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Modulation of chitinases and chitinase like proteins during macrophage differentiation and polarization

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ABSTRACT

Enzymatically active chitinases chitotriosidase (CHIT-1) and AMC-ase as well as Chitinase Like Proteins YKL-40, YKL-39 have specific patterns of association with immune response. Serum levels of CHIT-1 and YKL-40 mainly reflect the activation status of macrophages, and in particular lipid-laden macrophages. While chitotriosidase is a specific marker for lysosomal storage disorders, overexpression of YKL-40 was found in numerous of tumors, chronic and acute inflammations and during fibrosis progression. We compared the Chitinases variations during the differentiation and polarization of human macrophages. Gene expression analysis was investigated by real-time PCR from mRNA of human monocytes obtained from buffy coat of healthy volunteers, polarized to classically activated macrophages (or M1), whose prototypical activating stimuli are Interferon-gamma and lipopolysaccharide, and alternatively activated macrophages (or M2) obtained by interleukin-4 exposure. We found that CHIT-1 and YKL-40 were significantly modulated throughout monocyte-to-macrophage differentiation and polarization. In contrast, AMC-ase, YKL-39 showed slight modulation in the diverse stage of macrophages differentiation and/or polarization. In conclusion our results show that the expression of CHIT-1 AMC-ase, YKL-40 and YKL-39 is differentially regulated throughout monocyte-to-macrophage differentiation and polarization. Our finding suggests that whereas the immune function of AMC-ase and YKL-39 is restricted and selective, CHIT-1 and YKL-40 are protagonists in innate and acquired immunity.

KEYWORDS

Chitinases;
Chitinase like proteins;
Monocyte/macrophages;
M_1;
M_2;
Immuno-response.

INTRODUCTION

Chitinases are enzymes evolutionarily conserved belonging to the 18-glycosyl-hydrolase family, which are able to hydrolyze chitin and are part of the antiparasitic response against chitin containing organisms in lower life forms. Chitinases encompasses two genes encoding active chitinases, chitotriosidase

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(CHIT1) and acidic mammalian chitinase (AMC-ase or CHIA), and molecules named chitinase-like proteins (CLPs). CHIT-1 and AMC-ase represent an ancient gene duplication event, show sequence homology to bacterial chitinase[1] and possess true chitinase activity[2] whereas CLPs, which are the majority of the 18-glycosyl-hydrolase family members, are molecules that lack chitinase activity[3] as a result of mutations in their highly conserved enzymatic sites. The location of the AMC-ase (or CHIA) gene on human chromosome 1 (1q13.1e21.3) and the sequence homology and conservation of intron exon boundaries with CHIT-1, localized in chromosome 1q31-q32, confirms that these genes arose from a duplication event in an ancestor gene[2]. AMC-ase similarly to CHIT-1 gene consists of 12 exons and is relatively abundant in the gastrointestinal tract and lung indicating that may play a role as a food processor in stomach and is involved in lung inflammation[2,4]. Furthermore, the expression of AMC-ase in the lung suggests that the enzyme may have a dual function in digestion of chitinous substrates and host defense. AMC-ase is expressed in the epithelial cells and alveolar macrophages in patients with asthma and its production is driven by IL-4 and IL-13, indicating that it may play a role in the development of this disease[5]. In contrast, CHIT-1 is not an effector molecule in allergic inflammation but is rather regarded as a host-defense mechanism against chitin-containing pathogens[6,7]. CHIT-1, is a human analogue of chitinases from non-vertebrate species, that is used both as a diagnostic hallmark of Gaucher disease, and as a marker for monitoring the efficacy of various therapeutic approaches for treatment of GD[8]. The sources of secreted chitotriosidase are abnormal lipid-laden macrophages formed in tissues Gaucher disease patients[9]. Later higher levels of this enzyme was found in other pathological conditions including β-thalassaemia[10], sarcoidosis[11], multiple sclerosis[12], atherosclerosis and coronary artery disease[13], acute ischemic stroke[14], cerebrovascular dementia (CVD) and Alzheimer's disease (AD)[15], in the progression of nonalcoholic steatohepatitis[16,17], and in parasitic infections such as Plasmodium falciparum malaria[18].

