

Lung Dendritic Cells Express Higher Stress Proteins on Higher Allergen Dose Exposure and Contribute to Allergen Tolerance Induction

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Abstract

Different doses of allergen lead to different T lymphocyte responses which partly explain the phenomenon of low or high dose tolerance. Dendritic cells (DC) are responsible for driving the T lymphocyte responses to a variety of exogenous stimuli, but the mechanisms are not completely understood.

Elucidate the mechanisms of how DCs drive the differentiation of T lymphocytes in response to different doses of allergen.

Groups of male BALB/c mice (n=4-5) were sensitized intraperitoneally with sham or different doses (low: 10 µg, high: 1000 µg) of Der P1 (house dust mite allergen). They were exposed daily to aerosolized Der p1 allergen for 7 consecutive days. Different concentrations of Der p1 solution were nebulized: sham, low dose (15 µg/mL), or high dose (1500 µg/mL). Bronchoalveolar lavage fluid (BALF) was obtained from the lungs. Levels of IL-12 in BALF were measured. Lung tissue sections were then stained to detect the expression of Hsp70 by lung DCs.

Lung DCs exposed to a higher sensitizing doses of Der p1 allergen tend to express significantly higher levels of Hsp70 and secrete higher levels of IL-12 in BALF. There was a significant positive correlation between the levels of Hsp70 expression by lung DCs with IL-12 levels in BALF (r=0.581).

Higher exposure doses of allergen puts lung DCs under stressed condition thereby induced high expression of 'stress proteins' which may explain the mechanism of DCs drive the T lymphocyte response.

Experimental article (J Int Dent Med Res 2020; 13(2): 778-784)

Keywords: Dendritic cells, heat shock protein, IL-12, animal model, asthma.

Received date: 10 January 2020

Accept date: 29 February 2020

Introduction

Different exposure doses of allergen leads to different direction of T lymphocyte responses which partly explain the phenomenon of low or high dose tolerance¹⁻³. Dendritic cells are cells that responsible for driving the appropriate T lymphocyte responses to a variety of exogenous stimuli^{4,5}, but the mechanisms by

which they perform that task are not completely understood.

Other studies have shown that dendritic cells exposed to some stressful conditions from the environment (heat, ultraviolet radiation or heavy metals), pathological insults (infections or malignancies), or physiological stimuli (growth factors or cell differentiation) induce a marked increase in heat shock proteins (HSPs) synthesis, a phenomenon known as the stress response⁶⁻⁸.

These HSPs are shown to induce the production and release of a variety of pro-inflammatory cytokines, including IL-12, nitric oxide and C-C chemokines by monocyte, macrophage and dendritic cells. They also induce the maturation of dendritic cells which enable these cells to activate T lymphocytes as demonstrated by the up-regulation of MHC class

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I and II molecules, co-stimulatory molecules such as B7-1 and B7-2⁹⁻¹¹. Recently, it has been shown that HSPs play an important role in antigen direct and cross-presentation because they can bind antigenic peptides generated within the cells, form HSP-peptide complexes. When released into extracellular compartment the HSP-peptide complexes can be recognized and taken up by other dendritic cells via CD91-mediated endocytosis, resulting in representation of the antigenic peptide by dendritic cells to T lymphocytes with the peptide specific receptor^{9,12,13}.

Using cellular stress and homeostasis paradigm, it can be speculated that dendritic cells exposed to high dose of allergen are under stressful condition and thereby release or produce stress protein such as HSPs and certain cytokines. The present study aim to answer the following questions: can the mechanism by which dendritic cells drive the differentiation of T lymphocytes under different exposure doses of allergen be explained through the 'cellular stress concept', and are dendritic cells that exposed to high doses of allergen indeed under stress and thereby express or secrete high amount of 'stress proteins' which also act as 'danger signals' that used by dendritic cells to drive the differentiation of T lymphocytes?

