ERJ Express. **Published on July 22, 2010 as doi: 10.1183/09031936.00055210**

Title: Impaired Function of Regulatory T cells in Hypersensitivity Pneumonitis

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This work was funded by Canadian Institutes of Health Research (CIHR)

Running head: T regulatory cells in HP

Abstract

Hypersensitivity pneumonitis (HP) is characterized by a lung lymphocytosis. Most individuals exposed to HP antigens remain asymptomatic. The mechanisms involved in the impaired immune tolerance leading to HP are unclear. Normally, T regulatory cells (T_{reg}) control the immune response. Could T_{reg} suppressive function deficiency explain the uncontrolled inflammation in HP?

Bronchoalveolar lavage (BAL) and blood samples were obtained from normal subjects, asymptomatic individuals, and HP patients. BAL and blood T_{reg} were isolated. The ability of T_{reg} to suppress T cell proliferation and the role of IL-17 was verified.

BAL and blood T_{reg} cells from normal subjects suppressed the proliferative response of activated T cells by 47.1% and 42% respectively. BAL and blood T_{reg} cells from asymptomatic subjects had a slightly decreased activity and suppressed proliferation by 29.4% and 31.8% respectively. BAL and blood T_{reg} from HP patients were totally non functional and unable to suppress proliferation.

Low levels of IL-17 were detected in sera and BAL from both normal and asymptomatic individuals whereas measurable levels were found in patients.

T_{reg} may be involved in the antigen tolerance in asymptomatic subjects. A defective T_{reg} function, potentially because of increased IL-17 production, could account for the exacerbated immune response characteristic of HP.

Key words: Extrinsic allergic alveolitis, T lymphocytes regulation.

Introduction

Hypersensitivity pneumonitis (HP) is an inflammatory, granulomatous, and immunologically mediated pulmonary disease caused by an exacerbated immune response to repeated inhalations of various antigens, mostly organic in nature. Animal and vegetable proteins as well as bacteria, fungi, and chemical compounds can cause HP [1]. For example, farmer's lung, one of the most common forms of HP, is often caused by *Saccharopolyspora rectivirgula* actinomycetes found in poorly conserved hay, straw, or grain [2].

HP is characterized by a large influx of activated lymphocytes in distal bronchioles and alveoli. T cells can account for up to 60-80% of total bronchoalveolar lavage (BAL)-recovered cells from patients [3, 4]. Chest radiography and CT scan usually show diffuse ground-glass infiltrates and nodular and/or patchy air space opacifications, [5]. In the chronic stage of the disease, some patients can develop emphysema and/or lung fibrosis [6].

Fortunately, very few individuals exposed to HP antigens develop these clinical symptoms. The prevalence of farmer's lung is estimated at 0.5% to 3% in exposed farmers [7, 8]. Antigen exposed persons often have specific serum

antibodies and develop an asymptomatic lymphocytic alveolitis but seldom develop active disease [9]. They therefore seem to maintain a tolerance to the causative antigen.

Regulatory T cells (T_{reg}) are a unique population of $CD4^+$ T cells that play a pivotal role in the maintenance of the balance between the tissue-damaging and protective effects of the immune response. T_{reg} are generally defined by the extracellular expression of CD25, the high affinity α chain of the interleukin-2-receptor (IL2-Rα) [10] and by the intracellular expression of the forkhead/winged helix transcription factor (FoxP3). To date, FoxP3 is the most specific marker for T_{reg} and the best method to distinguish these cells from other T cells. In human, purification could be enhanced by excluding T cells that express CD127 and by including those that express CD39, CD73, LAG-3, and CTLA-4 but none of these markers are exclusive to T_{reg} [11]. IL-17 is a pro-inflammatory cytokine induced mostly by Th17 cells but also by NK cells, dendritic cells, $CD8^+$ T cells, and gamma-delta T cells. IL-17 promotes IL-1β, IL-6 and IL-17 production by neighbouring cells, triggering a pro-inflammatory environment [12]. Secretion of pro-inflammatory cytokines such as IL-1β and IL-6 can inhibit T_{reg} suppressive function [13, 14].

The aim of the present study was to verify the hypothesis that T_{reg} isolated from blood and bronchoalveolar lavage (BAL) fluids of normal and asymptomatic subjects efficiently suppress T helper cell activation while those from blood and

bronchoalveolar lavage fluids of patients suffering from active HP lose this suppressive function, allowing an exacerbated immune response and clinically significant lung inflammation. The second objective was to verify the potential role of IL-17, an inflammatory mediator known to affect T_{reg} function.

Materials and Methods

The study population included six patients with active HP, all males, (five with farmer's lung, and one with HP to *Paecilomyces* sp. (mean age: 48.5 yr; range: 31 to 69 yr); four asymptomatic, antigen-exposed (*Paecilomyces sp*) male wood workers who all had high serum IgG levels against the antigen; mean age: 38 yr; range: 31to 49 yr), and four not exposed control male subjects (mean age: 39 yr; range: 30to 45 yr). The diagnosis of HP was based on previously described criteria [15]. All patients were in the acute phase of the disease. The asymptomatic subjects had normal lung function and chest radiographs. As a group, the asymptomatic subjects (AS) were slightly younger than the subjects with HP. All subjects were nonsmokers, free of recent respiratory infections, and on no medication at the time of study. This project was approved by the Institut

universitaire de cardiologie et de pneumologie de Québec Research Center ethics committee and all participants signed an approved consent form.

Blood samplings and bronchoalveolar lavages

All patients and volunteers underwent a 150 ml blood sampling and a fiberoptic bronchoscopy with bronchoalveolar lavage (BAL). The wedged lung segment was lavaged with five aliquots of 60 ml each of normal sterile saline prewarmed to 37° C; the fluid was gently aspirated after each aliquot. Lavage fluid was recovered and centrifuged, and the resulting BAL cells were counted in a hemocytometer. Cell differential counts were performed after Diff-Quik (Dade Diagnostics, Aguada, PR) staining on glass coverslips.