YKL-40 (or chitinase-3–like 1) and YKL-39 (or chitinase-3–like 2) are the prototypes of enzymatically deficient CLPs. YKL-40 and YKL-39 are so called because their sequence contain a YKL motif, but, in contrast to YKL-40, YKL-39 is not a glycoprotein[19]. Biological activities of YKL-40 embrace regulation of cell proliferation, adhesion, migration and activation. YKL-40 is produced by variety of cells, including neutrophils, monocytes, and macrophages[20-22]. In addition, increased levels of YKL-40 protein and/or mRNA have been noted in patients with a wide spectrum of pathologies, including bacterial infections, rheumatoid arthritis, osteoarthritis (OA), giant cell arteritis, sarcoidosis, sclerodermia, diabetes, atherosclerosis, inflammatory bowel disease, and a variety of malignancies[20]. YKL-39, display anabolic properties in cartilage. It was originally identified as an abundantly secreted protein in primary culture of human articular chondrocytes[19]. YKL-39 mRNA is significantly up-regulated in cartilage of patients with OA[23]. Since the expression of YKL-39 was upregulated both in early degenerative and late stage of OA has been recognized as a biochemical marker for the activation of chondrocytes and the progress of the OA in human[24]. Proteomic analysis confirmed that YKL-39, is secreted by human osteoarthritic cartilage in culture[25]. Although chitinases and CLPs function in the inflammation is under examination, little is known regarding their regulation during macrophages full maturation and polarization. Macrophages play a crucial role in regulating the initiation, amplification, and resolution of innate immune responses. Several diseases including atherosclerosis, diabetes, cancer, and rheumatoid arthritis are associated with a deficiency or alteration in macrophage function[26]. Macrophages can be classically activated (i.e. M1) or alternatively activated (i.e. M2) based upon the response of these cells to the extracellular milieu (2). In the presence of interferon (IFN)-γ and lipopolysaccharide (LPS), macrophages become M1 activated while in the presence of T helper 2-type cytokines such as interleukin (IL)-4, macrophages undergo alternative activation or are skewed toward to M2 phenotype[27]. In this study we compared the modulation of CHIT-1, AMC-ase, YKL-

40 and YKL-39 during macrophage activation and their polarization in classical activated macrophages (M1) and alternative activated macrophages (M2).

MATERIALS AND METHODS

Cells

Human monocyte-derived macrophages (HMMs) were isolated from fresh buffy coat of healthy volunteers as previously described[28]. The buffy coat were diluted with phosphate-buffered saline (PBS) supplemented with 2.5mM EDTA and layered onto Ficoll-Hypaque gradients (Gibco, Invitrogen, Milan, Italy). After 30 min of centrifugation at 400 × g at room temperature, the mononuclear cells were collected, washed twice with PBS and placed in plastic Petri dishes at a concentration of $1 \times 10^6 - 2 \times 10^6$ cells/cm surface areas in Iscove's medium supplemented with 2mM glutamine, and 50 mg/ml of penicillin/streptomycin. After 2 h incubation, the non-adherent cells were washed out using PBS. The adherent cells (monocytes) were cultured in Iscove's medium supplemented with rHuman M-CSF 5ng/ml (Peproteck, BDA, Italy), 10% fetal bovine serum (FBS), 2mM glutamine, and 1% of penicillin/streptomycin (Invitrogen, Milan, Italy).

Macrophage differentiation

Macrophages were obtained by culturing monocytes for 7 days in Iscove's medium supplemented

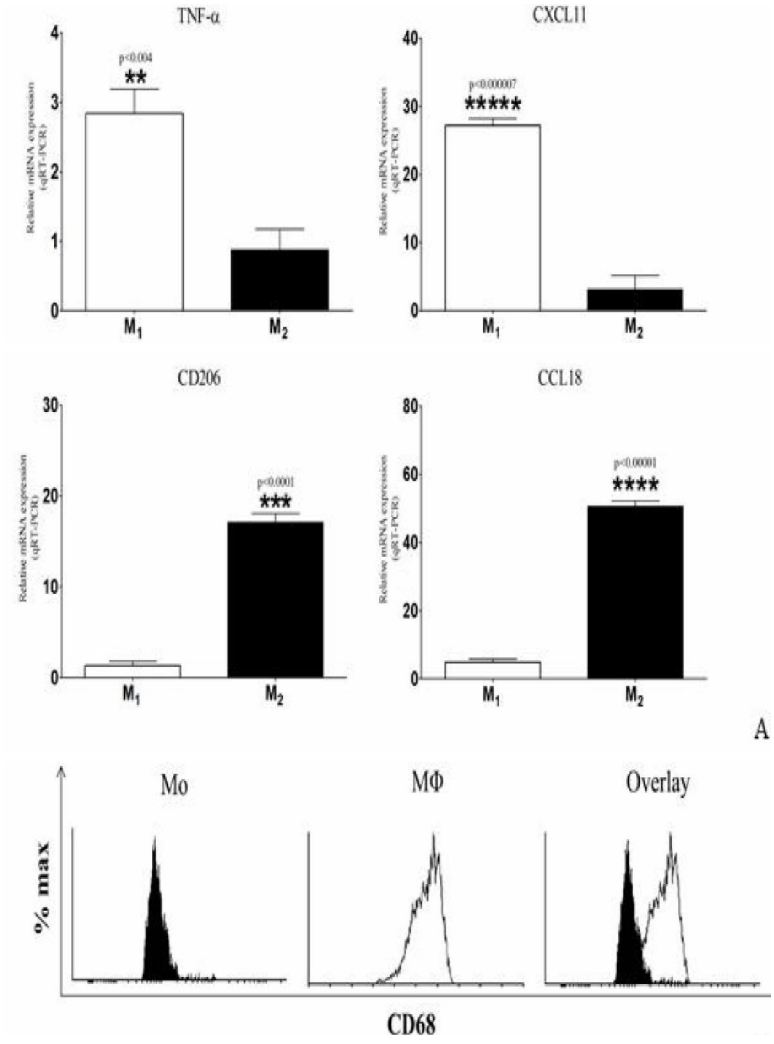

Figure 1 : A) Real-time PCR confirming macrophage polarization performed for two M1 markers (TNF-α and CXCL11) and two M2 markers (CCL18 and CD206). Statistical analysis was performed by Student's t test. B) Analysis by cytofluorimeter of marker CD68

