Severity of Asthma: The Role of CD25+, CD30+, NF-κB, and Apoptotic Markers

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Abstract

Objectives: We studied the role of the regulatory T cells CD4+CD25+ (Treg) and activated CD4+CD30+ cells in the pathogenesis of asthma and their association with apoptosis and NF-κB in patients with mild intermittent asthma (MA), severe persistent asthma (SA), and healthy volunteers (HV).

Methods: Peripheral blood lymphocytes (PBL) were extracted from asthmatic patients during exacerbations, and CD4+ cells were separated using Dynal beads. Immunostaining of whole PBL for NF-κB, Bax, and Bcl-2, and immunostaining of CD4+ cells for CD25+ and CD30+ cells were performed using immunocytochemistry.

Results: Treg cells were expressed at higher levels in MA than in HV and SA (P<.05), while CD30+ T cells were expressed at higher levels in both SA and MA than in HV (P<.05), although there was no remarkable difference between SA and MA (P>.05). Levels of NF-κB, Bcl-2, and Bcl-2/Bax increased, whereas those of Bax decreased, progressively, from MA to SA (P<.05). NF-κB levels correlated directly with the Bcl-2/Bax ratio and with CD4+CD30+ cells in SA and MA, whereas CD4+CD30+ cells correlated inversely with the Bcl-2/Bax ratio.

Conclusions: Unregulated Treg cells probably return inflammatory responses to normal values during exacerbations in MA; however, expression of Treg cells was extensively diminished in SA, leading to probable loss of suppressive control over underlying immune reactions. CD4+CD30+ cells were associated with the pathogenesis of asthma but not with severity. NF-κB seems to be the central inflammatory factor in SA, with a remarkable loss of PBL apoptosis, diminished Treg levels, and high CD30+ cell levels that probably induce NF-κB, which in turn blocks the proapoptotic potential of CD30 induction itself.

Keywords: Asthma. Apoptosis. Memory cells. CD45RO. Tρ1. Th2. IL-4. IFN-γ.

Resumen

Objetivos: Estudiamos las células T reguladoras CD4+CD25+ (Treg) y las células activadas CD4+CD30+ en la patogenia del asma y su asociación con la apoptosis y NF-κB en pacientes con asma leve intermitente (AL), grave persistente (AG), y voluntarios sanos (VS).

Métodos: Se extrajeron linfocitos de sangre periférica (LSP) de pacientes asmáticos durante las exacerbaciones y las células CD4+ se separaron usando bolas Dynal. Se realizó una inmunodetección de todos los LSP para NF-κB, Bax, y Bcl-2, y otra inmunodetección de las células CD4+ para células CD25+ y CD30+ empleando inmunocitoquímica.

Resultados: Las células Treg se expresaron con niveles más altos en AL, seguido de VS, y los niveles más bajos en AG (P<0,05), mientras que las células T CD30+ se expresaron con niveles más altos en AG y en AL, que en VS (P<0,05), aunque no existían diferencias destacables entre AG y AL (P>0,05). Los niveles de NF-κB, Bcl-2, y Bcl-2/Bax aumentaron, mientras que los de Bax disminuyeron, progresivamente, de AL a AG (P<0,05). Los niveles de NF-κB correlacionaron directamente con el cociente Bcl-2/Bax y con las células CD4+CD30+ en AL y AG, mientras que las células CD4+CD30+ correlacionaron inversamente con el cociente Bcl-2/Bax.

Conclusiones: Las células Treg no reguladas probablemente retoman la respuesta inflamatoria a valores normales en las exacerbaciones asmáticas en AL; sin embargo, la expresión de las células Treg estaba ampliamente reducida en AG, debido probablemente a una probable pérdida del control supresor sobre la reacción inmune subyacente. Las células CD4+CD30+ estaban asociadas a la patogenia del asma pero no a la severidad. El NF-κB parece que es un factor inflamatorio central en el AG con una marcada pérdida de la apoptosis de los LSP, disminución de Treg, y muchas células CD30+ que probablemente inducen el NF-κB que sucesivamente bloquea el potencial proapoptótico de la propia inducción CD30.

