3a/LPS stimulation. Selection of the most up-regulated genes. A: gene name, B: Ch1 (int-backgr), C: Ch2 (int-backgr), D: C/D. The numbers (in B and C) represents spot intensities after background subtraction.***

## *4.2 C3a/LPS stimulation modifies cytokine mRNA expression in HMC-1 and cbd-MC*

To examine LPS/C3a-induced cytokine production from mast cells more extensively, HMC-1 and cbd-MC were stimulated with C3a, a combination of C3a/LPS, and a combination of PMA/Ionophore. Total RNA was isolated, and microarray and PCR analysis were performed. IL-1β was up-regulated in both cbd-MC and HMC-1 after stimulation with LPS/C3a. (fig.7-8), TNF-α seemed to be expressed in both cell types, but our studies has failed to prove up-regulation after stimulation with LPS. IL-5 was only up-regulated in cbd-MC after stimulation with C3a and LPS/C3a. IL-10 was expressed in both cbd-MC and HMC-1, and was more strongly expressed in cbd-MC. IL-13 seemed to be up-regulated in both HMC-1 and cbd-MC after stimulation with LPS, and grossly up-regulated after stimulation with PMA/Ionophore. $PGD_2$-synthetase was constitutively expressed in both cell types, and was not regulated by C3a, LPS or PMA/Ionophore stimulation. Only Cbd-MC expressed $COX_2$. It seemed to be expressed at a constant level through stimulation. Lipocortin seemed to be constitutively expressed at a high level in both cells types, and stronger in cbd-MC. Our preliminary PCR and microarray analysis also indicated that HMC-1 cells stimulated with C3a and C3a+LPS down-regulated their expression of this cytokine( Results figs 8-12).