Materials and methods

Mice

Male inbred BALB/c mice of about 8-10 weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan) via PN Biofarma (Bandung, Indonesia). Food and water were provided ad libitum and mice were kept in a 12 h-light, 12 h-dark cycle in a specific pathogen free condition according to Federation of European Laboratory Animal Science Associations (FELASA) recommendation¹⁴. The local Ethical Committee (ACUC Veterinary Faculty Universitas Airlangga) approved the in vivo manipulations used in this study with certificate No. 150-KE.

Sensitization and Allergen Challenge Protocol

Sensitization and challenge were performed according to the method by Tournoy et al¹⁵ with some modifications. The lyophilized natural major house dust mite allergen (Der p1) was purchased from Greer Laboratories (Lenoir, N.C., USA). Groups of mice (n=4-5) were

sensitized intraperitoneally with sham (phosphate-buffered saline/PBS), or low dose (10 µg), or high dose (1000 µg) injection of major house dust mite allergen (Der p1) adsorbed to 20:1 ratio of aluminium hydroxide (Al(OH)₃) solution (Pierce Biochemicals, Thermo Fisher Scientific (Hong Kong) Ltd, Shatin, Hong Kong). Fourteen days later, the mice were exposed daily to aerosolized Der p1 allergen for 7 consecutive days (days 15-21). The mice were individually placed in a 50-ml plastic tube and exposed to aerosolized Der p1 allergen for 30 min. The aerosol was produced by Pulmo-Aide E0570 compressor nebulizer (DeVilbiss Corp., Somerset, PA, USA), driven by compressed air at 5-9 L/min. Different concentrations (doses) of Der p1 solution were nebulized: sham (PBS), low dose (15 µg/mL), or high dose (1500 µg/mL) solutions. The illustration for the protocol can be seen in Figure 1.

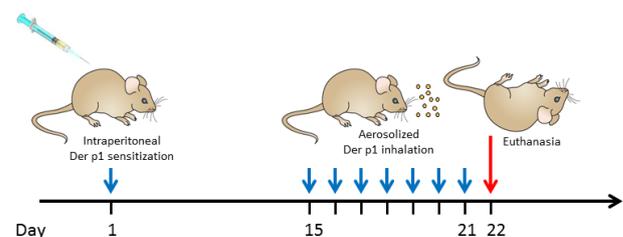


Figure 1. The scheme of our study protocol: mice intraperitoneal sensitization, aerosol inhalation exposure, and euthanasia.

Bronchoalveolar Lavage (BAL) and Measurement of IL-12

Mice were put under anesthesia at 24 h after the last challenge (day 22). BALF was obtained from the whole lungs by inserting a cannula into the surgically exposed trachea for cell analysis. BAL was performed via 3 intratracheal instillations with 0.3 ml Hank's balanced salt solution (HBSS) + 1% Bovine Serum Albumin (BSA), followed by 3 instillations with 1 ml of HBSS to collect cells for cytospin analysis as previously described^{15,16}. The BAL fluid of the first three fractions was centrifuged and the supernatant was used for cytokine (IL-12) detection and measurement. IL-12 levels in BALF were measured using standard indirect ELISA method. After staining with May-Grünwald-Giemsa, total and differential cell counts were done using standard morphologic criteria.

Preparation of Lung and Lymph nodes

Immediately after BAL, the mice were killed by right cardiac puncture and blood aspiration using an 18 gauge polyurethane catheter (Becton Dickinson, Madrid, Spain). The collected blood was used for measurement of serum cytokines. Pulmonary and systemic circulation were then perfused with saline/EDTA to remove the intravascular pool of cells. Paratracheal and parathymic intrathoracic lymph nodes were collected in steril petri dishes on ice for other studies. Lungs were carefully separated from thymic and cardiovascular remnants and removed in toto, including the main brochi and trachea. Left lung was separated for histological and immunohistochemical examination. Right lung was thoroughly minced using iridectomy scissors and incubated for 30 min in digestion medium in a humidified incubator at 37°C and 5% CO₂, according to a modified protocol¹⁷ and further used for other studies.

