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Research article

Alveolar macrophages in allergic asthma: An expression signature characterized by heat shock protein pathways

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ABSTRACT

The implication of alveolar macrophages (AM) in asthma, a T_h2 disease, has not been well characterized. Thus, the goal of this study is to better characterize AM phenotype of allergic asthmatic compared with normal subjects using genomic expression analyses. Microarray analyses were performed with AM isolated from bronchoalveolar lavage. Robust multiarray analysis (RMA) normalization and Smyth's moderated *t* test were used to select differentially expressed genes. Fifty differentially expressed genes were identified. Nineteen have been classified in categories linked to stress or immune responses and among them; nine are part of the heat shock protein (HSP) family. Difference of expression for three (*HSPD1, PRNP, SERPINH1*) of the five selected genes were validated using real-time reverse transcription–polymerase chain reaction. Enzyme-linked immunosorbent assay was used to measure the protein level of heat shock protein 60 (HSP60), the protein encoded by *HSPD1*, and showed difference in AM protein level between allergic asthmatic and control subjects. In summary, this study suggests that HSP gene family, particularly HSP60, is involved in AM functions in a context of allergic asthma. These results also support the involvement of AM immune functions in the development of an allergic asthmatic response.

1. Introduction

Asthma is a chronic disorder of the airways characterized by reversible airflow obstruction, airway inflammation, remodeling, and hyperresponsiveness [1]. Several functional aspects of AM justify their study in allergic asthma. These cells, distributed from the alveoli to the upper airways, are the main immune cells in the airway lumen and are in direct contact with exogenous agents. They participate in lung immune homeostasis through phagocytosis and the release of mediators in response to these agents [2]. They also modulate immune response by inhibiting antigen presentation by dendritic cells, T-cell activation, and B-cell antibody production [3,4].

Moreover, AM are stimulated in the course of innate and acquired immunity, including humoral immune responses associated with the production of antibodies and cellular immune responses characterized by the production of T_h1 (interferon (IFN)–γ, interleukin (IL)–12, IL-18) and T_h2 (IL-4, IL-13) lymphokines, as well as by IL-10, the main regulatory cytokine [5]. These types of activations induce different responses from these cells. Indeed, they can produce both pro- and anti-inflammatory mediators, as well as T_h1 and T_h2 cytokines, and consequently have the capacity to orientate the type of immune response triggered [5,6].

Differences have been observed in AM phenotype of allergic asthmatic subjects compared with healthy subjects. Indeed, allergic asthmatic AM show a lower phagocytic activity and a greater antigen presentation activity than control cells [7,8]. Moreover, the transfer of AM from allergy-resistant rats (Sprague-Dawley) to allergy-susceptible rats (Brown Norway) demonstrates the importance of AM in inhibiting the early phase reaction and airway hyperresponsiveness in response to allergen challenge. It also suggests a different phenotype for AM of allergy-susceptible rats [9].

Characterization of the expression profile of AM of asthmatic and control subjects should help to better understand the implication of this cell in asthma. Microarray analyses of gene expression have already demonstrated their effectiveness in the characterization of tissue or cellular phenotype in asthma and other complex traits [10]. One of its principal advantages is to allow the discovery of novel possible pathways as this technique looks at all genes independently of their respective known functions [10]. According to this, gene expression microarrays have been chosen to achieve the principal goal of this study, which was to characterize AM phenotype of allergic asthmatic subjects. Our results demonstrated

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Table 1
Clinical characteristics of the subjects involved in microarray analysis and real-time RT-PCR

	Microarrays and qRT-PCR ($n = 5$)[a]		Immunohistochemistry ($n = 4$)		ELISA ($n = 5$)	
	Ctrl	AA	Ctrl	AA	Ctrl	AA
Male:female ratio	3/2	3/2	2/2	2/2	4/1	4/1
Mean age, y (range)	26 (20–37)	29 (24–36)	25 (20–30)	28 (20–34)	28 (23–36)	24 (18–29)
Smoking status						
Never	4	4	3	4	5	4
Ex-smoker[b]	1	1	1			1
AM% (SD)	76 (10)	86 (10)	75 (9)	70 (6)	79 (16)	81 (9)
FEV_1% predicted (SD)[c]	94 (10)	103 (19)	96 (9)	90 (6)	102 (4)	102 (18)
PC_{20} mg/ml (SD)[d]	>16 to >128	2.13 (2.45)	>16 to 51.54	0.59 (2.54)	>32 to >128	1.35 (3.74)
Allergy	No	Yes	No	Yes	No	Yes