**Introduction**

Asthma is a chronic inflammatory lung disease that leads to significant morbidity, mortality, and financial burden [1]. Inflammation is both the central pathogenic feature and the principal clinical manifestation, and is responsible for airway obstruction and hyperresponsiveness [2]. According to the United States National Heart, Lung and Blood Institute, asthma can be classified as mild intermittent, mild persistent, moderate persistent, and severe persistent [3]. Allergic inflammation is a balance between immune tolerance, immune cell survival, cytokine and activation marker interaction, and the presence of allergens [4]. Malfusions of apoptosis play a role in the chronicity of many inflammatory diseases [5], including asthma, and immune cell recruitment can lead to adverse effects [6]. A significant increase in the percentage of proapoptotic Bcl-2 protein and a decrease in the percentage of proapoptotic Bax protein have been reported in asthmatic patients [7].

There are several reports on the existence of a unique CD4+CD25+ population of professional regulatory T cells (Treg) that actively prevent both the activation and the effector function of CD4+CD25- T cells [8-10]. These cells do not proliferate in response to antigenic stimulation in vitro, are naturally anergic, and can suppress activation and proliferation of CD4+CD25- cells in an antigen-nonspecific manner through cell-to-cell interaction [11]. Although natural Treg cells are important in maintaining immunologic tolerance in asthma, their role in the pathogenesis of allergic asthma remains unclear [12]. The decrease in the CD4+/CD25+ ratio and its function in peripheral blood lymphocytes (PBL) may be responsible for the pathogenesis of asthma [13]. Some studies show that the level of CD4+CD25+ T cells and mRNA expression of functional Treg molecules are suppressed in patients with allergic asthma compared with healthy controls, and that this suppression is significantly related to airflow allergy [14,15]. Findings on the role of Treg in asthma are inconsistent, and one report [16] demonstrated that Treg cells were overexpressed in atopic asthematics with normal functional status.

Another marker that is potentially involved in the pathogenesis of asthma is CD30. This transmembrane costimulatory molecule belongs to the tumor necrosis factor (TNF) receptor superfamily that is expressed on activated T cells. A recent study revealed a positive correlation between expression of CD30 and the development of allergic diseases [17]. CD30 was shown to be a marker of type 2 helper T (Th2) lymphocytes [18]. Th2 lymphocytes (key cells in the pathogenesis of allergic inflammation), and serum levels of soluble CD30 were found to be high in allergic patients [18]. In addition, the association between CD30 and apoptosis is complex and unclear. In Hodgkin lymphoma, overexpression of CD30 drives activation of the constitutive nuclear factor-kB (NF-kB), which is one of the most potent proapoptotic factors [19]. Furthermore, some authors point to the strong potential of CD30 to induce apoptosis in eosinophils, and show how both CD30 expression and eosinophil apoptosis increased in a time-dependent manner [20,21].

In this study, we explore the relationship between CD4+CD25+ Treg cells, activated CD30+ T cells, and the expression level of NF-kB in PBL, and compare them with PBL levels of proapoptotic Bax and antiapoptotic Bcl-2 molecules and their Bcl-2/Bax ratio in 3 groups; patients with severe persistent asthma (SA), patients with mild atopic asthma (MA), and healthy volunteers (HV). We evaluate the role played by Treg, activated CD30+ cells, NF-kB, and apoptosis in the severity of asthma. To our knowledge, this is the first study to explore this relationship in adults experiencing exacerbations.

**Methods**

**Participants**

Fifty-two asthmatic patients (36 MA and 16 SA) were enrolled from February 2007 to March 2008 in Selangor, Malaysia. Age ranged from 17 to 53 years (31 men and 21 women), and none were smokers or had other major illnesses. All patients were diagnosed by a respiratory medicine specialist and diagnosis was confirmed by lung function tests at the Central Kuala Lumpur Hospital. Twenty age-matched HV aged 23 to 55 years (12 men and 8 females) were also enrolled. The HV were nonsmokers and their chest radiographs and lung function tests showed no evidence of respiratory disease. Five mL of blood was withdrawn from asthmatic patients and controls for isolation of PBL. Samples were extracted from asthmatic patients within the first 12 hours of an exacerbation. Moreover, therapy was closely monitored to avoid discrepancies in therapy protocols and to reduce the likelihood of oral or inhaled therapy interfering with the study findings. All the patients and controls gave their written informed consent for interviewing and blood sampling. The study was conducted according to the requirements of the Declaration of Helsinki and approved by the local ethics committee.