T cells isolation from blood and BAL

Blood and BAL mononuclear cells were isolated by Ficoll gradient. Cells were plated for 2 h at 37°C in RPMI medium (GIBCO BRL, Burlington, ON, Canada) completed with 5% bovine fetal serum to separate monocytes (adherent) from lymphocytes (non adherent cells). Lymphocytes were harvested in supernatants. Effector T cells and regulatory T cells were isolated with the $CD4^+CD25^+$ Regulatory T Cells Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) and CD4, CD25, and FoxP3 expressions were verified with PE-Cy5-CD4 (BD biosciences, Mississauga, Canada), PE-CD25 (BD biosciences, Mississauga, Canada), and FITC-FoxP3 monoclonal antibodies (eBioscience Inc, San Diego, CA, USA) in an

EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA). The percentage of blood and BAL isolated $CD4^+CD25^+$ T_{reg} cells among $CD4^+$ T cells were calculated for each group of subjects.

In vitro suppression assays

Isolated T cells (50 x 10^4 cells) were left unstimulated or stimulated with 10 µg/ml of anti-CD3 (BD biosciences) and 4µg/ml of anti-CD28 antibodies (BD biosciences) in 96 wells round-bottom plates. Equivalent numbers of T_{reg} from the same subject were added or not. After a four day incubation, cells were pulsed with 1 µCi of [^{3}H] thymidine/well for 16 hours and proliferation was measured using a Packard Tri-Carb liquid scintillation counter 2100 TR (GMI Inc., Ramsey, MN, USA). Assays were done in triplicate or in duplicate when the number of T_{reg} was too small (assays with BAL fluids cells from normal subjects). Results are expressed as percentage of proliferation from control T effector cells exposed only to anti-CD3 and anti-CD28.

Cytokines detection

The concentration of IL-17A in sera and BAL fluids and the concentration of TNF-α in BAL were measured in triplicate using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean values ± S.E. for graphical representation. For comparisons between group means, a one-way analysis of variance (ANOVA) was performed. We considered a p value < 0.05 as a significant difference between groups.

Results

Bronchoalveolar lavage

Results of BAL total cell counts and differentials are illustrated in Figure 1a. Data are expressed in cells / ml of BAL. Exact number of cells that were recovered from the BAL of each group are presented in Table 1. Low cell counts were obtained for normal subjects. Asymptomatic subjects showed a moderate increase in total BAL cell counts and a slight increase of the percentage of lymphocytes (Figure 1b) compared to normal individuals. As expected, all BAL cell populations were increased in HP patients compared to other groups with a marked increase in lymphocyte counts and percentage.

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cell isolation

No significant difference was observed between healthy controls, asymptomatic subjects and HP patients with regard to the percentage of isolated $CD4^+CD25^+$ cells of the total $CD4^+$ T cells population from blood (Figure 2a) and BAL (Figure 2b). Isolated $CD4^+CD25^+$ cells were mostly $FoxP3^+$ and their frequency was

comparable between groups of subjects. The expression of FoxP3 on isolated $CD4^+CD25^+$ cells from blood and BAL was verified for each group and no statistical difference was observed between the groups (Table 2). Table 2 also shows the mean fluorescence intensity of Foxp3 which was significantly different between normal subjects and HP patients for blood ($p = 0.0006$) and BAL ($p = 0.0001$). No significant difference was observed between asymptomatic individuals and the other groups.

In vitro proliferation assays

Results of *in vitro* proliferation assays are presented for blood cells (Figure 3a) and BAL cells (Figure 3b). Results are expressed as a percentage of proliferation obtained from T effector cells stimulated with anti-CD3 and anti-CD28 alone. Blood and BAL T_{reg} from normal individuals efficiently suppressed T effector cell proliferation. When an equivalent number of T_{reg} from normal individuals was added, proliferation of activated T cells decreased to 58.0% of control proliferation for blood and 52.9% for BAL. BAL and blood T_{reg} from asymptomatic exposed subjects had retained their ability to suppress T cells proliferation. Proliferation of activated T cells decreased to 70.63% of control proliferation for blood and 68.2% for BAL when T_{reg} are added to effector T cells. T_{reg} of HP patients from both blood and BAL were unable to suppress activated T cell proliferation: 122.8% and 106.3% respectively.

IL-17 and TNF-α analyses

To study the mechanism by which the immune response could be exacerbated in HP, IL-17 was measured in the sera and BAL from the three study groups. No detectable levels of IL-17 were measured in the serum from normal individuals and asymptomatic subjects. Sera from patients with HP show a marked increase in IL-17 concentration (210.47 $\pm$ 95.24 pg/ml) (Figure 4a). No IL-17 was detected in BAL from normal individuals whereas an intermediate amount was found in BAL from asymptomatic subjects (192.37 $\pm$ 83.45 pg/ml). An increased level of IL-17 was detected in BAL from HP patients (580.07 $\pm$ 138.68 pg/ml) (Figure 4b). These results suggest that IL-17-secreting cells are activated in HP and could be related to T_{reg} loss of suppressive function and enhanced influx of pro-inflammatory soluble factors and cells. In order, to characterize the inflammatory response in HP patients and asymptomatic subjects, we also measured levels of TNF-α in BAL from each cohort (Figure 5). Normal subjects had small amounts of TNF-α in BAL (210.83 $\pm$ 46.26 pg/ml), whereas a slight increase is noted in BAL from asymptomatic individuals (1498.11 $\pm$ 163.31 pg/ml). HP patients show a marked increase of this mediator in BAL (3233.78 $\pm$ 282.51 pg/ml) compatible with a more intense inflammatory response.