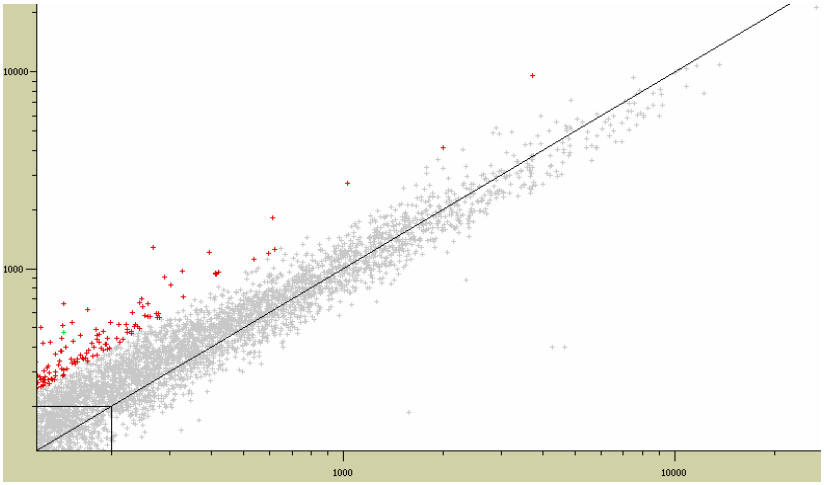
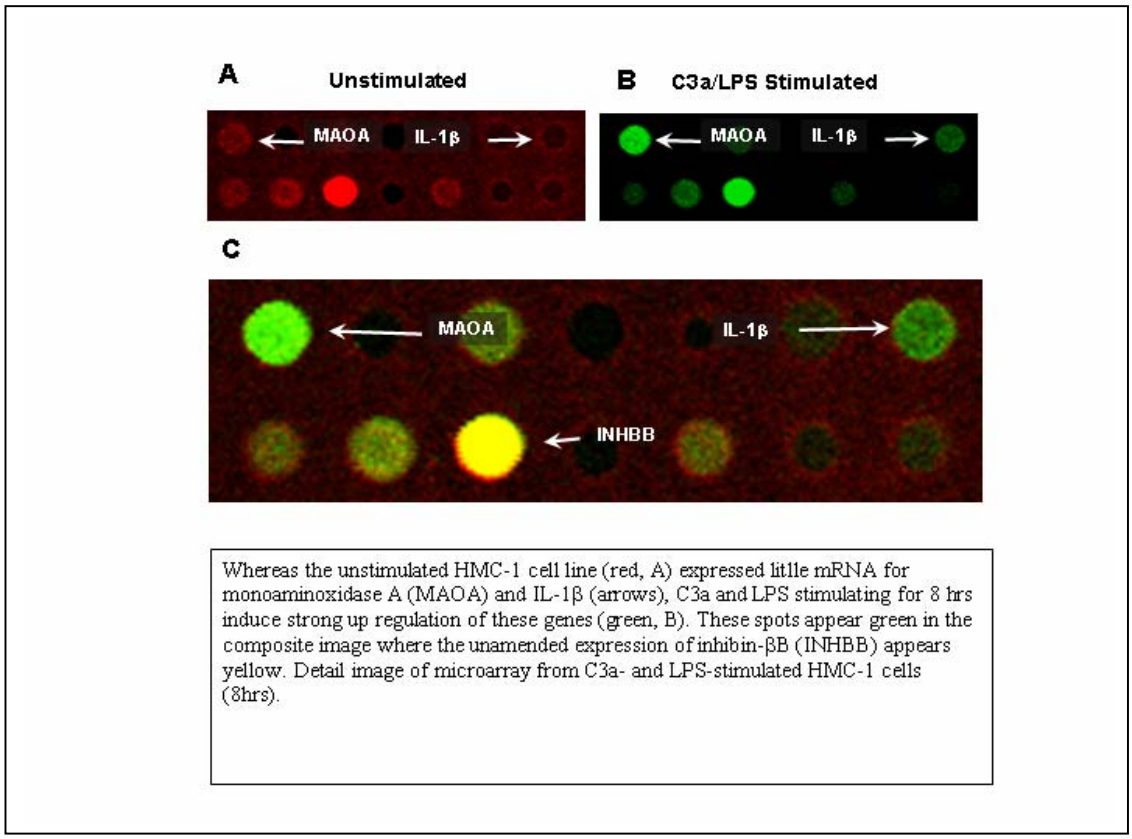
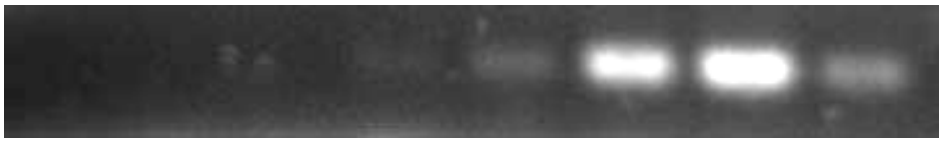



***Figure 7. Scatter plot showing genes expressed in the HMC-1 after C3a/LPS stimulation. Increased expression of genes is illustrated by increased distance to the bisecting line. Red dots represents genes which were more than 3 times up-regulated.***



IL-1β

| HMC | CBD | HMC | CBD | HMC | CBD | HMC | CBD |
|---|---|---|---|---|---|---|---|
| C | C | C3a | C3a | C3a / LPS | C3a/ LPS | PMA/ Ion | PMA/ Ion |

***Figure 8. PCR analysis confirmed the microarray analysis for IL-1β up-regulation following C3a/LPS stimulation in HMC-1 cells***