Histological examination

For histological examination, the left lung was removed and fixed in 4% paraformaldehyde. Then, the tissues were embedded in resin (Jung HistoResin Plus; Reichert/Jung, Heidelberg, Germany) and cut into 2-µm sections, which were stained with hematoxylin and eosin (H-E).

Measurement of Hsp70 expression by lung dendritic cells

For immunohistochemical analysis, some of the lung tissue sections were deparaffinized and underwent standard procedures for antigen retrieval¹⁸. Tissue sections were then double stained to detect the expression of Hsp70 by lung dendritic cells. Anti CD11c and anti-hsp70 monoclonal antibodies were used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). CD11c positive dendritic cells were stained with 3,3'-diaminobenzidine (DAB), while Hsp70 were stained with light violet blue (LV blue). Methyl green was used as counterstaining. Tissue sections for this process were mounted using 5% gelatin. Examination and interpretation of Hsp70 expression by lung dendritic cells was done by two independent observers. Expression scoring was done according to modification of the method of Soini et al¹⁹ and Pizem et al²⁰. Scores from both observers were checked for consistency.

Statistical Analysis

Data were analyzed with the statistical package SPSS 16.0 (SPSS Inc., Chicago, IL). Values are expressed as mean ± SD. To check the consistency of Hsp70 expression scoring by two independent observers paired t test and Pearson's correlation were used. Kolmogorov-Smirnov test was used to check the normality of data distribution, and Levine's test was used to check the homogeneity of data variant. The differences between the groups were tested using ANOVA. For data with abnormal distribution Kruskal-Wallis A and Kendall Tau analysis were used. Pearson's test was used to determine correlation between Hsp70 expression scores and IL-12 levels. P <0.05 was accepted as statistically significant.

Results

Lung Dendritic Cells Exposed to High Sensitizing and Inhalation Doses of Der p1 Allergen Express High Levels of Hsp70

The expression of Hsp70 in lung dendritic cells under different sensitizing and inhalation doses of Der p1 allergen is shown in Figure 2.

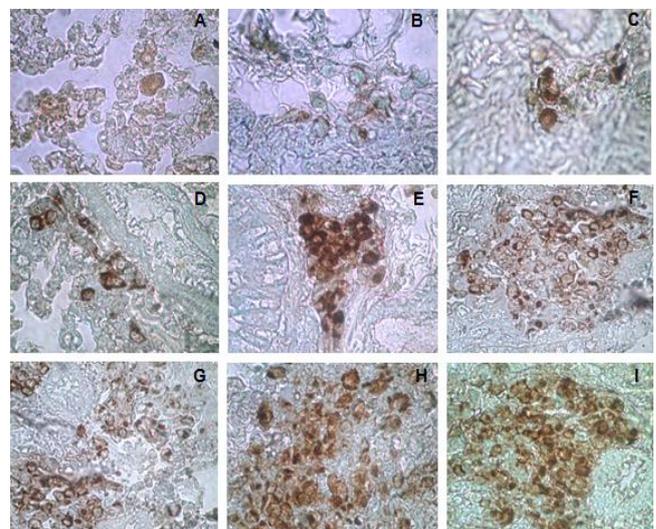


Figure 2. The representative expressions of heat shock protein Hsp70 by lung dendritic cells under different sensitizing and inhalation dose of Der p1 allergen.

The above figures shows the representative expressions of heat shock protein 70 (Hsp70) by lung dendritic cells in groups of mice (n=4-5) exposed to different sensitizing and inhalation

dose of Der p1 allergen as seen under light microscopy (1000x magnification) in immunohistochemistry (IHC) lung tissue sections. We use double staining technique to reveal lung dendritic cells (CD11c positive) which express Hsp70 (Hsp70 positive). (A: control mice, B: sham sensitization + low dose inhalation, C: sham sensitization + high dose inhalation, D: low dose sensitization + sham inhalation, E: low dose sensitization + low dose inhalation, F: low dose sensitization + high dose inhalation, G: high dose sensitization + sham inhalation, H: high dose sensitization + low dose inhalation, I: high dose sensitization + high dose inhalation).