AA, allergic asthmatics; AM, alveolar macrophages; Ctrl, controls; FEV_1, forced expiratory volume in 1 second; PC_{20}, provocative concentration of methacholine that induces a 20% fall in FEV_1; SD, standard deviation.
[a]For one sample of each phenotype, there was not enough RNA to do RT-PCR after microarray analysis.
[b]The ex-smokers are the subjects which smoked a maximum of six packages a year and which stopped smoking more than 5 years ago.
[c]Geometrical mean calculated for all controls and allergic asthmatic subjects of each group.
[d]Mean calculated for all allergic asthmatic subjects of each group. Only ranges are shown for control subjects because of noncontinuous data.

a possible implication particularly for HSP in the immune functions of AM in asthma physiopathology, as nine HSP were underexpressed by AM of allergic asthmatic subjects. Moreover, protein level of HSP60 suggests especially a role for this biomarker in immune functions of this cell type.

2. Subjects and methods

2.1. Evaluation of gene expression

2.1.1. Subjects and samples

Five control subjects without allergy or asthma and five allergic asthmatic subjects meeting the American Thoracic Society criteria for the diagnosis of asthma [11] participated in microarray and real-time reverse transcription–polymerase chain reaction (RT-PCR) studies. All subjects were recruited at respiratory specialized clinic and gave their consent for participation. The baseline evaluation included a questionnaire on health status and respiratory conditions, a physical examination, skin prick tests using 26 common aeroallergens (pets, house dust mites, dust, tree pollens, grass pollens, ragweed, molds, and feather) and the measurements of forced expiratory volume in 1 second (FEV_1) and of the dose of methacholine that resulted in a 20% fall in FEV_1 (PC_{20}). Clinical characteristics of the subjects are given in Table 1. Asthmatic subjects had positive skin prick tests (at least one positive response, *i.e.*, wheal diameter ≥3 mm at 10 minutes). Table 2 lists the tested allergens and the sensitization profile of the subjects. Asthmatic subjects presented asthma symptoms that required treatment with an inhaled short-acting β_2-agonist on demand or associated allergic rhinitis symptoms that required a histamine-antagonist on demand. The bronchoscopies and the bronchoalveolar lavages were performed for research purposes only; they were performed as previously described on local anesthesia and using five 60-ml boluses of prewarmed saline solution in the right middle lobe [12]. To improve homogeneity of the samples, bronchoalveolar lavages were performed at the same time of the day and only if subjects did not experience inflammatory or allergen response (*e.g.*, flu or hay fever) during the previous month. Bronchoalveolar lavages were not performed during the pollen season for the sensitized subjects. RNA was available for four subjects per phenotype to run real-time RT-PCR.

2.1.2. Genomic expression

AM were purified by adherence (2 hours) from bronchoalveolar lavage, giving purity >98%. Cell purity was verified using Diff-Quik staining. RNA was isolated using a silica gel-based RNeasy spin column protocol (Qiagen, Valencia, CA). Microarray analysis was performed with Affymetrix Genechip HG-U133A microarrays containing 22,283 oligonucleotide target probes (Affymetrix, Santa Clara, CA). Hybridization and scanning of image were performed at the McGill University and Genome Quebec Innovation Centre (www.genomequebec.mcgill.ca). To minimize technical variability, RNA processing steps (RNA extraction, probe labeling and chip hybridization) were performed in parallel for control and asthmatic samples. Detailed probe synthesis hybridization and washing protocol have been previously described [13]. The scanned images were analyzed using the Microarray Analysis Suite 5.0 (MAS5, Affymetrix) and the raw image files obtained (CEL format) were used for statistical analysis. Analyses were performed with the Affy and Limma packages available in Bioconductor (http://www.bioconductor.org), which uses R language (http://www.R-project.org) [14,15]. Quality tests to look at artifact or variability among microarrays were done with the Affy package. Probe intensities from the 10 chips were first normalized using robust multi-array analysis (RMA), which comprises background correction, quantile normalization, and median polish steps. Use of the RMA method in conjunction with p value and fold change cut-off (p value < 0.05 and absolute fold change >1.50) allowed us to not perform multitesting correction [16,17]. To even better decrease false discovery rate, Smyth's moderated t test was used instead of standard t test (Limma package) [16].