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with 10% fetal bovine serum (FBS) 2mM glutamine, 1% of penicillin/streptomycin (Invitrogen) and 5 ng/ml M-CSF in Petri dishes at a density 1×10^6-2×10^6 cells/cm2. Macrophage polarization was obtained as previously described [29]. In brief, the culture medium was removed and cells were cultured for an additional 18 h in RPMI 1640 supplemented with 5% FBS and LPS (50 ng/ml) plus IFN-γ (100U/ml) (for M1 polarization) or IL-4 (20 ng/ml) (for M2 polarization) (Peproteck, Milan, Italy). Five different cell types were generated: freshly isolated monocytes (Mono T0), cells at intermediate differentiation (3 days of culture: Mono T3), resting fully differentiated macrophages (7 days of culture: Macrophages), classical activated macrophages (M1), alternative activated macrophages (M2) (Figure 1). Macrophage polarization was confirmed by real-time PCR for two M1 markers (TNF-α and CXCL11) and two M2 markers (CCL18 and CD206) (Figure 1).

Gene expression analysis by real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Milan, Italy). For reverse transcription–polymerase chain reaction (RT-PCR), 2 μg of total RNA was reverse-transcribed with High-Capacity cDNA Reverse Trascription Kit (Applied Biosystems, Monza, Italy) in a 20 ml reaction solution. Real-time fluorescence PCR, based on SYBR Green, was carried out in a 30 mL final volume containing 1 SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy), 200 nM forward and 200 nM reverse primers (supplementary TABLE 1) and 20 ng of cDNA. Thermal cycling was performed for each gene in triplicate on cDNA samples in MicroAmp Optical 96-well reaction plate (Applied Biosystems, Monza, Italy) with MicroAmp optical caps (Applied Biosystems) using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Monza, Italy). Amplification was carried out with the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles each of 95°C for 15 sec and 60°C for 1 min. Data are presented as mean % ± S.D. of at least three independent experiments. Differences were analyzed by Student t test, with $p<0.05$ being considered statistically signiûcant.

Flow cytometry

Flow cytometric measurements were performed using a four color FacsCalibur (BD Biosciences, San Jose, CA). Cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA), as per manufacturer instruction. Intracellular staining for CD68 was performed using PE-conjugated mouse anti-human CD68 (BD Biosciences, San Jose, CA). Data were analysed using Cyflogic software, version 1.2.1

Statistical analysis

Data are expressed as mean ± standard error (SE). Significance was assessed by one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

TABLE 1 : Primer pairs used in real-time PCR analysis

Genes	Forward	Reverse	Product size	Cycles
AM-CASE	CAAGCCCAGGCTGATGGTCACTGC	GTACAGATCTCGTAGTAAGCCC	402	30
CHIT-1	ACCCTGTTAGCCATCGGAGCTGG	TGCACAGCAGCATCCACCTTGAGG	470	25
YKL-40	GCCATCAAGGATGCACTCGCTGCA	GAAGAAATTCCCTTGCCAGGCTT	327	30
YKL-39	TCTTGACTGCGGGCGTATC	CCCAAGACCCATGGAAGTCA	119	30
CD206	AAGGCGGTGACCTCACAAG	AAAGTCCAATTCCTCGATGGTG	47	30
CCL18	CTCCTTGTCCTCGTCTGCAC	TCAGGCATTCAGCTTCAGGT	248	30
CXCL11	GCCTTGGCTGTGATATTGTG	TGATTATAAGCCTTGCTTGCTTCG	234	30
SOD2	GCTGACGGCTGCATCTGTT	CCTGATTTGGACAAGCAGCAA	100	30
TNF-α	AGCCCATGTTGTAGCAAACC	TGAGGTACAGGCCCTCTGAT	134	30
GAPDH	CTGCACCACCAACTGGAGGCTGG	AGGTCCACCACTGACACGTT	272	30

RESULTS

Levels of tumor necrosis factor (TNF)-α and superoxide dismutase (SOD2) during in vitro differentiation and polarization of peripheral blood monocytes

We detected the transcriptional levels of TNF-α and superoxide dismutase (SOD2) in order to ascertain that monocytes macrophages were not activated during differentiation. Over the first 7 days of differentiation, the levels of both TNF-α and SOD2 were significantly down-regulated, confirming a non-activation of the monocyte. We observed a reduction of TNF-α expression after five days of culture, whereas decreased significantly on the seventh day (p=0.015). The reduction of SOD2 expression ranged from 3.99 to 3.31 fold on the fifth and the seventh day respectively (Figure 2). The polarization of macrophages in M1 induced by LPS (50ng/ml) and IFN-γ (100U/ml) resulted in an increase of both TNF-α and SOD2 expression (16.5 fold, p =0.009; 2.19 fold p =0.006; respectively) (Figure 2). In contrast, both TNF-α and SOD2 expression was reduced in M2 polarized macrophages (Figure 2).