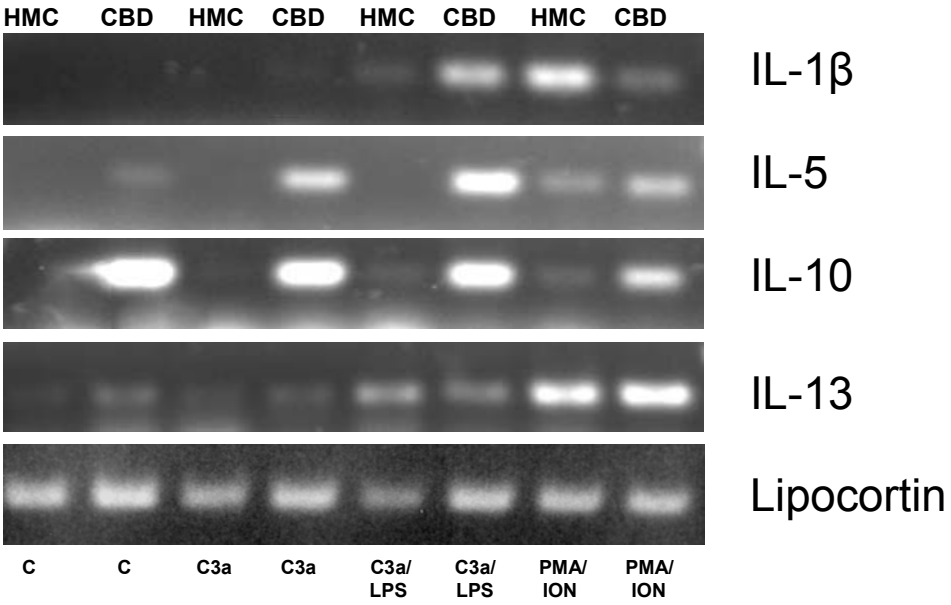
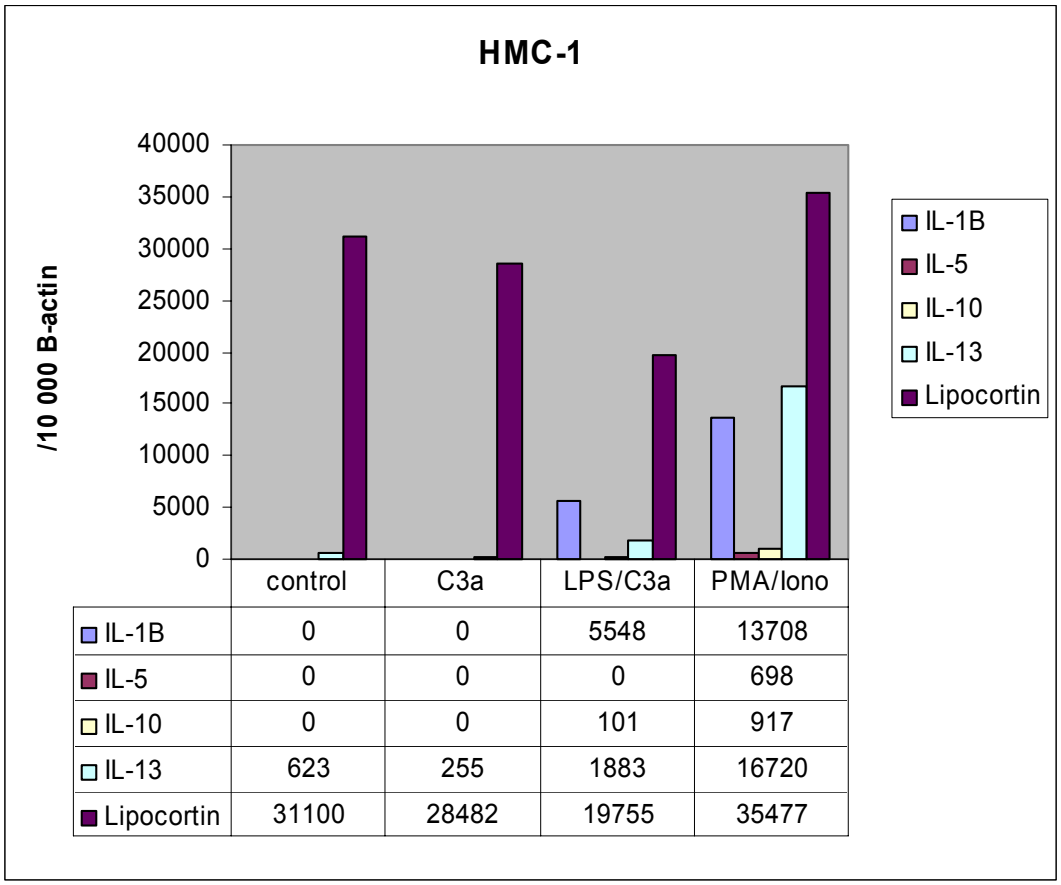