Table 2
Allergen sensitization profiles of allergic asthmatic individuals

Methods	Subjects	Allergen sensitization profiles[a]
Evaluation of gene expression		
Microarrays and real-time RT-PCR	1[b]	P, G, R, T, H, D, F
	2	P, G, R, T, H, D
	3	P, G, R, T, M, H, D
	4	P, G, R, T, F
	5	P
Evaluation of protein level		
Immunohistochemistry	1	P, G, R, T, M, H, D
	2	P, G, R, T, M, H, D
	3	P, G, R, T, M, H, D, F
	4	P, T, D
ELISA	1	P, G, R, T
	2	P, G, R, T, H, D
	3	P, H
	4	P, R, T, M, H, D, F
	5	P, R, T, H, D, F

[a]List of allergens tested: P, pets (cat, dog, cow, horse); G, grass pollens (cocklebur, grass, plantain, ryegrass, timothy, wormwood); R, ragweed; T, tree pollens (ash, birch, elm, maple, oak, poplar, mix of trees); M, mold (Alternaria, *Aspergillus fumigatus*, Hormodendrum, Penicillium); H, house dust mites (*D farinae*, *D pteronyssinus*); D, dust and F, feather.
[b]This sample was only used for microarray analyses because there was not enough RNA to do real time RT-PCR.

2.1.3. Relative quantification of genetic expression by real-time RT-PCR

Five genes (chemokine CC beta receptor 1 (*CCR1*), heat shock 60-kDa protein 1 (*HSPD1*), 2,5-oligoadenylate synthetase 1 (*OAS1*), prion protein (*PRNP*), and serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (*SERPINH1*)) were selected to be tested using real-time RT-PCR. First, RNA integrity was evaluated using capillary electrophoresis. Sensiscript RT (Invitrogen, Burlington, ON, Canada) with oligodT(20) primers was used for cDNA synthesis. The SYBR Green RT-PCR reaction was performed in 72-wells in the Rotor-Gene 3,000 (Corbett Research, Sydney, Australia) with 1.5 ng of genomic DNA and 1.5–2.5 mM $MgCl_2$, buffer 1X (1.5 mM $MgCl_2$), 0.8 mM dNTPs, 0.4 μM gene-specific primers (see Supplementary material), 0.5X SYBR Green, and 1 U of Taq DNA polymerase (Qiagen) for a final volume of 20 μl. For *SERPINH1,* QuantiTect SYBR Green PCR Kit was used with QuantiTect Primer Assay (Hs-SERPINH1_1-SG; Qiagen). Each sample was run in triplicate with a negative control and averaged for final RNA quantification. A standard curve was done with three serial dilutions in triplicate for each selected gene and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was used as housekeeping gene. Quantification obtained from standard curves of each gene was normalized to the relative amount of *GAPDH* using the two standard curves method implemented in the Rotor-Gene 6 software (version 6.0). Measures of expression for the two phenotypes expressed as mean ± SEM were compared by Student's unilateral *t* test. A *p* value <0.05 was considered significant.

2.2. Genetic association study

2.2.1. Subjects and samples

The sample comprised 253 families (probands and their relatives) originating from Saguenay–Lac-Saint-Jean for a total of 1275 DNAs. A proportion of 37.0% of relatives were affected by asthma and 51.3% were atopic. All information concerning sample recruitment and clinical evaluation of the subjects were described in a recent report [18]. Clinical characterization is available in Table 2 of the Supplementary material. (Also see the Supplementary material for a description of the methodology.)

2.3. Evaluation of protein level

2.3.1. Subjects and samples

Cells were obtained from bronchoalveolar lavage fluids of nine control subjects without allergy and asthma as well as nine allergic asthmatic subjects. All subjects gave their consent for the study. Clinical evaluation and bronchoalveolar lavage procedure were done as described earlier there. Tables 1 and 2 list clinical characteristics and allergen sensitization profiles of the subjects. Asthmatic subjects presented asthma symptoms that required treatment only with an inhaled short-acting β_2-agonist on demand or associated allergic rhinitis symptoms that required a histamine-antagonist on demand.

2.3.2. Validation of protein expression by immunocytochemistry

Protein expression was observed using ABComplex/AP method (Dako Cytomation, Mississauga, ON, Canada) as previously described [19]. Slides were fixed with PLP-sucrose, and coloration was done using Fast Red and Mayer's hematoxylin (Dako Cytomation). The anti-HSP60 antibody used for this experimentation was the H1830-77 from US Biological (Swampscott, MA) and the secondary antibody was the polyclonal rabbit anti-mouse (E0464) from Dako Cytomation. Isotype-matched immunoglobulin was used as control (0311D, Becton Dickinson).

2.3.3. Quantification of protein level by ELISA

AM were purified by adherence (2 hours) from bronchoalveolar lavage, giving purity >98%. Concentrations of HSP60 were measured in cell lysates using HSP60 ELISA Kit (Stressgen Bioreagents, Ann Arbor, MI) according to the manufacturer's protocol. Protein level was expressed as mean ± SEM. Differences of expression were analyzed by Student's *t* test and considered significant when $p < 0.05$. The sensitivity of the assay has been determined to be 3.125 ng/ml.

This study was approved by the Centre de santé et de services sociaux de Chicoutimi and the Laval Hospital Human Ethic Committees.