Comparison of CHIT-1, AMC-ase, YLK-40 and YKL-39 expression during in vitro differentiation and polarization of peripheral blood monocytes.

The comparison of the two chitinase (CHIT-1 and AMC-ase) and of CLPs (YLK-40 and YKL-39) modulation was investigated using quantitative real time RT-PCR on primary human monocyte-to-macrophage maturation and subsequent polarization into M1 or M2 cells. The levels of CHIT-1, AMC-ase, YKL-40 and YKL-39 during the differentiation of monocytes into macrophages and subsequent macrophages polarization into M1 and M2 showed significant differences. After 3 days of culture the levels of CHIT-1 expression were increased by 1.56 fold, compared to the day 0, a more substantial increase of 2.87 was observed for YKL-40 expression compared to the day 0 (Figure 3). On the contrary no significant variation was observed for AMC-ase and YKL-39 expression compared to the day 0. On the fifth day the expression levels of CHIT-1 and YKL-40 increased significantly by 15.12 and 10.89 fold, respectively, compared to the day 0 (Figure). On the seventh day, when monocytes were completely differentiated into macrophages as confirmed by cytoflorometric data (Figure 1B), the expression of both CHIT-1 and YKL-40 underwent to a further increase, by 15.75 and 13.97 fold, respectively compared to the day 0 (Figure 3).

In contrast, on the fifth and seventh day no significant variations were detected in AMC-ase and YKL-39 expression compared to the day 0 (Figure

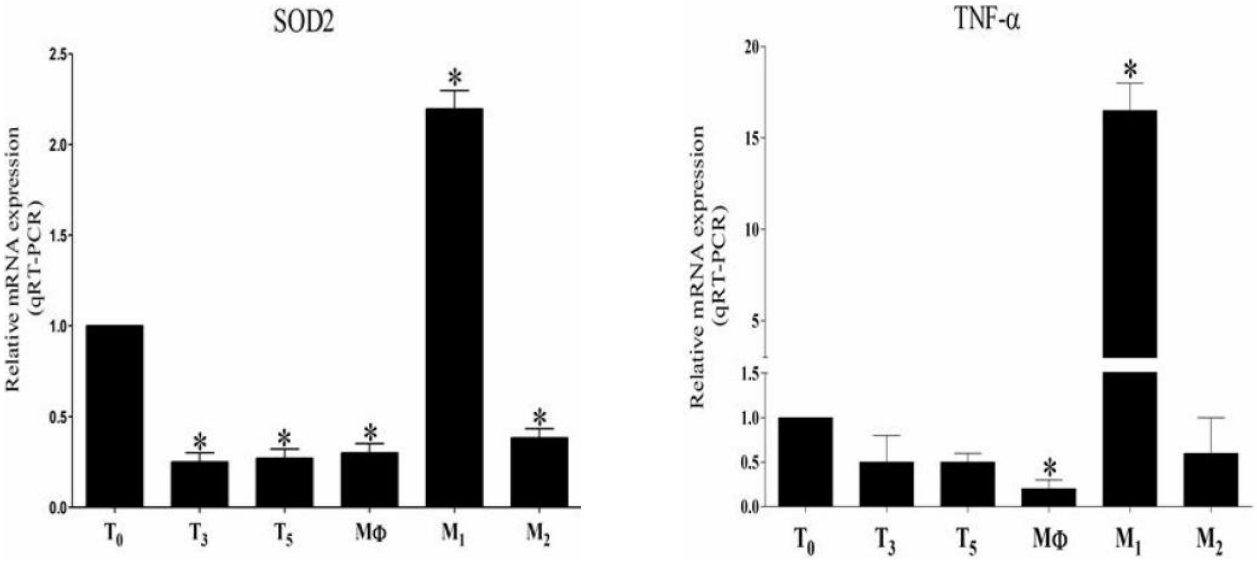

Figure 2 : Real-time PCR confirming the non-activation of the monocyte/macrophages. A) variation of SOD2 expression and B) TNF-α expression on monocyte/macrophages at day 0, 3, 5, 7 (M Φ), classical activated macrophages (M1), alternative activated macrophages (M2). All RT- PCR values are given as ΔCt values. Statistical analysis was performed by Student's t test. *P < 0.05 of differentiated or polarized cells versus day 0 cells

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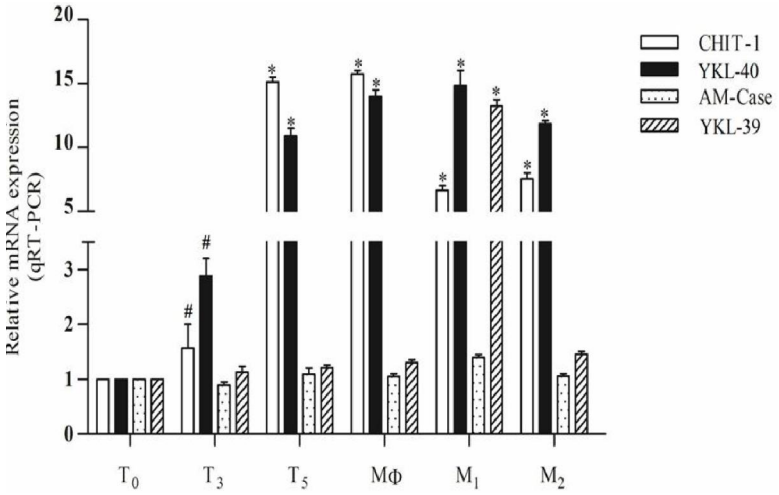