Discussion

Regulatory T cells are rare cells. Studying these cells in humans with a rare disease is a challenge. Characterization of T_{reg} is less evident in humans than in mice where definition of $CD4^+CD25^+Foxp3^+$ cells as T_{reg} is well accepted. In humans, other markers such as CD39, GITR, CTLA-4, as well as the absence of

CD127, is expected. However, due to very low number of isolated T_{reg} in BAL and blood, and the necessity to have a sufficient number of cells to carry out lymphosuppressive studies with confidence, the presence of all these markers could not be verified for this study. The fact that cells identified by $CD4^+CD25^+Foxp3^+$ marker clearly showed lymphosuppressive properties suggests that these are indeed immune regulatory cells. The message of this paper is not the precise identification of pure T_{reg} but that these cells have lost their normal lymphosuppressive function in acute HP.

Although the number of subjects per group was relatively small, which is understandable considering the orphan nature of the disease, the difference in T_{reg} function between patients with HP and the other 2 groups is striking and conclusive. Moreover, the fact that all proliferation assays and cytokine detection were performed in triplicate or in duplicate depending on the number of T_{reg}, gives more accuracy to our data and confirm the reproducibility of the data.

The results of this study suggest that the immune response to HP-causing antigens is modulated by T_{reg} cell function. Although the percentage of T_{reg} among $CD4^+$ T cells was similar for the three groups, those from asymptomatic antigen-exposed individuals and from healthy control subjects efficiently suppressed the proliferation of effector T cells while those from HP patients had no suppressive activity. Moreover, even if the three groups had a similar percentage of $Foxp3^+$ expression, T_{reg}, fluorescence intensity of the Foxp3

marker was clearly decreased in HP patients compared to normal individuals, whereas cells from asymptomatic subjects showed an intermediate fluorescence. This dysfunctional phenotype of T_{reg} from HP patients could explain the large accumulation of lymphocytes in the lungs in these patients while the intermediate activity in asymptomatic subjects could be sufficient to control the disease.

These findings indicate that the normal T_{reg} lymphosuppressive function in asymptomatic subjects is able to attenuate the magnitude of the cellular immune response to inhaled antigens. The presence of significant levels of antigen specific antibodies in the serum is indicative of an antigenic exposure. The suppression, although incomplete, since these subjects have a mild increase of lung inflammatory cells, may be sufficient to prevent the disease *per se*. The clinical significance of the low grade inflammation in asymptomatic subjects is supported by a follow-up study of dairy farmers who were presenting a slight increase in BAL lymphocytes but remained asymptomatic and still had normal lung function after 20 year [9].

Similarly to the low occurrence rate of HP and inherent mechanisms of immune tolerance to SR antigen in most exposed farmers, we have shown that in a *Saccharopolyspora rectivirgula* (SR)-induced murine model of HP, the lymphocyte counts decrease with time and that T_{reg} suppressive functions appear after 10 weeks of repeated SR exposure (data not shown). Similar observations

of attenuated inflammatory response were reported previously in mice exposed to SR antigen [16].

In the SR-induced murine model of HP, the immune response seems to mimic the response of asymptomatic individuals. Other animal models studies have also shown that continued antigen challenge results in the waning of the pulmonary response rather than progression of the disease [17, 18]. These observations are also noted in humans where symptoms may be less severe with recurrent exposures [19].

Lung tolerance to a continuous exposure to antigens is well documented and has been attributed to a change in the phenotype of antigen-presenting dendritic cells. A decrease in MHC class II and co-stimulatory molecules and impairment of effector T cells stimulation can lead to a tolerogenic dendritic cell phenotype producing IL-10 and/or TGF-β and induce a T_{reg} phenotype [20]. The low grade continuous inflammation may be sufficient to induce the mechanisms of control of inflammation in asymptomatic exposed subjects.

In patients with HP, the normal T_{reg} lymphosuppressive function is impaired, thus lymphocytes are free to proliferate, to accumulate in the lung, and to maintain an inflammatory environment. The events leading from the asymptomatic stage to the development of the disease that can explain this T_{reg} loss of function are still

unclear. As pointed out earlier the prevalence of HP is very low and most subjects exposed to the causal antigens do not develop the disease. It has been suggested that some factors can trigger HP by breaking the homeostasis between immune tolerance and inflammatory mechanisms. Potential promoting factors for HP include a concomitant viral or bacterial infection, an increase in antigen load, and a genetic predisposition. Patients suffering from acute bouts of HP often report initial symptoms suggestive of a respiratory viral infection. Dakhama *et al.* reported the presence of viral antigens in the lungs of HP patients [21]. We have previously reported that a viral infection could trigger an exacerbated immune response to SR antigen in a mouse model of HP [22]. In that viral-induced HP murine model, Sendai, a paramyxovirus viral infection leads to dendritic cell maturation and up-regulation of MHC class II and CD86 [23]. Indeed, some studies have shown that over expression of co-stimulatory molecules such as MHC class II, CD80, and CD86, decreases the suppressive effects of T_{reg} and promote effector T cell activation [24]. Similarly, Ahn *et al*. reported that dendritic cells can reverse the suppressive effect of T_{reg} independent of cytokines production [25].

Defective T_{reg} cells have been reported in other inflammatory diseases such as arthritis and multiple sclerosis [26, 27]. In a recent article, Park *et al*. demonstrated that T_{reg} are indeed implicated in *Saccharopolyspora rectivirgula*-induced murine HP [28]. The authors showed an increased severity of HP in mice depleted of $CD25^{+}$ cells. These mice present a higher level of TGF-β and

IFN-γ in the lung, an increased number of inflammatory cells in bronchoalveolar lavage, more severe lung damages, and higher level of serum SR-specific IgG. An adoptive transfer of $CD4^+CD25^+$ cells restores this inflammatory environment and decreases IFN-γ production by T cells in the lung. These results demonstrate that $CD4^+CD25^+$ cells play a protective role in HP by reducing IFN-γ production by T cells. However, in this study, no analysis of T_{reg} function was performed. The results of the present study demonstrate that in active human HP, the regulation of the effector arm of immune response is no longer under the control of T_{reg} cells. As a consequence, the exacerbated cellular influx and inflammatory mediators are most probably responsible of the acute phase of the disease. HP patients are not protected against inflammatory process because the $CD4^+CD25^+$ cells have lost their suppressive function and can no longer control T effector cell proliferation.