3. Results

3.1. Gene expression study

AM gene expression of allergic asthmatic and control subjects showed significant ($p < 0.05$) differences in the expression of 50 genes (58 transcripts), which were classified into nine biologic function categories (Table 3). Nineteen of these genes (38%) were classified into categories linked to stress or immune responses; immune signaling molecules, and immune and stress responses. Nine members of the HSP family are underexpressed in allergic asthmatic compared with control subjects.

Because of the large quantity of genes tested simultaneously, before going further with analyses of differentially expressed genes, results have to be validated by another technique to avoid false positives. Consequently, five genes (*CCR1, HSPD1, OAS1, PRNP,* and *SERPINH1*) were selected from the two immune and stress categories to be tested using real-time RT-PCR. They were selected according to known functions suggesting a possible involvement in asthma pathogenesis (Table 3). Considering that nine HSP genes are found differentially expressed, a particular interest was given to this family. Indeed, among the nine HSP genes, two genes that were not documented in alveolar macrophages but have known functions possibly linked to asthma development were selected. Of the five genes selected, only *CCR1* has been shown to be expressed in AM [20], but a possible difference of expression linked to asthma has not been documented yet. Real-time RT-PCR showed expression of all five genes in AM from control and asthmatic subjects and confirmed the difference in gene expression between asthmatic and control for three of them: *HSPD1, PRNP* and *SERPINH1* (Fig. 1). Real-time RT-PCR data showed that these three genes were significantly underexpressed in AM of asthmatic subjects (2.16-, 1.52-, and 3.63-fold, respectively; $p < 0.05$), as observed in microarray data (2.08- and 1.52-fold for two *HSPD1* transcripts, and 1.75- and 1.80-fold for *PRNP* and *SERPINH1,* respectively; $p < 0.05$, Table 3).

3.2. Genetic association study

To determine whether the difference of expression for these three genes in asthmatic subjects can be explained by genetic variants, genetic association tests were performed between tagging single nucleotide polymorphisms (tagSNPs) distributed in each of these genes and asthma (see Supplementary material). Family-based association testing revealed no significant association for asthma, suggesting that no tagSNP seems to be responsible or in linkage disequilibrium with SNP responsible of the lower expression observed for the three genes in asthmatic subjects (data not shown).

3.3. Protein expression study

HSPD1, one of the three validated gene, encodes HSP60. This HSP has been recently linked to the regulation of immune response [21–24], but its involvement in AM functions has not yet been investigated. Therefore, this gene was selected for subsequent analyses to compare its protein and genetic expression in alveolar macrophages of control and asthmatic subjects. The immunocytochemistry data showed similar expression of HSP60 protein in AM

Table 3
Genes differentially expressed in alveolar macrophages of allergic asthmatic subjects in comparison with control subjects