Figure 3 : Real-time PCR to detect CHIT-1, AMC-ase, YKL-40 and YKL-39 expression during human monocyte/ macrophages differentiation and polarization. T0, T3, T5 (monocyte/macrophages at day 0, 3, 5); M Φ 7 (macrophages at day 7), M1:classical activated macrophages, M2: alternative activated macrophages. All RT- PCR values are given as ΔCt values. Statistical analysis was performed by Student's t test. #P < 0.05 and *P < 0.001 of differentiated or polarized cells versus day 0 cells

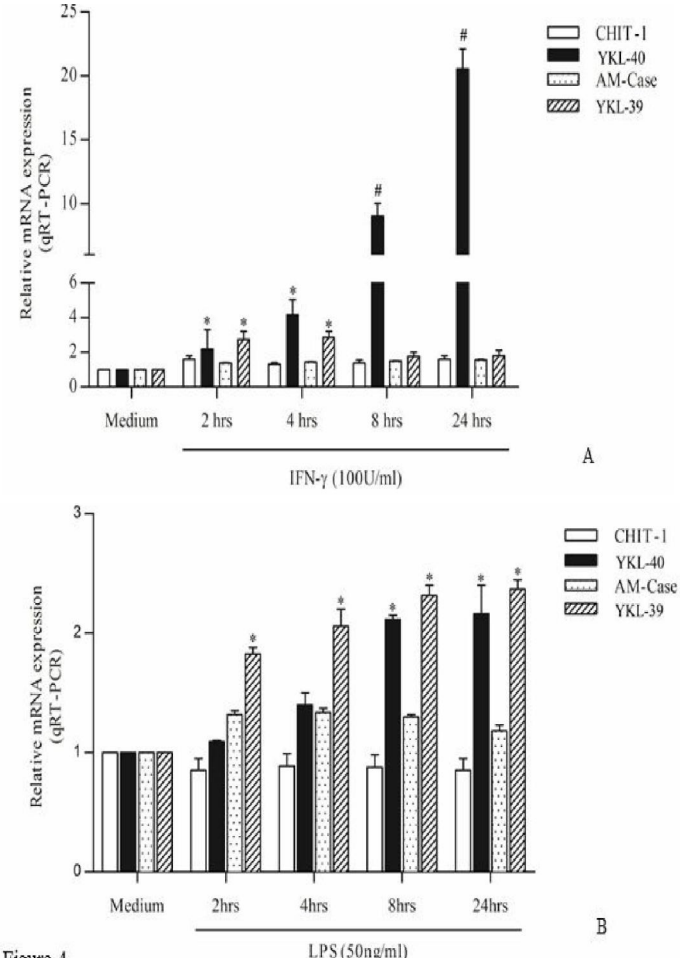

Figure 4 : Detection of CHIT-1, AMC-ase, YKL-40 and YKL-39 mRNA level by real time RT-PCR in undifferentiated monocytes, untreated and treated for 2, 4, 8, and 24 hrs with (A) IFN-γ(100 U/ml) (B) LPS (50 ng/ml). Statistical analysis was performed by Student's t-test. *P<0.05; #P < 0.001 of CHIT-1, AMC-ase, YKL-40 and YKL-39 expression of treated cells versus control

3). As M1 polarization occurred, CHIT-1 levels were increased by 6.62 fold compared to the day 0 as opposed to the day 0, but in comparison with day 7 it decreased significantly (Figure 3). In contrast, following polarization in M1 macrophages the levels of YKL-40 and YKL-39 significantly increased by 14.86 and 14.02 fold compared to day 0, whereas AMC-ase expression showed a modest increase by 1.39 fold compared to the day 0. YKL-39 expression levels were constant all through macrophages differentiation. The expression of YKL-40 presented a decreasing trend in comparison to M1, even if it maintained high levels (11.82 fold) compared to day 0. Vice versa CHIT-1 was more expressed in M2 than in M1 showing a fold increase by 7.52 compared to day 0 (Figure 3). Concerning, AMC-ase expression in M2, no variation was detected compared to the day 0 monocytes.