**Isolation of PBL**

PBL were isolated from the heparinized whole blood of all asthmatic patients to prepare a pure population of lymphocytes. Separation was by Ficoll-Hypaque density sediment centrifugation (Sigma, St. Louis, Missouri, USA). The final concentration of the lymphocyte suspension was adjusted to 1 x 10^6 cells/mL [22].

**Positive Selection of CD4+ T Cells**

The Dynal CD4 Positive Isolation Kit (Cat. no. 113.31D) (Dynal Biotech, Smestad, Norway) for magnetic beads was used for the positive selection of CD4+ T cells from the isolated total PBL cells of all asthmatic patients. Dynabeads were mixed with the isolated PBL in a tube. After a short incubation, the bead-bound CD4+ cells were separated using a magnetic particle concentrator (MPC-S, (Dynal Biotech) for 20-µL to 2-mL samples. The positively isolated cells were then detached from the beads by adding DETACHaBEAD (Dynal Biotech). CD4+ cells were separated and purified according to the manufacturer’s instructions (version 2 of the manual) [23]. The resulting cells were >99% pure.
Antibodies

Rabbit polyclonal antibodies for the immunocytochemistry assay were obtained from Abcam plc (Cambridge, UK). Antihuman Bax antibody (ab10813), antihuman Bcl-2 (ab18210), antihuman NF-κB p65 (ab31481), antihuman CD30 (ab36749), and rabbit polyclonal antihuman CD25 (sc-666) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Biotinylated goat secondary antibodies (ab6702) against rabbit immunoglobulins were from Abcam plc.

Immunocytochemistry for PBL

Immunocytochemistry was performed according to the instructions of the manufacturer of the primary and secondary antibodies (Abcam plc), with modifications. Two hundred microliters of lymphocyte suspension was aliquoted into wells of a cytocpin and centrifuged at 650 rpm for 6 minutes on silane-coated slides. Slides were dried in a desiccation chamber overnight, then fixed in 2% paraformaldehyde (BDH, Poole, Dorset, UK) for 20 minutes at room temperature and washed 3 times in phosphate-buffered saline (PBS) (BDH). Slides were incubated in 95°C preheated antigen retrieval buffer, 100 mM Tris (Sigma), 5% (w/v) urea (Merck, Darmstadt, Germany), pH 9.5, for 10 minutes in a water bath. After 3 washes with PBS, cells were permeabilized for 15 minutes at room temperature in blocking buffer, 3% bovine serum albumin (Merck) in PBS, plus 0.1% Triton X-100 (Sigma) followed by blocking of nonspecific binding in blocking buffer for 1 hour at room temperature.

One hundred microliters of rabbit polyclonal antibodies diluted 1:50 in blocking buffer were added to the immunocytochemistry slides, which were then incubated in a humid chamber overnight at 4°C. The following day, slides were rinsed gently with PBS-0.05% Tween 20 (Merck) and placed in a fresh PBS-Tween bath for 1 minute. One hundred microliters of the biotinylated goat anti-rabbit secondary antibodies diluted 1:600 in PBS was added to the bath, which was then incubated in a humid chamber for 1 hour at 37°C. After washing with PBS-Tween 20 solution, 2 drops of streptavidin-horseradish peroxidase reagent (Dako, Glostrup, Denmark) was applied, and slides were placed in a humid chamber and incubated for 30 minutes at 37°C. The prepared diaminobenzidine substrate chromogen solution (Dako) was then applied, and slides were incubated in the dark for 20 minutes at room temperature. Mayer hematoxylin stain was used as a counterstain, and slides were dehydrated and mounted with DPX mounting fluid (BDH). Immunostained cells that were moderate-to-intense dark brown in color were considered positive, and other cells were considered negative (Figure 1). Under the light microscope, the number of brown stained cells out of 200 countable total cells was calculated to measure the percentage of the positive PBL expressing Bax, Bcl-2, CD25, CD30, and NF-κB p65.