Gene[a]	Cytoband[b]	ACCNUM	Probe set	Flc[c]	p	Function[d]
Cell growth and proliferation						
CDKN1B	12p13.1–p12	NM_004064	209112	1.56	0.024	Cell cycle [1]
RREB1	6p25	NM_001003699	203704_s_at	1.61	0.007	Cell differentiation [41]
Genetic transcription and translation						
DICER1	14q32.13	NM_177436/NM_030621	212888	1.52	0.036	RNA interference [3]
MRPL18	6q25.3	NM_014161	217907_at	−1.66	0.008	Mitochondrial ribosome [4]
PAIP1	5p12	NM_006451	210283_x_at	1.59	0.015	Translational initiation [5]
PHF3	6q12	NM_015153	217954_s_at	1.59	0.020	Transcription factor [6]
RNF6	13q12.2	NM_005977	203403_s_at	1.55	0.001	Transcription regulation [7]
SFRS3	6p21	NM_003017	202899_s_at	−1.55	0.015	mRNA splicing factors [42]
SP3	2q31	XM_092672	213168_at	1.55	0.008	Transcriptional regulation [42]
UPF3A	13q34	NM_023011	214323_s_at	1.50	0.006	Export of spliced mRNA [10]
Immune signaling molecules						
* *CCR1*	13p21	NM_001295	205098_at	1.69	0.009	Binds CC chemokines [11];
			205099_s_at	1.73	0.014	Expressed by AM [12]
CYTIP	2q11.2	NM_004288	209606_at	1.93	0.024	IL-2, IL-12-stimulated Tcell [13]
* *OAS1*	12q24.2	NM_016816	202869_at	1.79	0.002	Antivirus activity [14]; Severe acute respiratory syndrome [15]
* *PRNP*	20pter-p12	NM_000311/NM_183079	215707_s_at	−1.75	0.002	Phagocytosis [16]; Superoxide dismutase activity [17]
SAMSN1	21q11	NM_022136	220330_s_at	1.50	0.006	B cell activation [18]
Immune and stress responses						
BAG3	10q25.2–q26.2	NM_004281	217911_s_at	−2.08	0.028	HSP70 and HSC70 partner [19]
DNAJB1	19p13.2	NM_006145	200666_s_at	−2.33	0.006	HSP70 partner [20]; IL-10
DNAJB4	1p31	NM_007034	203811_s_at	−1.72	0.018	production [21]
DUSP1	5q34	NM_004417	201044_x_at	−1.87	0.046	Induced by heat stress [22] and
DUSP6	12q22–q23	NM_022652/NM_001946	208891_at	1.80	0.030	IL-10 [23]; MAPK pathway [24]
GADD45B	19p13.3	NM_015675	209305_s_at	−1.57	0.038	Damage, growth stresses [25]
HSPA1A	6p21.3	NM_005345	200800_s_at	−2.42	0.002	Anti-inflammatory [26]; AM and asthma [27]
HSPA1B	6p21.3	NM_005346	202581_at	−2.96	0.004	
HSPA6	1q23	NM_002155	213418_at	−7.00	0.007	
			117_at	−5.60	0.008	
HSPCB	6p12	NM_007355	214359_s_at	−1.56	0.018	Anti-inflammatory [28]; childhood asthma [29]
* *HSPD1*	2q33.1	NM_002156/NM_199440	200806_s_at	−2.08	0.004	Anti-inflammatory [26,30–33];
			200807_s_at	−1.52	0.008	Asthma [34]
HSPH1	13q12.2–13.3	NM_006644	206976_s_at	−1.69	0.028	Regulation of inflammation [35]
			208744_x_at	−2.35	0.009	
* *SERPINH1*	11q13.5	NM_001235	207714_s_at	−1.80	0.014	Collagen-specific [36]
STIP1	11q13	NM_006819	212009_s_at	−1.89	0.039	HSP70 and HSP90 partner [37]
Intracellular signaling molecules						
CALU	7q32	NM_001219	214845_s_at	−1.55	0.002	CA2+ binding proteins [38]
INSIG1	7q36	NM_005542/NM_198336/ NM_198337	201625_s_at	−1.62	0.028	Cholesterol biosynthesis [39]
			201627_s_at	−1.74	0.042	
PEX6	6p21.1	NM_000287	204545_at	−1.60	0.025	Peroxisomal targeting [40]
YWHAZ	8q23.1	NM_003406/NM_145690	200641_s_at	−1.63	0.012	Insulin pathway [41]
Metabolic enzymes						
GNPTAB	12q23.2	NM_024312	212959_s_at	1.63	0.020	Lysosomal hydrolases [42]
LIMK1	7q11.23	NM_002314	208372_s_at	−1.57	0.002	Actin cytoskeletton [43]
MAT2A	2p11.2	NM_005911	200769_s_at	−1.70	0.025	S-adenosylmethionine synthesis [44]
NQO2	6pter-q12	NM_000904	203814_s_at	1.69	0.003	NADH, NADPH oxydation [45]
RNASE6	14q11.2	NM_005615	213566_at	1.83	0.012	Degradation of RNA [46]
SCD	10q23–q24	NM_005063	211162_x_at	−1.75	0.002	Fatty acid desaturase [47]
			211708_s_at	−1.82	0.002	
TPP1	11p15	NM_000391	214196_s_at	−1.54	0.047	Lysosomal exopeptidase [48]
Structural proteins						
DOCK4	7q31.1	NM_014705	205003_at	1.52	0.022	Adherens junctions [49]
HIST2H2AA3 HIST2H2AA4	1q21–q23	NM_003516/NM_001040874	214290_s_at	−1.54	0.014	Nucleosomes formation [50]
MFN1	3q26.23	NM_033540	207098_s_at	1.64	0.019	Mitochondria morphology [51]
Transmembrane proteins						
APP	21q21.2–q21.3	NM_000484	200602_at	2.02	0.032	Transcriptional activation, amyloid plaques [52]
LDLR	19p13.3	NM_000527	202067_s_at	−1.61	0.045	LDL endocytosis [53]
			217173_s_at	−1.55	0.020	
MSR1	8p22	NM_002445	208423_s_at	−1.60	0.011	Negative molecule transport [54]
			211887_x_at	−1.63	0.003	
SLC16A3	17q25	NM_001042422/NM_004207/ NM_001042423	202855_s_at	−1.90	0.045	Lactic acid, pyruvate transport [55]
SLC7A5	16q24.3	NM_003486	201195_s_at	−1.62	0.041	Neutral amino acids transport [56]