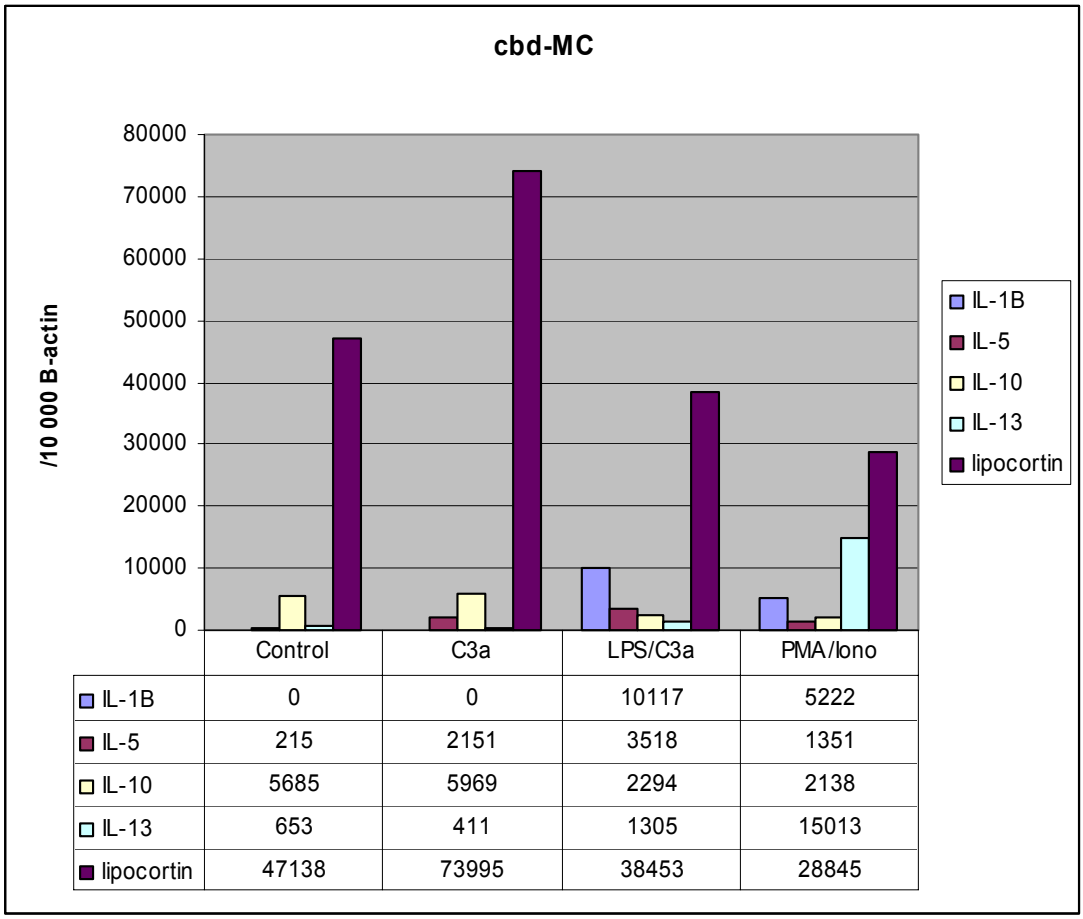