Interpretation and scoring were done by two independent observers, both showed high consistency ($t=1.922$, $p=0.063$; $r=0.974$, $p\leq 0.001$). The relative expression scores for each group of interventions were measured and shown in Figure 3.

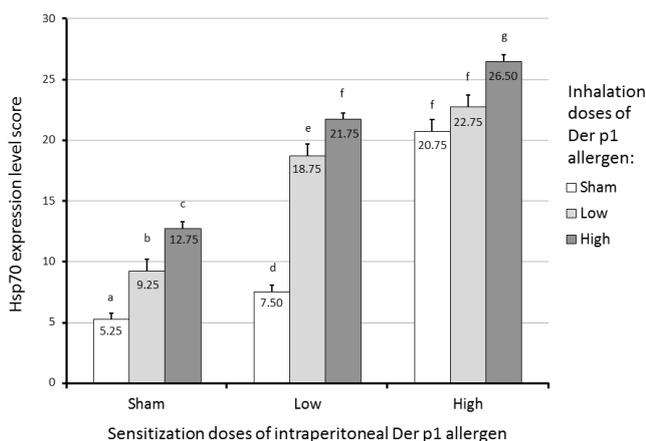


Figure 3. Relative Hsp70 expression by lung dendritic cells under different sensitizing and inhalation dose of Der p1 allergen.

Mean values of relative heat shock protein 70 (Hsp70) expression in each group of mice ($n=4-5$) and the respective standard error bars were presented. Groups marked with different alphabet (a,b,c,d,e,f, and g) showed significantly different expression score ($p<0.05$). Groups with the same alphabetical marks had non-significant difference in Hsp70 expression.

Lung dendritic cells that were exposed to a higher intraperitoneal sensitizing doses of Der p1 allergen tend to express significantly higher levels of Hsp70 (Figure 3). This tendency was

also seen if we modulate the inhalation dose of Der p1 allergen (low dose inhalation, grey bars, and high dose inhalation, dark grey bars). Coupling effects were seen, as the higher the sensitizing doses and the higher the inhalation doses, the higher the expressions of Hsp70. The non-significant difference of Hsp70 expression between sham inhalation and low inhalation in groups with high sensitization dose (20.75 vs. 22.75 respectively, $p>0.05$), indicates that the sensitizing dose is more important in determining the Hsp70 expression by lung dendritic cells than the inhalation dose.

Higher Level of IL-12 was Detected in BALF in Group of Mice Exposed to Higher Sensitizing and Inhalation Doses of Der p1 Allergen

Similar to the expression levels of Hsp70 by lung dendritic cells, higher intraperitoneal sensitizing doses of Der p1 allergen tend to result in increased levels of IL-12 in BALF (Figure 4). The same tendency was also seen if we modulate the inhalation dose of Der p1 allergen (low dose inhalation, grey bars, and high dose inhalation, dark grey bars). Coupling effects were also seen, as the higher the sensitizing doses and the higher the inhalation doses, the higher the levels of IL-12 in BALF, particularly in groups which received high sensitization doses of intraperitoneal Der p1 allergen (143.54, 204.46, and 328.04 respectively, $p<0.05$).