Table 3
Continued

Gene[a]	Cytoband[b]	ACCNUM	Probe set	Flc[c]	p	Function[d]
Vesicle transport						
EXOC5	14q23.1	NM_006544	218748_s_at	−1.73	0.029	Targets exocytic vesicles [57]
RAB5C	17q21.2	NM_004583/NM_201434	201156_s_at	−1.56	0.035	Docking, fusion of vesicles [58]

[a]Genes marked by an asterisk were selected to be tested by real-time RT-PCR.
[b]Gene location obtained from National Center for Biotechnology Information public database Build 128 (http://www.ncbi.nlm.nih.gov).
[c]Fold changes (Flc) are indicated for each probe set significantly under- or over-expressed ($p < 0.05$; absolute Flc > 1.5) by alveolar macrophages of allergic asthmatic subjects compared with control subjects. Positive data indicate genes that are overexpressed; negative data indicate genes that are underexpressed by alveolar macrophages of allergic asthmatic subjects.
[d]References that allow classification of differentially expressed genes in function categories:
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of control and asthmatic subjects (90% ± 5% and 90% ± 4% of positive cells for control and asthmatic subjects, respectively). This result was not surprising, as HSP60 is constitutively expressed in cells for its chaperone activity [25]. However, the result suggested a difference in each cellular protein level instead of a difference in the number of cells expressing the protein (Fig. 2). Thus, HSP60 protein level in cell lysates of AM from control and asthmatic subjects was measured using ELISA. HSP60 level was significantly higher in AM of asthmatic subjects (35.0 ± 14.5 ng/ml) compared with controls (15.4 ± 10.8 ng/ml) (Fig. 3). This represents a 2.28-fold increase, which contrasts with the 2.16-fold decrease of *HSPD1* mRNA level observed in AM of asthmatic subjects (Fig. 1).

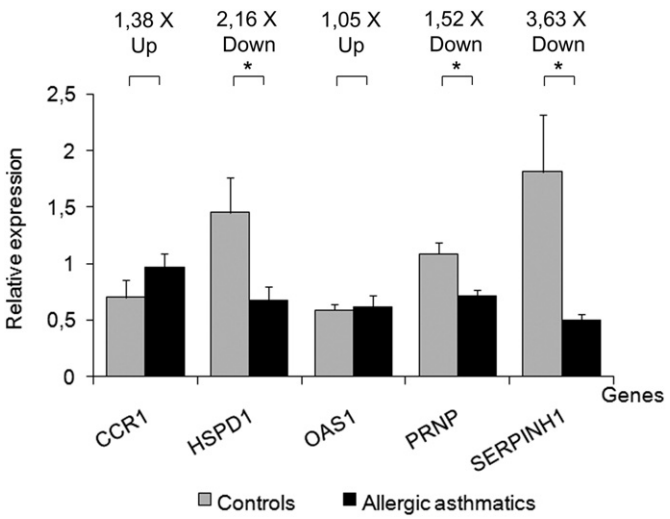

Fig. 1. Expression of the five selected genes using real-time RT-PCR. Chemokine (C-C motif) receptor 1 (*CCR1*), heat shock 60-kDa protein 1 (*HSPD1*), 2,5-oligoadenylate synthetase 1 (40–46 kDa) (*OAS1*), prion protein (*PRNP*) and serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (*SERPINH1*) mRNA expression by alveolar macrophages (AM) isolated from bronchoalveolar lavage of control (gray bars) and allergic asthmatic (black bars) subjects. Measure of the mRNA expression by real-time RT-PCR was done in triplicate with negative control and normalized to *GAPDH* expression using two-standard curves method ($n = 4$). Data are expressed as mean ± SEM values. *HSPD1*, *PRNP*, and *SERPINH1* mRNA are significantly ($^*p < 0.05$) underexpressed in AM of asthmatic subjects compared with controls.

4. Discussion

Genomic expression profiling of AM presented in this study corresponds to the basic state of asthmatic subjects (unchallenged) and should help in understanding the implications of this cell type in the development of airway allergic asthmatic response. Interestingly, 38% of the differentially expressed genes are part of the immune signaling response and the stress and immune responses categories (Table 3), supporting the hypothesis of an implication of AM in the development of asthma physiopathology. Moreover, the nine genes in the HSP family that are underexpressed in allergic asthmatic subjects suggest an implication for this gene family in particular. Indeed, even if endogenous HSP have been first known for their chaperone functions, studies have now demonstrated implications for some of them (HSP60, 70, 90 and 110) in the immune response (for a review, see Pockley et al. [26]). More precisely, endogenous HSP60 and HSP70 have shown anti-inflammatory properties in studies in rheumatoid or juvenile arthritis and in animal models [26].