Statistical Analysis

The statistical analysis was performed using SPSS version 10 (SPSS Inc, Chicago, Illinois, USA) and MS Excel 2000. After the normal distribution pattern of the immunocytochemistry readings was verified, the mean (SD) and parametric t tests were used to evaluate the significance of differences. Pearson's correlation coefficient was used to measure the correlation between the variables studied.

Results

Expression of Bax, Bcl-2, CD4+CD25+, CD4+CD30+, and NF-κB

The results of the immunocytochemistry assay were visually accurate and easily interpreted. Isolated whole PBL cells were examined for cellular expression of Bax, Bcl-2,
Figure 2. Mean (SD) percentages of the expression of the markers in the peripheral blood lymphocytes of severe asthmatic patients, mild asthmatic patients, and healthy volunteers. A, Bax was significantly lower in severe asthmatic patients than in the other groups. B, Bcl-2 was significantly higher in severe asthmatic patients than in the other groups. C, The Bcl-2/Bax ratio was significantly higher in severe asthmatic patients than in mild asthmatic patients, and higher in mild asthmatic patients than in the healthy volunteers. D, NF-κB was significantly higher in severe asthmatic patients than in both mild asthmatic patients and healthy volunteers. E, CD25+ expression was significantly higher in mild asthmatic patients than in severe asthmatic patients and healthy volunteers, and in severe asthmatic patients it was significantly lower than in healthy volunteers. F, Expression of CD30+ from the CD4+ pool was higher in both severe asthmatic patients and in mild asthmatic patients than in the healthy volunteers, with no significant differences between severe asthmatic patients and mild asthmatic patients.
and NF-κB, while the pure CD4+ T cells underwent CD25 and CD30 staining. The mean (SD) percentage of positive PBL for Bax was lower in SA (11.48 [9.6]) than in both MA (29.41 [7.4]) and HV (35.23 [7.5]) (P<.05). In contrast, the mean (SD) percentage of the Bcl-2-positive PBL was higher in SA (33.57 [8.4]) than in both MA (16.38 [5.4]) and HV (8.34 [4.8]) (P<.05). Accordingly, the mean Bcl-2/Bax ratio was calculated and found to be significantly higher in SA (2.9 [1.6]) than in MA (0.55 [0.1]) (P<.05), and in MA than in HV (0.23 [0.11]) (P<.05) (Figure 2). For NF-κB expression in PBL, the mean (SD) percentage of positive expression of NF-κB in SA (32.6 [7.4]) was much higher than in MA (7.6 [3.7]) and HV (3.9 [2.5]) (P<.05), and no significant differences were found between NF-κB expression in MA and in HV (P>.05) (Figure 2).

For the cellular expression of CD4+CD25+ and CD4+CD30+ cells, the mean (SD) percentage of positive expression was calculated using the CD4+ pool rather than the whole PBL pool to yield more precise results for comparison. Surprisingly, the mean percentage of positively expressed CD25+ in the CD4+ pool was found to be significantly higher in MA (58.5 [8.3]) than the moderately low level in HV (41.3 [4.9]) (P<.05) and the very low level in SA (21.65 [8.3]) (P<.01). The level in SA was also significantly lower than in HV (P<.05) (Figure 2). For CD4+CD30+ cells, there was no significant difference in the mean percentage of positive expression of CD30+ form the CD4+ pool in SA (57.6 [7.5]) or in MA (51.5 [9.4]), although both were higher than in HV (30.7 [6.1]) (P<.05) (Figure 2). There was no relationship between the immunostaining marker studied and age or sex in the SA and MA groups.