A recent explanation for the defective T_{reg} activity is that the presence of TNF impairs the suppressive function of T_{reg} [29]. Anti-TNF therapy is successful in restoring T_{reg} function in patients suffering from diabetes [26]. In the thymus, TNF could act on T_{reg} and cause the loss of their suppressive function. Recent observations suggest that T_{reg} are very sensitive to TNF. Thymic $CD4^+CD25^+$ cells derived from healthy donors show a higher expression of TNFRII than $CD4^+CD25^-$ cells [30]. Since TNF is increased in patients with HP (Figure 5) [31], it could potentially be a mechanism by which T_{reg} lose their immunosuppressive function in this disease.

The finding that IL-17 is increased in HP can also explain, in part, the impaired T_{reg} function as well as the exacerbation of the immune response characterizing the disease. IL-17 is a pro-inflammatory cytokine mostly produced by Th17 cells. IL-17 promotes IL-6 and IL-1β production by neighbouring cells, triggering a pro-inflammatory environment [12]. These conditions, as well as interaction with pathogens, promote dendritic cells maturation and secretion of pro-inflammatory cytokines. Production of pro-inflammatory cytokines, in particular IL-6, IL-1β, and TNF, released by mature dendritic cells can subvert T_{reg} immunosuppressive function [13, 14]. Moreover, recent work demonstrated that, in the absence of pro-inflammatory signal, FoxP3 can abrogate RORγt, a transcription factor for Th17 cells, and drive T_{reg} differentiation. In a pro-inflammatory environment this inhibition is abrogated, IL-17-secreting Th17 cells differentiation is initiated, and T_{reg} suppressive function is inhibited [32]. Moreover, in an inflammatory environment, T_{reg} can differentiate in Th17 cells and secrete, effector-like cells, IL-17 [33]. Th17 cells are involved in many autoimmune, inflammatory, and infectious diseases [34-36]. A recent study reported the lung accumulation of $CD4^+$ T cells that produce IL-17 in HP patients [37]. Inflammatory mediators, such as TNF and IL-6, are involved in HP [38-40]. Recently, A/H1N1 influenza A virus infection has been found to promote an increase in pro-inflammatory mediators involved in the development of Th17 cells [41]. We hypothesize that a HP promoting factor, a viral infection for example, could trigger the maturation of

dendritic cells and the production of IL-17 by immune cells contributing to the attenuation of T_{reg} suppressive function.

Another study by Ito *et al.* indicates that maturation of dendritic cells by Sendai virus leads to an up-regulation of co-stimulatory OX40 ligand (OX40L) expression level [42]. Since T_{reg} cells express high levels of OX40, a member of TNF-receptor family [43] and that binding of OX40 with its ligand on mature dendritic cells blocks T_{reg} suppressive function [44, 45] one might expect that such a mechanism would also affect the regulatory function T_{reg} cells. Ligation of OX40 to its ligand does not affect the expression of FoxP3 in T_{reg} [43]. The molecular explanation for this T_{reg} loss of function is still unknown. Moreover, OX40-OX40L binding promotes T cells proliferation and increases the production of several cytokines [44]. Hence, in HP, mature dendritic cells could potentially inhibit T_{reg} function through OX40-OX40L binding. On another hand, mature dendritic cells could promote the inflammatory environment by activating T effector cells that trigger the lymphocytosis.

Similarly to other inflammatory and autoimmune diseases, a defect in T_{reg} function is involved in the pathology of HP. The events leading to the breakdown of the maintenance of the immune homeostasis to HP antigens need to be further clarified. However, we hypothetised that impaired T_{reg} function is probably caused by multiple interdependent immune events (Figure 6). Maturation of dendritic cells by a cofactor, such as a viral infection, promotes a pro-

inflammatory milieu which can in turn abrogate T_{reg} function in a cytokine-dependant or independent way.

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Table 1: Number of cells (x 10^6) recovered from BAL of normal individuals, asymptomatic subjects, and HP patients.

Total cells recovered from BAL	Normal (n = 4)	Asymptomatic (n = 4)	HP patients (n = 6)
Total inflammatory cells	29.82	35.63	134.57
Macrophages	24.72	28.58	72.13
Lymphocytes **T_{reg}**	3.97 0.12	7.20 0.17	51.00 2.81
Neutrophils	0.37	0.24	11.84

Eosinophils	0	0	0

Table 2: Percentage of Foxp3 expression and Foxp3 cell mean fluorescence intensity for normal individuals, asymptomatic subjects, and HP patients.

	% of Foxp3 Expression	Mean Fluorescence Intensity
Normal		
Blood	92.9	45.3
BAL	90.23	58.25
Asymptomatic		
Blood	88.75	15.88
BAL	92.5	13.8
HP patients		
Blood	86.0	13.24
BAL	88.3	7.99

Figures legends

Figure 1: a) Total and differential BAL cell counts from normal individuals, asymptomatic subjects, and HP patients are expressed per millilitre of recovered BAL (mean ± SEM). Inflammatory cells counts and lymphocyte counts of the HP patient group were significantly higher than control subjects: Total cells (0.928×10^6 vs 0.152×10^6; $p = 0.036$; Lymphocytes (0.285×10^6 vs 0.021×10^6; $p < 0.018$). b) Percentage of cell subpopulations recovered in BAL fluid (mean ± SEM) from normal individuals, asymptomatic subjects, and HP patients.

Percentage of lymphocytes from HP subjects is higher than those from normal individuals (37.9% vs 13.3%; p = 0.045). There is an increase in lymphocyte percentage from asymptomatic subjects (20.17%) but no significant difference neither from normal individuals (p = 0.45) nor from HP patients (p = 0.21). n = 4-6.