Two studies have shown HSP differential gene expression in asthma. A study by Youssef et al. demonstrated that many HSPs are more expressed by stimulated basophils, and that histamine released by basophils is increased in allergic asthmatic subjects [27]. Another study has performed gene expression profiling and genome-wide analysis in a familial sample of asthma using lymphoblastoid cell lines. Their goal was to look at genes that combine heritability for expression levels and peak lod score for association with asthma. Genes involved in the response to unfolded proteins, which include chaperonins and HSP [28], were found among the genes targeted in their study. Data from our study, jointly with these two other studies, support the implication of the HSP gene family in asthma through different cell types.

Differences of expression observed in microarray results may be explained by different biologic mechanisms. In some cases, it can be the effect of mutations in regulatory regions of the differentially expressed genes. However, the family-based association analysis done in this study for the three validated genes by real-time RT-PCR (*HSPD1*, *PRNP*, and *SERPINH1*) indicated that tagSNP do not seem to explain differences of gene expression observed (data not shown). However, as these differences of expression have been validated by a second technique, it suggests that they probably are the result of

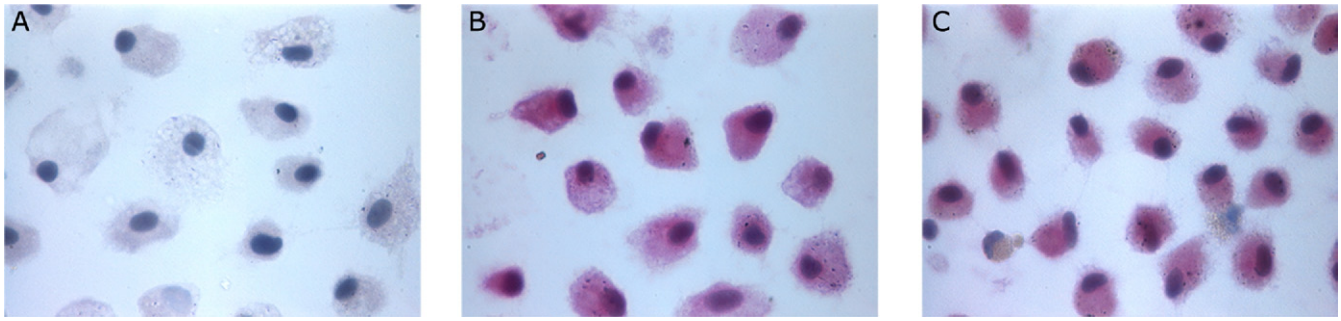

Fig. 2. HSP60 protein expression by alveolar macrophages of control and allergic asthmatic subjects. Bronchoalveolar cells of four control subjects and four allergic asthmatic subjects were fixed with PLP-sucrose and immuncytochemistry was done using ABComplex/AP method and Fast-Red coloration with Mayer's hematoxylin counterstaining (magnification ×1,000). Immunocytochemistry for HSP60 expression of (A) isotypic negative control, (B) control subjects, and (C) allergic asthmatic subjects. Results shown are representative. There was no difference in positive alveolar macrophage cell count for HSP60 between control and asthmatic subjects (90% ± 5% and 90 ± 4% of positive cells for control and asthmatic subjects, respectively).

other mechanisms as modulation above in their respective biologic pathway or epigenetic regulation [29].

Real-time RT-PCR was selected to validate genomic expression data because this technique usually confirm about 87% of microarray results done using RMA analyses [30]. It is consistent with the confirmation of the underexpression of three of the five genes (*HSPD1, PRNP,* and *SERPINH1*) in allergic asthmatic compared with control AM, according to the small number of genes tested (Fig. 1). Consequently, it is interesting to note that genetic expression of none of the validated genes has been documented in AM yet. Moreover, they all have functions possibly linked to the implication of AM in asthma pathogenesis.

The first differentially expressed gene, *PRNP,* encodes PRP, a prion protein. Cellular PRP, in opposition to scrapie PRP, is involved in the modulation of cell apoptosis [31], negatively modulates peritoneal macrophage phagocytosis activity [32], and has superoxide dismutase activity [33]. These functions have a direct impact on the number of inflammatory cells in the airways and then in the regulation of airway inflammation in asthma.