Correlations Between Bcl-2+, Bax+, Bcl-2/Bax, NF-κB+, CD4+CD25+, and CD4+CD30+

The correlation coefficient (r) was calculated to ascertain the behavior of marker expression in PBL. Expression of Bax was found to correlate inversely with Bcl-2 in MA (r=-0.65), in SA (r=-0.43), and in HV (r=-0.47) (P<.05). NF-κB was significantly correlated with the Bcl-2/Bax ratio in SA (r=0.83) and in MA (r=0.58) (P<.05), but not in HV. The CD30+ percentage in CD4+ cells was significantly correlated with NF-κB expression in SA (r=0.57) and in MA (r=0.33) (P<.05), but not in HV. On the other hand, the CD30+ percentage in CD4+ cells was significantly inversely correlated with the Bcl-2/Bax ratio in MA (r=-0.41) (P<.05), but not in SA or HV. No significant correlation was found for the expression of CD25+ in CD4+ cells with other variables in this study.

Discussion

Asthma is a series of complex, overlapping individual diseases or phenotypes, each defined by an interaction between genetic and environmental factors [24]. The findings of this study are consistent with those of Borish and Culp [24], who showed that the network of allergic and inflammatory factors that play a role in asthma inflammation vary greatly with the progress and stage of the disease. NF-κB exerts a key role in perpetuating inflammatory reactions in asthma, and levels of NF-κB expression increase with severity. NF-κB expression levels in PBL were generally higher in SA than in MA patients, although there was no significant difference between the NF-κB level in HV and in MA patients, even though PBL cells were taken during exacerbations in MA patients. This striking difference in NF-κB expression indicates that inflammation in MA, unlike SA, relies on different pathways in which NF-κB does not play a key role. The high level of NF-κB expression in SA only explains why the Bcl-2/Bax ratio was much higher in SA than in MA patients, as many recent studies have shown that NF-κB stimulates the expression and activity of Bcl-2, which itself acts as a potent antiapoptotic molecule and inhibits the proapoptotic Bax molecules [25-27]. The results of this study are consistent with those of other recent studies, which revealed that Bcl-2 levels or the Bcl-2/Bax ratio is higher in asthmatics than in healthy individuals [28-30]. In asthma, where inflammation is the major problem, lymphocyte persistence may play a key role in the pathophysiology of chronic persistent airway inflammation [31]. As the antiapoptotic role of NF-κB in the PBL of asthma patients becomes more defined, the expression level of NF-κB correlates positively with the Bcl2/Bax ratio in both MA and SA patients. In addition, Bcl-2 was inversely correlated with Bax.

The percentage of CD4+CD25+ and CD4+CD30+ cells was measured as a percentage of CD25+ or CD30+ from CD4+ pools, and not out of the total number of PBL isolated. It was thought that this could minimize the interference arising from variations in the CD4+ cell pools in the different study groups, as increased numbers of CD4+ T lymphocytes have been found in asthmatic patients who show signs of activation [11]. Therefore, when the number of CD4+ cells increases or decreases, the percentage of CD25+ or CD30+ cells measured from CD4+ cells only would not be affected. In this study, the mean percentage of CD25+ in CD4+ cells was significantly higher in MA than in both SA and HV, and in HV it was higher than in SA, where the lowest percentages of CD25+ were found in CD4+ cells. Since there is no dispute about the suppressor effect of CD4+CD25+ cells on other T cells, this finding proves that the number of Treg cells in mild asthma tends to increase, probably as an immunoregulatory step. Nevertheless, no similar behavior of Treg cells was found in patients with severe persistent asthma, where Treg expression was by far the lowest. This might provide a clue as to the possible role of Treg cells in curbing allergic inflammation and limiting asthma exacerbations to a status similar to that of the MA group. This may also explain why the immune and inflammatory reactions do not resolve after exacerbations in severe persistent asthma. The low level of Treg cells in SA might be attributed to either inhibitory signals or loss of activation, as previous reports found that the inhibitory properties of human CD4+CD25+ cells are activation-dependent, yet antigen-nonspecific [19,32]. Given that T cell receptor (TCR)-mediated activation of NF-κB is crucial for Treg development [33], it seems that the elevated expression of NF-κB in SA failed to activate Treg development, maybe because activation of the high levels of NF-κB in SA was not driven by the TCR pathway. Further research is necessary to discover the exact network of mediators that controls the low levels of Treg cells in severe asthma, despite high levels of NF-κB. Many studies show that expression of Treg cells in asthma is
lower than that observed in normal individuals [12,13,14], and the consequent loss of control of Treg suppression can participate in the pathogenesis of asthma. On the other hand, one study [16] revealed that expression levels of Treg cells are normal in atopic asthma patients and higher than in healthy individuals. This discrepancy in the results of previous studies might be clarified by the observation in the present study that Treg cell levels increase during exacerbations of mild or moderate asthmatic patients, but decrease sharply in cases of severe persistent asthma, thus indicating that Treg cells might suppress allergen-induced exacerbations but not the severe persistent inflammation of asthma.