***Figure 9.***
***Cytokine mRNA expression by HMC-1 and CBD mast cells after 2 hours stimulation.***
***HMC-1/CBD cells ($5x10^5$ cells per ml) were grown under conditions as described in Materials and Methods. Control cells (C) were harvested after 2 hours. Stimulation was with rhC3a (500ng/ml), rhC3a (500ng/ml) + LPS (2µg/ml), or PMA (50ng/ml) + Ionophore ($10^{-6}$M).***




| | control | C3a | LPS/C3a | PMA/Iono |
|---|---|---|---|---|
| IL-1B | 0 | 0 | 5548 | 13708 |
| IL-5 | 0 | 0 | 0 | 698 |
| IL-10 | 0 | 0 | 101 | 917 |
| IL-13 | 623 | 255 | 1883 | 16720 |
| Lipocortin | 31100 | 28482 | 19755 | 35477 |

***Figure 10. Alterations in mRNA expression in HMC-1 cells following stimulation by C3a, LPS/C3a and PMA/Ionophore. Columns representing mRNA copies per $10^4$ β-actin based on pixel volume interpretations of bands shown in fig. 5***




| | Control | C3a | LPS/C3a | PMA/Iono |
|---|---|---|---|---|
| IL-1B | 0 | 0 | 10117 | 5222 |
| IL-5 | 215 | 2151 | 3518 | 1351 |
| IL-10 | 5685 | 5969 | 2294 | 2138 |
| IL-13 | 653 | 411 | 1305 | 15013 |
| lipocortin | 47138 | 73995 | 38453 | 28845 |

***Figure 11. Alterations in mRNA expression in cbd-MC cells following stimulation by C3a, LPS/C3a and PMA/Ionophore. Columns representing mRNA copies per $10^4$ β-actin based on pixel volume interpretations of bands shown in fig.5***