SERPINH1 encodes HSP47, a collagen-specific chaperone. This protein is associated with inflammatory diseases, such as pulmonary fibrosis, vascular and rheumatoid diseases, and cancer [34]. However, its possible immune functions, such as those recently

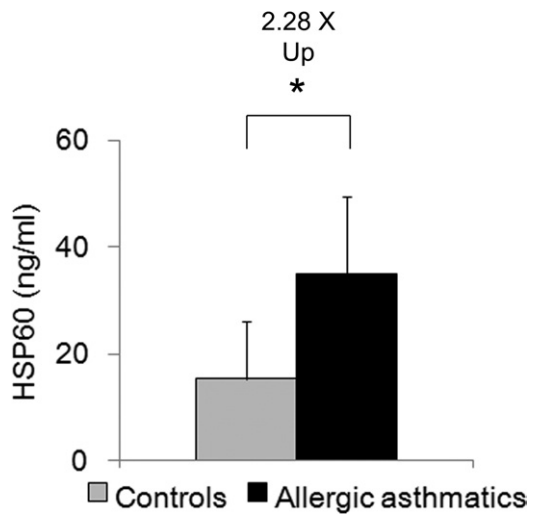

Fig. 3. Protein level of HSP60 in alveolar macrophages (AM) of control and allergic asthmatic subjects. AM were purified from bronchoalveolar lavage and lysed to measure HSP60 by ELISA. Protein levels for control (gray bars) and allergic asthmatic (black bars) subjects are shown ($n = 5$). Data are expressed as mean ± SEM values. Level of HSP60 is significantly higher ($^*p < 0.05$) in AM of asthmatic subjects compared with controls.

documented for other members of the HSP family, have not been studied yet.

Finally, the second HSP validated gene, *HSPD1,* encodes HSP60. This protein has a regulatory effect on T cell immune functions in three principal ways. First, it stimulates secretion by T cell, regulatory T cell (Treg), and B cell, of the anti-inflammatory cytokine IL-10 [21–23]. Second, HSP60-stimulated Treg also inhibits two pro-inflammatory cytokine secretion by T cell; tumor necrosis factor (TNF) and IFN-γ [23]. Third, HSP60 has the capacity to inhibit T-cell chemotaxis [24]. It may also have an autocrine effect on AM, as direct binding of this protein on a specific receptor on this cell type has been demonstrated [35]. Consequently, all of these functions support an implication for this protein in immune roles of AM in asthma pathogenesis. Indeed, it suggests that HSP60 may downregulate the immune response, thereby preventing asthma development.

For all of the reasons mentioned above, HSP60 was selected to document its protein level in asthmatic and control AM and to compare it with the difference in gene expression observed. The sample sizes used for the gene expression profiling and the protein level analyses may seem limited. However, they were sufficient to achieve statistical significance between phenotypic groups in both experiments [36,37]. Furthermore, the use of both robust normalization and cut-offs for *p* value and fold change in gene expression profile allowed detecting differentially expressed genes with the used sample size [13,15,38]. Moreover, the relative uniformity within and between the asthmatic groups is a major strength of the study. Indeed, the good similarity for lung function for all allergic asthmatic subjects included in this study, and the fact that they have comparable medication and allergen sensitization profiles (Tables 1 and 2), eliminates confounding with regard to asthma medication dosage and atopic status.

As observed in this study, genetic expression and protein level for a specific gene are often contrasting (up to 50% of the time) [39]. There are three principal reasons for mRNA and protein level discrepancy: post-transcriptional mechanisms, the *in vivo* half-life of proteins, and the errors and noises in mRNA and protein measurement techniques [39]. Interestingly, Gustavsson et al. have evaluated the half-life of HSP60 in HELA cells to be 37.5 ± 2.7 hours [40]. According to this long half-life, a decrease in *HSPD1* mRNA expression might be visible in protein level only several hours later. Moreover, it is documented that protein level can be regulated at transcriptional or at translational level [39]. Proteins turnover that are regulated at transcriptional level shows correlation of 0.89 between protein levels and transcript abundance. However, this correlation is approximately only 0.20 for proteins regulated at the translational level [39]. These data suggest the possibility that HSP60 level can be regulated at the translational level, which could

be consistent with such a long half-life and could explain the discrepancy observed between mRNA and protein level in this study.

However, according to regulatory functions of HSP60, an increase in this protein level should limit the immune response. It is surprising to observe a more than two fold increase in AM of allergic asthmatic subjects compared with controls. It is possible that the immune response triggered in asthmatic subjects induces an increase in AM HSP60 production to regulate AM functions, but that HSP60 might not be able to achieve its regulatory role in allergic asthmatic AM. However, to understand the effect of a difference in intracellular level of HSP60 in AM, functional studies need to be performed. Indeed, until now, only effects of extracellular HSP60 on immune cell functions have been observed [21–24].

In conclusion, genomic expression profiling performed in this study suggests that HSP gene family is involved in AM functions in a context of allergic asthma, and also supports a role for AM immune regulatory functions in the development of allergic asthma response. Moreover, protein level analysis targets HSP60 as a novel biomarker specific to AM activities. Obviously, it will be necessary to perform functional research to clearly determine the role and impact of the HSP60 in AM activities in allergic asthma biology.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.humimm.2009.07.11.005.

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