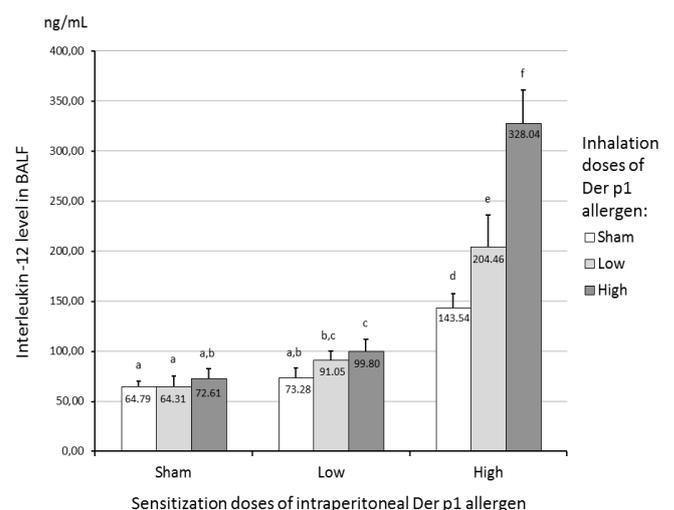


Figure 4. Interleukin-12 levels in bronchoalveolar lavage fluid under different sensitizing and inhalation dose of Der p1 allergen.

Mean values of interleukin-12 (IL-12) levels in each group of mice (n=4-5) and the respective standard error bars were presented. Groups marked with different alphabet (a,b,c,d,e, and f) showed significantly different IL-12 levels compared to other groups ($p < 0.05$). Groups with the same alphabetical marks had non-significant difference in IL-12 levels.

The expression levels of Hsp70 by lung dendritic cells significantly correlated with IL-12 levels in BALF

There was a significant positive correlation between the levels of Hsp70 expression by lung dendritic cells with IL-12 levels in BALF ($r = 0.581$; $p \leq 0.001$; Figure 5). This result implies that the stressed lung dendritic cells expressed high levels of stress protein which also associated with the levels of IL-12, which in turn contribute to the induction of Th1 type immune response or a shift from the initial Th2 type immune response.

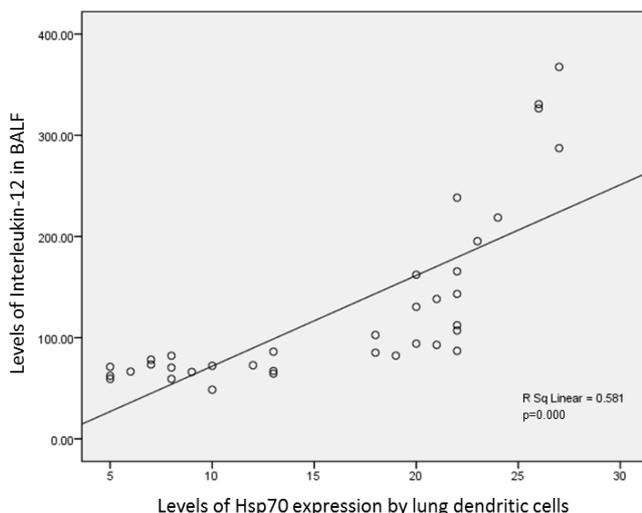


Figure 5. Correlation between levels of Hsp70 expression by lung dendritic cells with interleukin-12 levels in bronchoalveolar lavage fluid (BALF).

The higher the heat shock protein 70 (Hsp70) expression levels by lung dendritic cells, the higher the IL-12 levels in BALF ($r = 0.581$, $p \leq 0.001$).

Discussion

One of the mechanisms by which dendritic cells drive the differentiation of T lymphocytes

can be explained through the 'cellular stress concept'. As has been shown in the present study, dendritic cells that exposed to a high sensitization and inhalation dose of allergen (Der p1) are indeed under stress, as demonstrated by high level of 'stress protein' (Hsp70) expression compared to dendritic cells that exposed to 'usual' or low sensitization and inhalation dose.

'Cellular stress concept' stated that any cell exposed to a variety of environmental stressors, including extremes of temperature, ultraviolet radiation, toxins, or infections undergo some damage in the cellular component which interfere with cellular essential functions. In response to this, the 'stressed' cell utilize certain cellular stress response that serve as an adaptive purpose to protect itself from unfavourable environmental conditions, both through short term mechanisms that minimize acute damage to the cell's overall integrity, and through longer term mechanisms which provide the cell a measure of resiliency against similar adverse conditions. One of the mechanisms that has been extensively studied is the expression heat shock or heat stress proteins (HSPs)⁶. Traditionally, HSPs are regarded as intracellular molecules which only released into extracellular compartments upon necrotic cell death. However, recently it has been shown that HSPs can be actively released extracellularly in response to a number of stressful conditions²⁰. Our study result showed that exposure to high dose of allergen can be viewed as one of the stressful conditions.