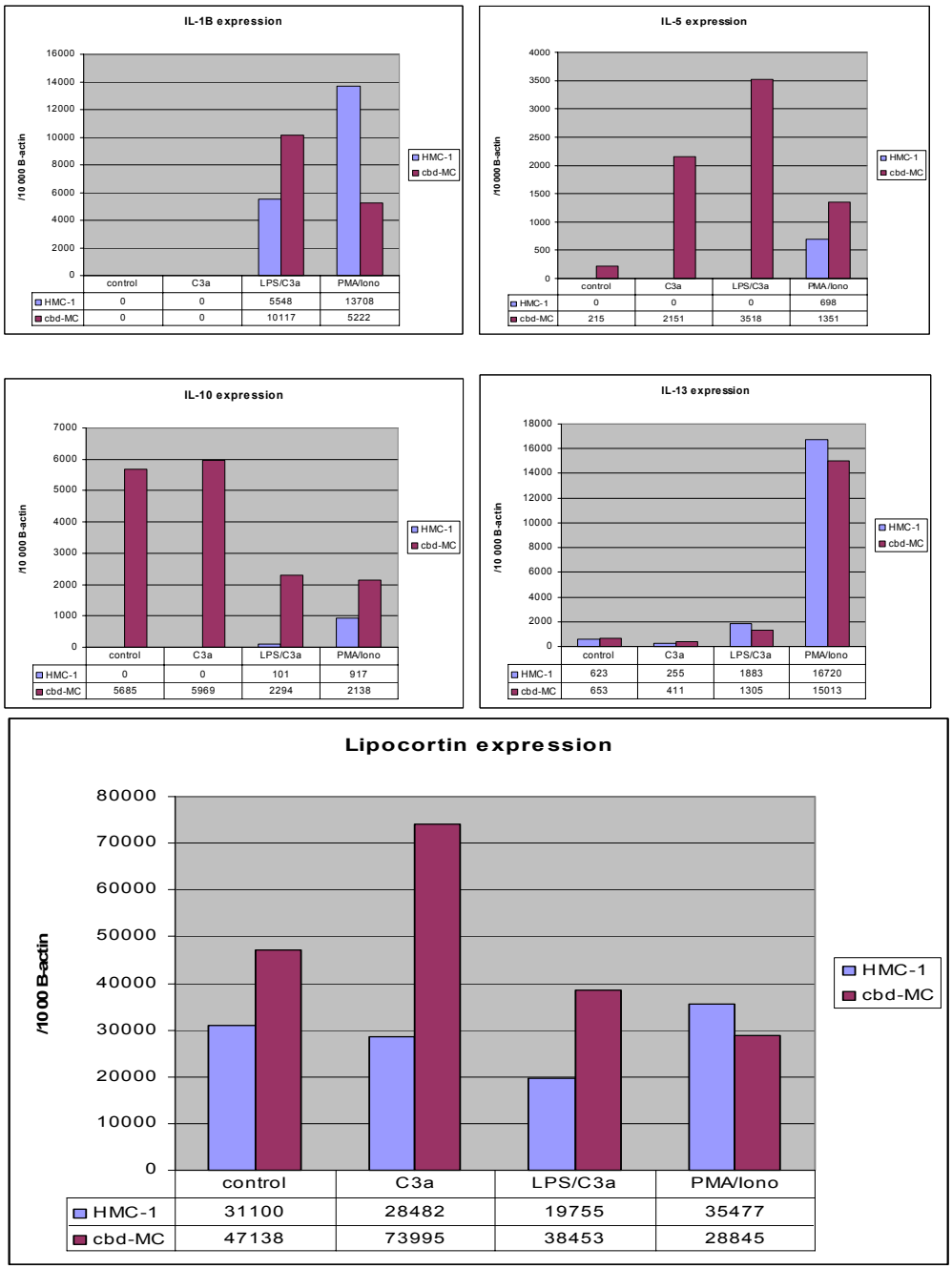



***Figure 12. Mast cells were stimulated with C3a, LPS/C3a and PMA/Ionophore. mRNA expression of a panel of cytokines were quantified . mRNA tended to be more highly expressed in cbd-MC. Columns expressing mRNA copies per $10^4$ β-actin based on pixel volume interpretations of bands shown in figure 9.***

## *4.3 HMC-1 express the C3a receptor*

HMC-1 ($1{,}0x10^6$ cells/ml) were stimulated with C3a at different concentrations (100 or 1000ng/ml) Flow cytometri were executed as described in *Materials and Methods*. Our results indicate that C3a-receptor is expressed on HMC-1 cell surfaces in equal amounts to positive controls(C-kit). No internalization of the C3a-receptor could be seen after 20 hours.

HMC-1 and cbd-MC ($1x10^6$cells/ml) were further stimulated with IL-4(100ng/ml) and IFN-γ (15ng/ml). Flow cytrometric analysis revealed that this did not affect C3a-receptor expression, and PCR confirmed that pre-stimulation by IL-4 or IFN-γ did not affect gene expression of C3a-reseptor (C3aR) in HMC-1 cells.

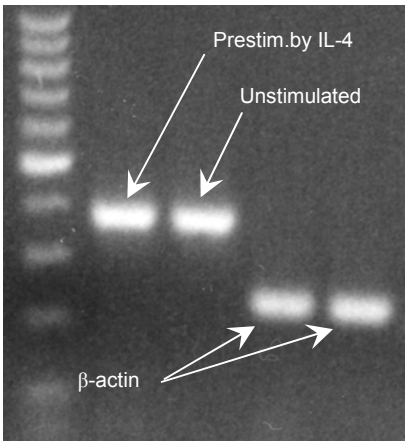



***Figure 13.: Pre stimulation with IL-4 in HMC-1 cells did not affect expression of C3a-reseptor (C3aR). β-actin confirms equal amounts of cDNA in both samples***

# 5. Discussion

## *5.1 LPS induce $T_H2$ cytokines from mast cells*

Stimulation of cord blood derived mast cells with lipopolysaccharide (LPS) from E. coli or peptidoglycan (PGN) from S. Aureus has recently been shown to induce the release of $T_H2$ associated cytokines: TNF-α, IL-1β, IL-5, IL-10 and IL-13, but not IL-4 from the cells. LPS and PGN interact with mast cells through TLR-4 and TLR-2 respectively (5, 10- 13, 50). These findings are in agreement with our preliminary studies on gene expression and mRNA expression following LPS stimulation. In addition to this, we have shown that cbd-mc constitutively express cyclo-oxgenase-2 (COX-2) and that they produce lipocortin. Following this project we will focus on a few of these in our continuing studies; IL-1β, IL-5, IL-10, IL-13, lipocortin, $PGD_2$ and TNF-α.

## *5.2 C3a induce airway hyper responsiveness*

Asthma is a chronic inflammatory disease of the lung, resulting in airway obstruction. C3a is released to the bronchoalveolar lavage fluid (BAL) after allergen challenge in asthmatic lungs and the inflammatory infiltrate of eosinophils and neutrophils correlates highly with the amount of anaphylatoxin present in BAL (18). A deletion in the C3a receptor gene prevent broncho-allergen induced smooth muscle contraction and recruitment of inflammatory cells in a murine model of asthma. Human asthmatics generate significant levels of C3a following intra-bronchial allergen deposition, but not saline. Thus C3a may be involved in the pathogenesis of asthma (16). Both mast cells and eosinophils express the G-protein coupled C3a receptor and are thought to be key players in asthmatic responses. Activation of this receptor leads to a transient mobilization of intracellular calcium ($Ca^{2+}$) (15). C3a promotes mast cell degranulation, resulting in release of histamine and leukotrienes, both of which can induce bronchoconstriction. The importance of non-IgE dependent mast cell activation in airway hyper responsiveness is further illustrated in IgE-deficient mice, which can develop antigen-induced airway hyper responsiveness (14, 17).