Figure 2: Percentages of $CD4^+CD25^+$ T cells of the total $CD4^+$ T cells from blood (a) and BAL (b) for normal individuals, asymptomatic subjects, and HP patients. No significant difference was observed. n = 4-6.

Figure 3: T_{reg} function test studies. a) Blood and b) BAL activated T cells (anti-CD3/CD28) were placed in culture with an equivalent number of blood or BAL T_{reg} cells from normal individuals, asymptomatic subjects, and HP patients. Results are expressed as percentage of control activated T cells proliferation (mean ± SEM). Blood T_{reg} from normal (58.0%; p = 0.02) and asymptomatic (68.2%; p = 0.029) subjects efficiently suppressed T cells proliferation compared to HP patients (122.83%). BAL T_{reg} from normal (52.88%; p = 0.003) and asymptomatic (70.6%; p = 0.025) subjects efficiently suppressed T cells proliferation compared to HP patients (106.3%). n = 4-6.

Figure 4: Concentration of IL-17 in serum (a) and BAL (b) from normal individuals, asymptomatic subjects and HP patients. Results are expressed as

concentration (pg/ml) of IL-17 (mean ± SEM). No or little IL-17 was detected in the serum and BAL from healthy controls and asymptomatic individuals. A marked increase of IL-17 concentration was observed in serum of patients with HP (210.47 ± 95.24; $p = 0.0369$) and BAL (580.07 ± 138.67; $p < 0.017$). n = 4-6.

Figure 5: Concentration of TNF-α in BAL from normal individuals, asymptomatic subjects and HP patients. Results are expressed as concentration (pg/ml) of TNF-α (mean ± SEM). Small amounts were detected in BAL from healthy controls and intermediate levels were found in asymptomatic individuals. A marked increase of TNF-α concentrations were observed in the BAL of patients with HP (3233.78 ± 282.51; $p < 0.002$). Different letters design groups with significant statistical difference where $p < 0.05$. n = 4-6.

Figure 6: Hypothetic mechanisms involved in the regulation of the immune response in asymptomatic subjects and in T_{reg} loss of immunosuppressive function in HP. In asymptomatic subjects, tolerogenic dendritic cells maintain an immunosuppressive environment which promotes efficient T_{reg} inhibitory function. Maturation of dendritic cells by an HP promoting factor causes pro-inflammatory mediators production by many immune cells that inhibit T_{reg} function. Increased co-stimulatory (OX40L, B7 molecules) and MHC class II molecules on mature dendritic cells increase their antigen presentation capacity and priming of T cells but also inhibit T_{reg} function. IL-17 production by dendritic cells and Th17 cells could promote molecular expression of transcription factor such as RORγt that

abrogate T_{reg} cells function. All these mechanisms could result in a T_{reg} loss of function allowing a pro-inflammatory environment and development of the disease.