Functionally, stress-inducible proteins can be grouped into seven classes. The predominant class of HSPs, the molecular chaperones, comprises five major and broadly conserved families-Hsp100s, Hsp90s, Hsp70s, Hsp60s, and small HSPs⁶. One of the most highly conserved chaperones is the Hsp70 protein. Under physiological conditions, this protein are involved in the de novo folding of proteins, but during and following stressful conditions, Hsp70 functions as a chaperone which enable the cell to cope with harmful aggregations of denaturated proteins. Thus, its extracellular expression confers protection against stresses that induce protein damage²¹. These phenomenon were observed in cultured cells, animal models, and human tissues, resulting from various stressful stimuli^{22,23}. The mechanisms of active HSP release are somewhat controversial because initially researchers cannot identify any signal peptide

that targets this protein for classical secretion. Recent findings suggest that HSP was released by a non-classical protein transport pathway that requires intact membrane lipid rafts for efficient release. Others also demonstrated that HSP were secreted within exosomes²⁴⁻²⁶.

One of the proposed functions of extracellular HSP is immune activity modulation. Many of these effects are mediated by cell surface receptors such as c-type lectin receptors and scavenger receptors expressed on a wide range of cell types²⁷. Secreted Hsp70 were shown to induce significant cytokines release including IL-1 β , TNF- α , and IL-12 by macrophages and dendritic cells²⁸⁻³⁰. Our study confirmed these findings and showed that lung dendritic cells exposed to a higher dose of Der p1 allergen not only express higher level of Hsp70 but also secrete higher level of IL-12. Our study found a significant positive correlation between the levels of Hsp70 expression by lung dendritic cells with IL-12 levels in BALF. It is of particular interest that other studies also observed a significant increase in Hsp70 (and thus, antibodies against Hsp70) in subjects who were exposed to environmental stresses such as carbon monoxide, heat, and dust³¹. Although not specifically mentioned in their report, we know that house dust mite like *Dermatophagoides pteronyssinus* is the dominant component of dust in the living environment³². Hsp70 was also reported to be present in the skin of allergic contact hypersensitivity model³³, indicating that Hsp70 is also an important mediators of allergic inflammation. All of those findings, either directly or indirectly, supported our study results.

As stated previously, extracellular HSP had been reported to activate macrophages, and dendritic cells via a receptor-mediated process^{28,34}. Activated and mature macrophages or dendritic cells then express some costimulatory signals that are needed for naïve T lymphocytes priming and activation³⁵. In our study, the expression of Hsp70 and the secretion of IL-12 can be viewed as the third signals that drive T lymphocyte differentiation.

In conclusion, using our approach with differential sensitizing and inhalation doses of allergen, we elucidate one of the mechanisms by which dendritic cells sensing the 'danger' or the 'stressful condition' in the microenvironment, and make use the 'stress protein' they express or secrete to alert naïve T lymphocytes dan drive

their appropriate direction of differentiation and ultimately determine the immune response toward immune tolerance to allergen. With this 'cellular stress concept', we can prove that the more 'stressed' the dendritic cells, the more 'stress protein' they secrete, and.

Acknowledgements

The authors express our uttermost gratitude to Prof. Peterhans Jan van den Broek, MD, PhD for reviewing and giving his helpful advice so that this final manuscript can be perfected. We also thank Heni Endrawati SSi., and Wahyuda Ngatiril Lady SSi., for expert technical assistance; Fitri Armania SSi., Aulia Rahmi MD., Dearikha Kharina MD., Ami Maghfiroh SSi., Budi Siswoyo, and Ali Sabeth for animal handling and sacrifice. No data were used to support this study.

Declaration of Interest

All of the authors declare that there are no potential conflicts of interest in relation to this manuscript writing and publication.

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