C3a is found in tissue from asthmatic patients, and regulation of IL-1β, IL-5, IL-13 and Lipocortin following C3a stimulation would indicate an important role for C3a in the development of the disease. In our studies following this report we will investigate the relation between C3a stimulation and cytokine expression further.

## *5.3 Regulation of $T_H2$ associated cytokines*

### 5.3.1 Mast cells regulate IL-1β in response to LPS/C3a stimulation

IL-1β is up-regulated in both cbd-MC and HMC-1 after stimulation with LPS/C3a. IL-1 is (together with TNF-α) considered to be the primary pro-inflammatory cytokine. They induce other cells to release a cascade of secondary cytokines amongst which are the chemokines- a subfamily of cytokines that attract and activate motile inflammatory cells. It is important particularly in the systemic responses of inflammation (e.g. fever), and often works synergically with TNF-α. IL-1β induces proliferation of activated B and T cells, $PGE_2$ and cytokines from macrophages, neutrophil- and T-cell-adhesion to endothelia, IL-6, IFN-$\beta_1$ and GM-CSF, fever, acute phase proteins and bone resorption by osteoclasts. The induction of bone resorption may indicate an important role of mast cells in tissue degeneration following periodontal disease.

### 5.3.2 Mast cells are important in recruitment of eosinophils

Our studies indicate that IL-5 is up-regulated in cbd-MC after stimulation with C3a and LPS/C3a. This cytokine is proliferative and chemotaktic on eosinophils, induce B cell proliferation, and stimulate IgM and IgA production. IL-4, -5, -6, -10 and -13 are associated with $T_H2$ type responses. IL-5 is predominantly involved in the late-phase of allergic reactions (51). In situ hybridization studies reveal an increase in the number of cells expressing IL-5 mRNA during the allergen induced late-phase reaction (52). These cells include eosinophils, mast cells and T cells, all of which produce variable amounts of IL-5 in human cutaneous late phase reaction. The strong association between IL-5 levels and asthma may be explained by the multiple effects of IL-5 on eosinophils in asthmatic patients. Eosinophils cultured in the presence of IL-5 display increased transmigration mediated by endothelial ICAM-1, and $\beta_2$-integrins on the surface of eosinophils (53). Serum levels of IL-5 during asthmatic exacerbation are significantly elevated as compared with those in remission (54). Thus, local IL-5 production may be important in recruiting eosinophils to the inflammatory sites.

### 5.3.3 Mast cells inhibit the $T_H1$ immune response

The high IL-10 expression in cbd-MC, but not in HMC-1, suggested a maturation dependent expression. This indicates that mast cells are able to produce and secrete high levels of this cytokine, which is known to down-regulate $T_H1$ responses (26). C3a and LPS did not change the level of mRNA IL-10 expression, suggesting that these cells produce it constitutively and may be able to excrete it in response to different stimuli. LPS has been reported to up-regulate IL-10 expression in MC/9 and BMMCs from mice (10) while HMC-1 secrete it constitutively (26). IL-10 is known to inhibit IFN-γ secretion from $T_H1$ cells. This underlines the fact that mast cells are associated with the $T_H2$ responses.

### 5.3.4 Bacterial products induce mast cells to produce IL-13

IL-13 was up-regulated in both HMC-1 and cbd-MC after stimulation with LPS, and grossly up-regulated after stimulation with PMA/Ionophore (5, 26) indicating that mast cells respond to bacterial inflammation with the production of this cytokine. IL-13 inhibits mononuclear phagocyte inflammation, and induce proliferation and differentiation of B cells. IL-13 is critical in the development of allergic asthma, although its mode of action is less clear (7).