Effect of IFN-γ and LPS on the expression of CHIT-1, AMC-ase, YLK-40 and YKL-39 in monocytes

Afterwards we detected the actions of CHIT-1, AMC-ase, YLK-40 and YKL-39 in undifferentiated monocytes under treatment with M1 polarizing molecules IFN-γ and LPS. The cells were treated at different time points (2, 4, 8, 24 h), afterwards we measured the expression levels of CHIT-1, AMC-ase, YLK-40 and YKL-39 by Real Time PCR. We observed that the treatment with IFN-γ (100U/ml) and LPS (50ng/ml) did not produce a significant increase in the expression of CHIT-1. In fact, IFN-γ (100U/ml) barely induced CHIT-1expression by 1.67 fold at 2 h, by 1.21 and 1.34 at 4 and 8 h respectively and by 1.59 at 24 h compared to the control (Figure 4 A). The expression of CHIT-1 under the effect of with LPS (50ng/ml) was reduced by 0.31 fold within 2 h compared to the control and held steady within 24 h (Figure 4 B). We found that the treatment with IFN-γ (100U/ml) powerless modulated AMC-ase expression. As shown in Figure 4A IFN-γ increased AMC-ase expression by 1.37, 1.48, and 1.50 fold over that of the control within 4, 8 and 24 h respectively. As well, the treatment with LPS (50ng/ml) was able to modulate only slightly AMC-ase expression. As shown in Figure 4B LPS increased AMC-ase expression by 1.32, 1.33, 1.30, and 1.2 fold over that of the control within 2, 4, 8 and 24 h, respectively. In contrast the treatment with IFN-γ (100U/ml) led to a significant increase in the levels of YKL-40 expression (2.16 fold at 2h; 4.16 fold at 4h; 9.01 fold at 8h; 20.53 fold at 24h). A similar trend was observed in monocyte treated with LPS (50ng/ml); the fold increases compared to control cells were by 1.45, 2.08 and 2.23 fold, at 4, 8 and 24 h respectively (Figure 4B). The treatment with IFN-γ (100U/ml) increased YKL-39 expression by 2.69 and 2.83, over that of the control within 4 and 8 h, respectively (Figure 4 A). Similarly, the treatment with LPS (50ng/ml) increased YKL-39 expression by 1.87, 2.12, 2.39 and 2.41 fold over that of the control within 2, 4, 8 and 24 h, respectively (Figure 4B). These results suggested that undifferentiated monocytes reflected the chitinases modulation observed in M1 polarized macrophages.

Effect of IL-4 on the expression of CHIT-1, AMC-ase, YLK-40 and YKL-39 in monocytes

In order to examine the behavior of CHIT-1, AMC-ase, YLK-40 and YKL-39 in undifferentiated monocytes under stimulation with IL-4, used as M2 polarizing cytokine, we treated monocytes at different time points (2, 4, 8, 24 h). The levels of CHIT-1 expression enhanced significantly by 5.30 fold at 2h and 5.47 fold at 4h, showing thereafter a significant reduction at 8 and 24 h (1.04 fold and 5.66 fold, respectively). The pattern of expression of AMC-ase was found to be similar to the one detected for CHIT-1. We found that the expression of AMC-ase underwent 2.25 and 2.55 fold increase within 2 and 4h, respectively, as compared with the control, showing thereafter at 8 h a 1.31 fold decrease (Figure 5) relative to treatments at 2 and 4 h, this reduction of AMC-ase expression was significant at 24 h (2.68 fold, $p=0.04$) compared to 2 and 4 h treatments. The treatment with IL-4 (20ng/ml) decreased significantly the expression of YKL-40 and YKL-39 throughout the time. The expression of YKL-40 reached its minimum expression at 8 and 24 h with a reduction by 6.82 fold and 8.53 fold respectively, and the expression of YKL-39 underwent a 6.02, 8.12, 9.16 and 9.03 fold decreases within 2, 4, 8

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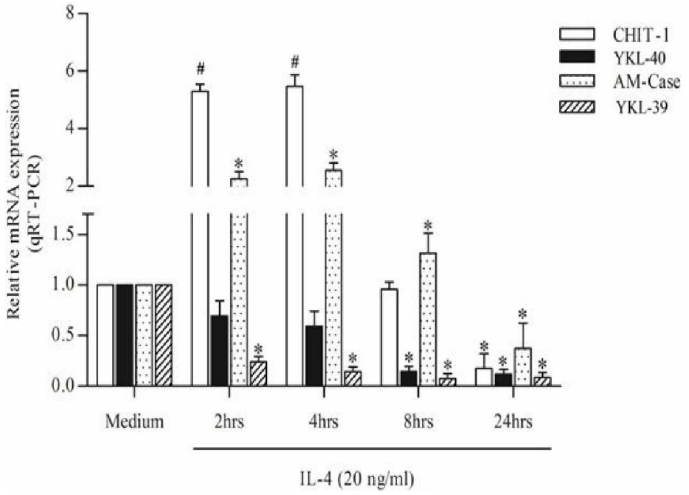

Figure 5 : Detection of CHIT-1, AMC-ase, YKL-40 and YKL-39 mRNA level by real time RT-PCR in M2-alternative activated macrophages, untreated and treated for 2, 4, 8, and 24h with IL-4 (20ng/ml). Statistical analysis was performed by Student's t-test. *P<0.05; #P < 0.001 of CHIT-1, AMC-ase, YKL-40 and YKL-39 expression of treated cells versus control

and 24 h respectively compared to the control cells (Figure 5).