Figure 1

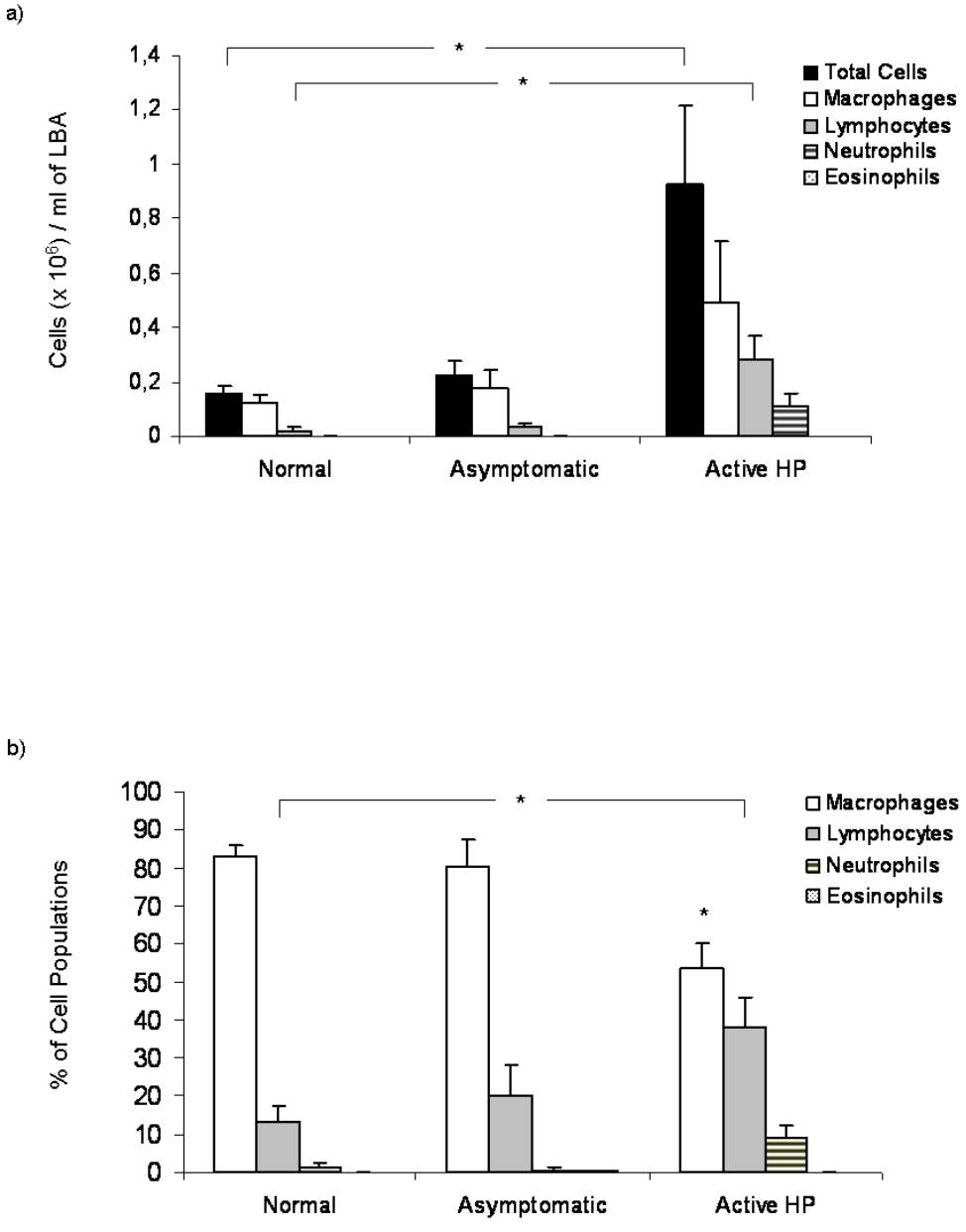


Figure 2

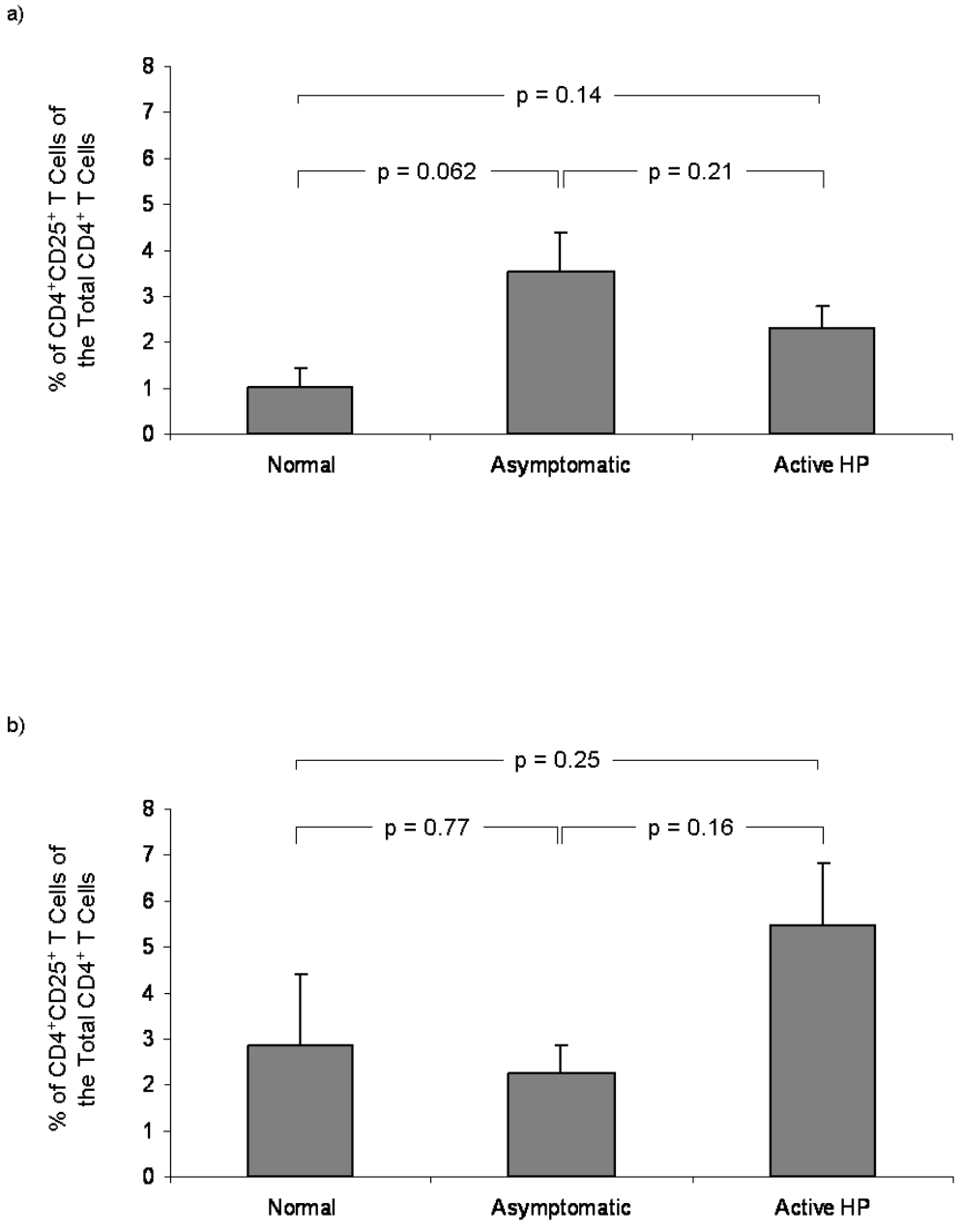
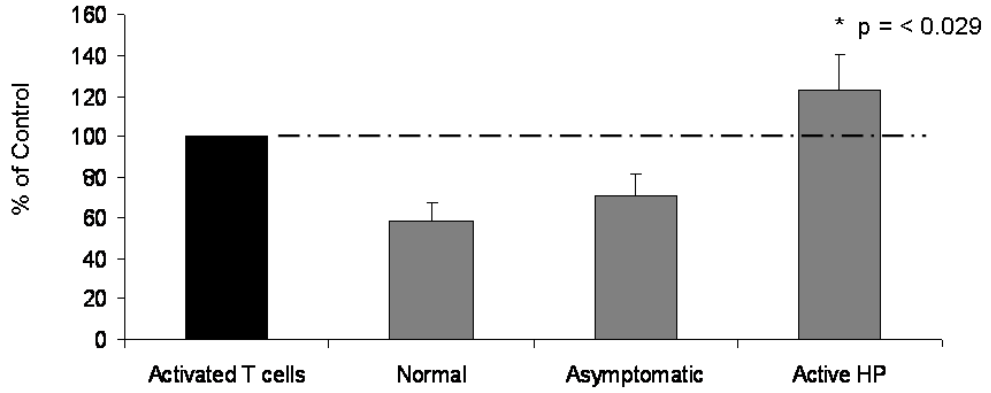

a)
% of CD4+CD25+ T Cells of the Total CD4+ T Cells
p = 0.14
p = 0.062
p = 0.21
Normal
Asymptomatic
Active HP
b)
% of CD4+CD25+ T Cells of the Total CD4+ T Cells
p = 0.25
p = 0.77
p = 0.16
Normal
Asymptomatic
Active HP