### 5.3.5 Mast cells do not regulate TNF-α in response to LPS

We did not see up-regulation of TNF-α mRNA following stimulation with LPS, but recent studies have shown that mast cells secrete this cytokine following such stimulation (5, 26). This may indicate that mast cells secrete stored TNF-α when challenged with bacterial toxins. TNF-α is involved in tumor cytotoxicity, cachexia, induction of late phase proteins, anti-viral and anti-parasitic activity, activation of phagocytic cells, induction of IFN-γ, TNF-α, IL-1, GM-CSF and IL-6, and endotoxic shock. It induces adhesion molecules on endothelial cells and subsequent transmigration of inflammatory leucocytes (7). Release of pre-fabricated TNF-α following LPS stimulation may be one of the body's responses to bacterial infection.

## *5.4 Regulation of eicosanoid production*

Both HMC-1 and cbd-MC expressed $PGD_2$-synthetase. Mast cell activation induced new synthesis of arachidonic acid metabolites ($LTC_4$, $LTB_4$ and $PGD_2$). Eicosanoids mediate bronchoconstriction through interaction with the TP-receptor (main receptor for prostanoid $TXA_2$), vasodilatation, venular permeability and inhibition of platelet aggregation through the

DP-receptor (main receptor for prostanoid $PGD_2$). They also mediate other early and late phase symptoms of asthma and regulate immune cell activation (7, 34, 42, 43).

Lipocortin seems to be constitutively expressed in both HMC-1 and cbd-MC, but stronger in the latter. Lipocortin modulates the inflammatory response through inhibition of Phospholipase-A which is an important converting enzyme in the synthesis of eicosanoids from arachidonic acid. The eicosanoid production seems to be more complexly regulated than first assumed. While stimulation with C3a induced a dramatic up-regulation of lipocortin, stimulation with C3a in combination with LPS seemed to inhibit lipocortin synthesis. The initial up-regulation following complement activation appeared annulled by interaction with E-coli LPS. This suggested a diverse regulation of eicosanoid production in mast cells. Whereas, C3a-induced lipocortin up-regulation would result in down-regulated eicosanoid production, C3a /LPS induced lipocortin inhibition would have the opposite effect and stimulate the production of these mediators. This lipocortin mediated regulation of mast cell eicosanoid production could be important in modulating other inflammatory conditions such as periodontal disease.

### *5.5 Mast cells as important regulators of airway remodeling in asthma*

The plasminogen activator (PA) system has an important role in controlling endogenous fibrosis and regulating extra cellular matrix (ECM) proteolysis relevant to tissue remodeling. The tissue-type PA (tPA) convert plasminogen to plasmin, which enhances proteolytic degradation of the ECM via at least three different mechanisms. First, plasmin removes glycoprotein from the ECM before matrix metalloprotease (MMP)-dependent degradation of collagen. Second, plasmin activates MMPs directly to degrade ECM components. Third, plasmin degrades ECM by inhibiting MMP inhibitors. Because of the high concentration of plasminogen in virtually all tissues, the production of small amounts of PA can result in high local concentrations of plasmin. An important mechanism in the regulation of PA activity is inhibition of tPA by three major inhibitors; PAI-1, PAI-2 and PAI-3. Only PAI-1 and PAI-2 are relevant to lung fibrosis, and PAI-1 is 20- to 100-fold more efficient than PAI-2. Airway remodeling in asthma is characterized by subepithelial fibrosis due to extensive deposition of ECM. An increase in PAI-1 can block fibrinolysis by MMPs and thereby promote fibrin and collagen deposition. The presence of fibrin and collagen is the main feature of airway remodeling. HMC-1 was induced to express mRNA for plasminogen activator inhibitor-1 (PAI-1) after C3a/LPS stimulation. This in agreement with previous reports from Cho et al. who found increased release of PAI-1 after PMA/ionophore activation of the HMC-1 cell line. This suggest that mast cells can play an important role in airway remodeling of asthma, and inhibition of PAI-1 activity can represent a therapeutic approach in the management of airway remodeling. (55).

# 6. Conclusion

Microarray based screening for changes in mRNA expression followed by PCR-based conformation showed that the anaphylatoxin C3a in it self and in combination with bacterial LPS induced gene regulatory event. Several pro-inflammatory genes and enzymes involved in the production of inflammatory mediators were up-regulated after stimulating. The microarray generated more information than we could include in this report and thus more work will be needed to clarify the effect C3a stimulation has on mast cells.

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