DISCUSSION

In this study we compared the modulation of the expression of CHIT-1, AMC-ase, YLK-40 and YKL-39 during HMMs differentiation and polarization. We found that the effective maturation of monocytes into macrophages is characterized by different patterns of chitinases expression. We observed that both CHIT-1 and YKL-40 expression exponentially increase of in the course of the time, but, whereas CHIT-1 showed a peak of expression between the fifth and the seventh day of culture, YKL-40 showed a peak of expression on the seventh day of culture and in polarized M1 macrophages. In contrast, the pattern of AMC-ase expression during the diverse stage of HMMs differentiation and/or polarization was barely modulated. As well to AMC-ase, YKL-39 expression did not show significant changes during HMMs differentiation. M1 polarized macrophages, resemble to Th1 cells. Similarly to Th1 cells they secrete proinflammatory cytokines and an array of cytotoxic molecules that help the clearance of invading pathogens and stimulate the acquired immune response. A diverse pattern of AMC-ase CHIT-1, YLK-40 and YKL-39 expression was detected also in undifferentiated monocytes treated with IFN-γ or LPS. We found that the treatment with IFN-γ and LPS barely enhanced AMC-ase expression, suggesting that AMC-ase acts precociously but weakly against infections and therefore may have a function in innate immunity. The major sites of expression of AMC-ase are airway epithelial cells. Our finding indicates that AMC-ase, unlike CHIT-1, is not selectively expressed and highly regulated in activated macrophages, nevertheless its slight increases in M1 stage and its modulation following treatment with pro-inflammatory stimuli indicates that the antimicrobial pathway in HMMs involves also a slight activation of AMC-ase. In undifferentiated monocytes the treatments with IFN-γ or LPS were ineffective on CHIT-1 expression. Previously we reported the stimulatory effect of IFN-γ, and LPS on the expression of CHIT-1 in macrophages[30]. This discrepancy can reflect a long-range contribution exerted by CHIT-1 operant with macrophages differentiation. CHIT-1 exerts a central role in an expanding spectrum of disorders ranging from granulomatous disease such as sarcoidosis 13 to a large amount of infections such as tuberculosis[31] and leprosy[32].

Concerning YKL-40 expression in undifferentiated monocytes, we found that the treatment with LPS significantly induced YKL-40 expression after 8 h of treatment. As well, IFN-γ treatment enhanced significantly YKL-40 expression at 8 h and reached the higher expression at 24 h. The modulation of YKL-

40 following treatment with pro-inflammatory stimuli in monocytes and its strong increases in M1 stage indicates a vigorous activation of YKL-40 during the early phases of immune response against pathogens invasion. Similarly to CHIT-1, YKL-40 is selectively expressed in activated macrophages suggesting that CHIT1 and YKL40 exert similar biological functions. In this contest a great attention must be given to the fact that both CHIT-1 and YKL-40 produced by differentiated macrophages, can be detrimental for the host tissues and can be implicated in the progression of a number of chronic inflammatory diseases. This concept is also supported by our previous findings in which we observed that overproduction of CHIT-1 could exert deleterious effect in many degenerative disorders[15]. Instead, a functional polymorphism in the CHIT-1 gene protects from NAFLD progression[33]. As well, genetic variation within the CHIT-1 gene was strongly associated with human longevity and with several phenotypes of healthy aging[34].

Regarding the effect of LPS on the expression of YKL-39 in undifferentiated monocytes we found that it was able to enhance YKL-39 expression after 2 h of treatment and went on over the time. Instead IFN-γ treatment enhanced YKL-39 expression at 2 h and reached the higher expression at 4 h, suggesting that monocytes recruited to the sites of inflammation can be themselves a source of YKL-39. In contrast to CHIT-1 or YKL-40, YKL-39 was found to be express only by synovial fibroblasts during OA[19]. Later, was reported that YKL-39 was slightly expressed in macrophages differentiated in the presence of IFN-γ or IL-4[35]. The demonstration of low levels of YKL-39 transcripts in macrophages is consistent, in part, with our results. Here we present the evidence that M1 macrophages and undifferentiated monocytes in response to IFN-γ or LPS are able to produce YKL-39. It has been shown that Th1 cells predominate in the synovium of patients with OA[36]. Consequently, IFN-γ which is one of the prevailing cytokine in OA tissues is able to induce the production of YKL-39 by monocyte-derived macrophages. In patients with OA, the prevalence of autoantibodies to YKL-39 and other auto-antigens on early stages of disease implied that the autoimmune response occurs during the initial phase of cartilage degeneration[37]. This finding suggests that synovial fibroblasts do not represent the sole source of YKL-39 in OA.

Alternative M2 macrophages, basically reproduce the Th2 anti-inflammatory response, they express phagocytic receptors, anti-inflammatory cytokines and release products that promote tissue regeneration and healing. It is believed that Th2 inflammation originally evolved to deal with parasites, whereas allergy and atopic asthma arise as a consequence of poorly controlled Th2 responses elicited independently of parasitic infection.