Figure 3

a)


160
140
120
100
80
60
40
20
0
% of Control
* p = < 0.029
Activated T cells
Normal
Asymptomatic
Active HP

b)

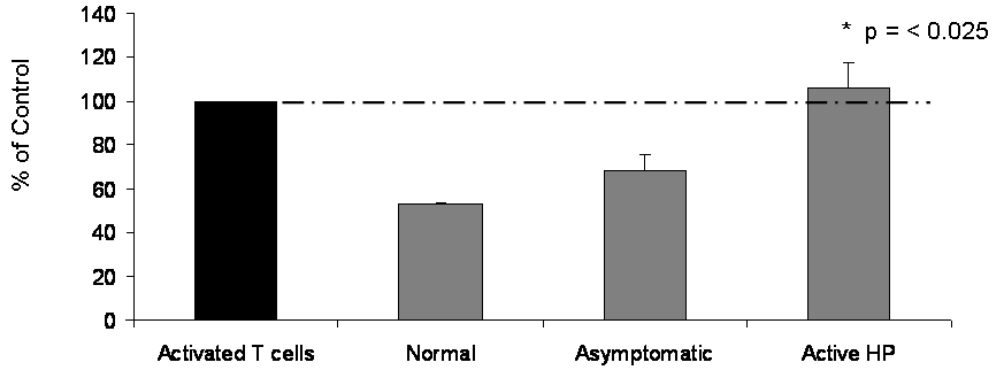

140
120
100
80
60
40
20
0
% of Control
* p = < 0.025
Activated T cells
Normal
Asymptomatic
Active HP

Figure 4

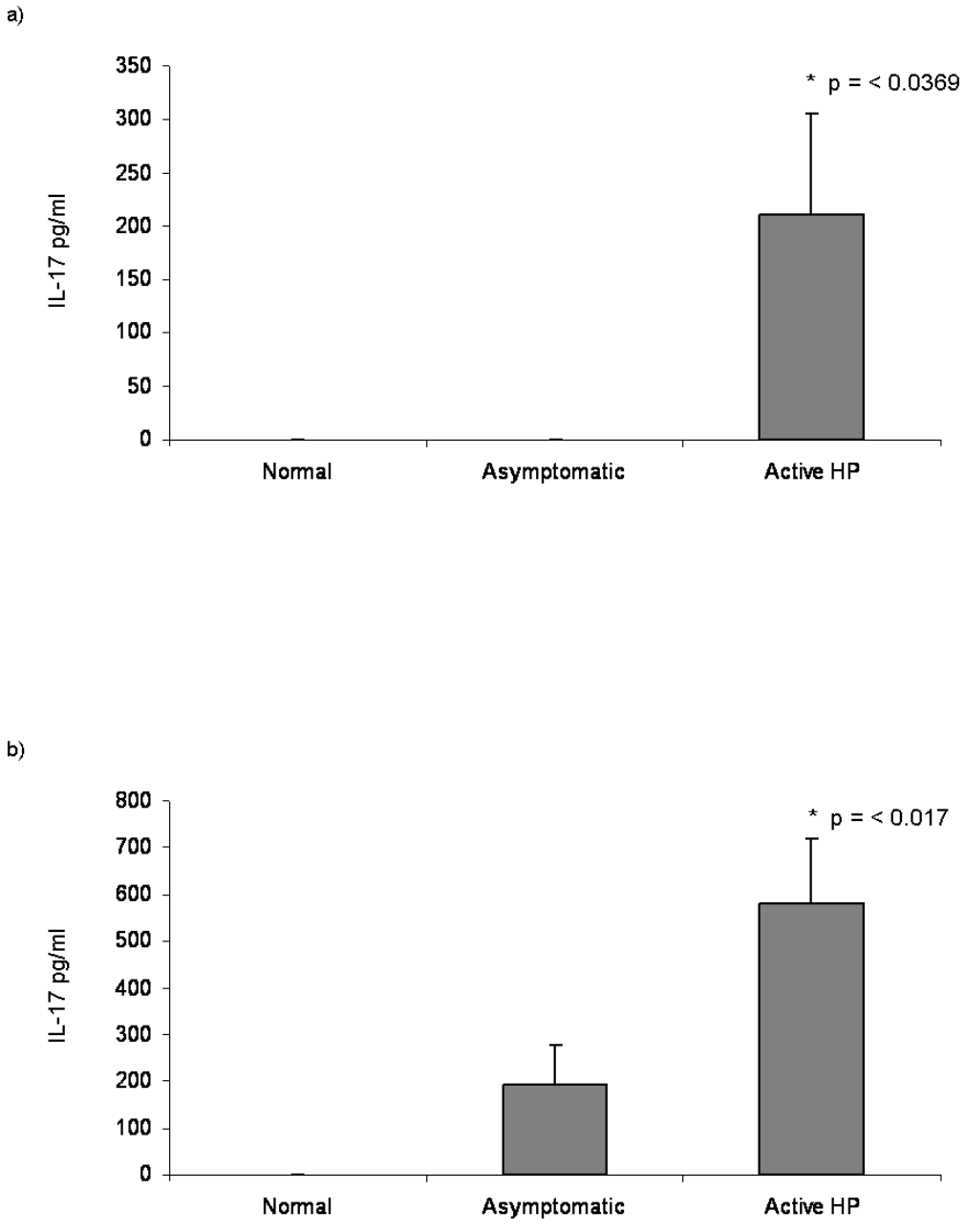

a)
350
300
250
200
150
100
50
0
IL-17 pg/ml
* p = < 0.0369
Normal
Asymptomatic
Active HP
b)
800
700
600
500
400
300
200
100
0
IL-17 pg/ml
* p = < 0.017
Normal
Asymptomatic
Active HP