Despite in M2 macrophages the levels of AMC-ase were unchanged compared to the levels observed in undifferentiated monocytes, IL-4 treatment in undifferentiated monocytes increases significantly AMC-ase expression in particular at 2 and 4 h, confirming that the expression of AMC-ase is induced by Th2-type response. AMC-ase is specifically upregulated in response to Th2 inflammation in the lung[5]. Furthermore, inhibition of AMC-ase may inhibits this inflammation[5]. Additionally, this result clarifies why AMC-ase increased secretion is closely associated with pathophysiological conditions dominated by Th2 type cells such as allergy and asthma[5]. Chitin has been shown to induce inflammatory cell recruitment[38] while the immunological actions of chitin would normally be limited in mammals by chitinase-mediated chitin degradation, interference with chitinase enzymatic activity would likely result in chitin accumulation. Therefore, both CHIT1 and AMC-ase may be required to ensure full degradation and clearance of chitin. The level of chitinase activity in the lung and the predominance of one enzyme over the other may influence the size and quantity of chitin degradation products, which has been shown to determine the inflammatory outcome[39].

Interestingly, in undifferentiated monocytes IL-4 treatment induced a significant increase on CHIT-1 expression, suggesting that expression of CHIT-1 is also induced by Th2-type response. The Th2 cytokine IL-4 promotes immune responses to parasites. This finding explains why CHIT-1 increased secretion is closely associated with pathophysi-

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ological conditions dominated by T-helper type 2 cells including infections with fungal pathogens and malaria parasites, fibrosis, allergy and asthma. Additionally, IL-4 is a well appreciated antagonist of the M1 response and macrophage pro-inflammatory properties[40]. IL-4 not only promotes fibrosis through TGFβ production[41] but also activates PPAR-γ[42] and PPAR-δ[43] to induce monocyte/macrophage alternative activation. Interestingly, IL-4 leads a M2 phenotype in regressive atherosclerotic lesions[44], providing in such a way a potential explanation of why CHIT-1 activity is associated with the presence of atherosclerosis[45]. Macrophages are paradoxically involved in both generation of fibrosis and its resolution; moreover M2 generates a positive feedback loop during resolution of inflammation. It is unclear what are the events influencing M2 differentiation and interrupting tissue repair/remodeling as well fibrotic outcomes. This finding could support the idea that increased levels of CHIT-1 in this stage could be involved in the modulation of the extracellular matrix affecting cell adhesion and migration during the tissue remodeling processes that take place in fibrogenesis[16,17]. These same fibrotic changes occur over time in the lung fibrosis as a result of chronic and acute inflammation.

Although in M2 macrophages YKL-40 maintained high levels of expression, IL-4 treatment in undifferentiated monocytes was ineffective on YKL-40. Some reports have suggested that YKL-40 may play a causative role in Th2 cell-mediated inflammation[46]. What is evident is that YKL-40 is enhanced in differentiated and polarized macrophages, therefore it seems to have pleiotropic effect in inflammation. The involvement of YKL-40 and CHIT-1 in human disease show a good deal of similarity. YKL-40 has also been identified as a protein expressed during conditions of tissue remodeling[19], atherogenesis[47], cell-proliferation and-survival[48], in the modulation of the extracellular matrix affecting cell adhesion and migration during the tissue remodeling processes that take place in fibrogenesis[20]. Increased serum levels of YKL-40 have been reported in patients with several inflammatory disorders including inflammatory bowel disease49, asthma50 and rheumatoid arthritis[51]. Furthermore, high YKL-40 levels in serum were an independent prognostic parameter for shorter survival disease severity, poorer prognosis, and shorter survival in many human neoplasias[52], suggesting that YKL-40 may be a growth factor of cancer cells and/or may protect them from undergoing apoptosis[48]. Both CHIT-1 and YKL-40 are rarely detectable in healthy individuals. Of note, both CHIT-1 and YKL-40 may have a specific affinity with certain pathogens.

Similarly to YKL-40, IL-4 treatment was ineffective on YKL-39 in undifferentiated monocytes. A study of Gratchev et al. showed that the combination of IL-4 and TGF-β had strong stimulatory effect on the expression of YKL-39 in macrophage cultures[53]. Nevertheless they reported that IFN-γ has inhibitory effect on the production of YKL-39 by monocyte-derived macrophages, which is in contrast with our results.

In conclusion our data showing the different modulation of AMC-ase, CHIT-1 YKL-40 and YKL-39 production, during macrophages differentiation and polarization suggest that all these chitinases play different rules in the immune response. Specifically, they indicate that CHIT-1 and YKL-40 are up-regulated in response to inflammatory stimuli in innate response but, as macrophages shift towards the M1-M2 phenotype, they can be having a crucial role in phagocytosis, bacterial killing and initiation of the adaptive immune response. Moreover, since M2 cells scavenge debris, phagocytize apoptotic cells after inflammatory injury, orchestrate tissue remodeling and repair through the production of extracellular matrix proteins, it is conceivable that CHIT-1 and YKL-40 may exert an additional role during Th2-driven immune response. In contrast, the slight modulation of AMC-ase and YKL-39 expression showed that the function of these chitinase is much more restricted and selective than the one exerted by CHIT-1 and YKL-40. Further investigations are indispensable to understand the physiological activity of chitinases and CLPs as well as the mechanisms of their cell-type specific secretion in the context of the whole organism in order to develop cell-type specific therapeutic approaches.

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