Figure 5

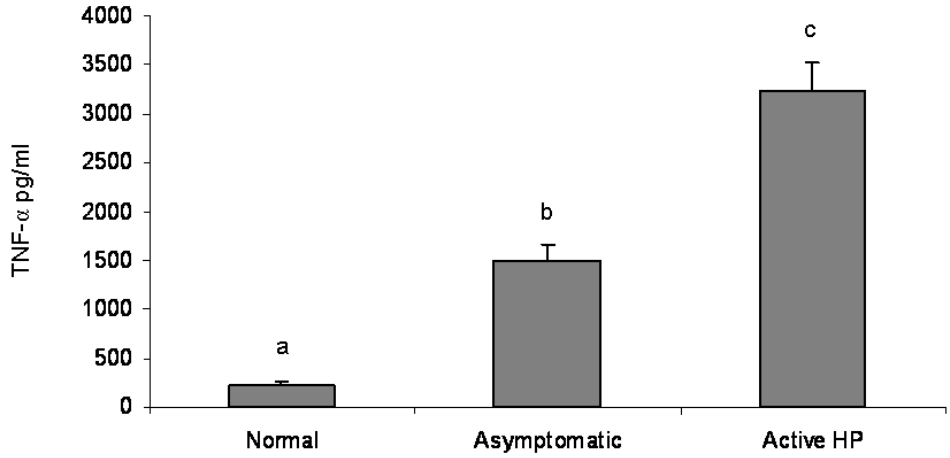
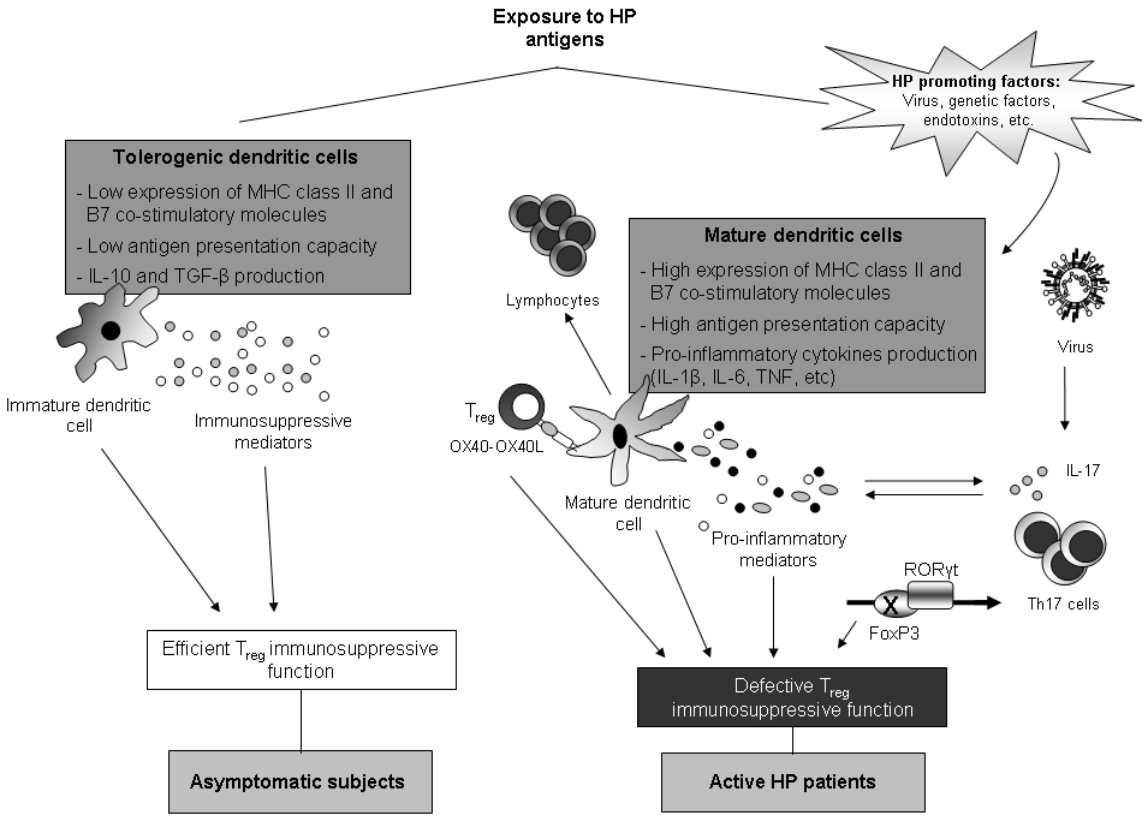

4000
3500
3000
2500
2000
1500
1000
500
0
TNF-α pg/ml
a
b
c
Normal
Asymptomatic
Active HP

Figure 6


Exposure to HP antigens
HP promoting factors: Virus, genetic factors, endotoxins, etc.
Tolerogenic dendritic cells
- Low expression of MHC class II and B7 co-stimulatory molecules
- Low antigen presentation capacity
- IL-10 and TGF-β production
Mature dendritic cells
- High expression of MHC class II and B7 co-stimulatory molecules
- High antigen presentation capacity
- Pro-inflammatory cytokines production (IL-1β, IL-6, TNF, etc)
Lymphocytes
Virus
Immature dendritic cell
Immunosuppressive mediators
Treg
OX40-OX40L
Mature dendritic cell
Pro-inflammatory mediators
IL-17
RORγt
Th17 cells
FoxP3
Efficient Treg immunosuppressive function
Defective Treg immunosuppressive function
Asymptomatic subjects
Active HP patients