We also found that CD30+ in CD4+ cells were expressed in higher percentages in both SA and MA than in HV, with no remarkable differences between SA and MA, thus indicating that CD30+ T cells are significantly associated with asthma. This also indicated that the expression of CD30+, unlike CD25+, was not highly correlated with the severity of asthma. This finding is supported by evidence from previous reports that CD30+ expression is associated with the pathogenesis of asthma, and that expression levels are higher in asthmatic patients [17,18,34]. However, cellular expression of CD30+ seems to be different from that of the soluble form of CD30, which has been shown to be associated with the severity of asthma [18]. However, the exact role of CD30 in the pathogenesis of asthma and inflammation is still confusing. One report stated that allergen-specific CD4+CD30+ secreting TNF-α cytokines are present in the circulation of atopic donors following exposure to allergens, thus demonstrating that CD30 is a marker of TNF-α cells [18]. Interestingly, in this study, the correlation of the expression level of CD30+ T cells with that of NF-κB and the Bcl-2/Bax ratio could point to the complex role of CD30 in asthma. CD30+ T cells were significantly and directly correlated with NF-κB in both SA and MA, and inversely with the Bcl-2/Bax ratio in MA only. This finding provided evidence that CD30 markers could induce apoptosis in the MA group, where no severe persistent inflammation had yet occurred and low levels of NF-κB were present. At the same time, CD30 appeared to be directly correlated with a potent antiapoptotic factor, NF-κB, in both MA and SA. Since CD30 expression was not much higher in SA than in MA, NF-κB was most probably triggered in SA by factors other than CD30, and NF-κB itself might counteract the proapoptotic potential of CD30 in SA. The findings of the present study are consistent with those of another report [19], which showed that CD30 signals lead to activation of NF-κB. Our findings also support those of other authors, who showed that CD30 acts as a strong proapoptotic factor for PBL [20,21].

Conclusions

The current study enables us to infer that CD30+ are associated with the pathogenesis of asthma but not with its severity. These cells were expressed at higher levels in MA and SA than in HV. In addition, CD30 seems to induce apoptosis in MA but not in SA, maybe due to the stronger antiapoptotic effect of NF-κB at high levels. Given the sharp increase in NF-κB levels in SA, it is probably the leading inflammatory factor in SA rather than in MA, and it appears to suppress apoptosis in SA. Therefore, NF-κB was associated with both pathogenesis and severity of asthma. As for Treg cells, they might play an important role in curbing exacerbations in patients with MA, in whom CD25+ T cells are expressed at higher levels than in SA and HV, whereas their level was lowest in SA, a situation that could lead to complete loss of regulation of lymphocytic immune reactions. Inflammatory reactions in severe persistent asthma seem to be perpetuated by the lack of abundant Treg cells, apoptosis was extensively suppressed by the very high levels of NF-κB, and CD30 lost its proapoptotic effect, as it did not increase in SA at the same level as NF-κB, with no consistent assumption on the role of its proinflammatory effect. In contrast, in mild intermittent asthma, apoptosis was still functioning satisfactorily, Treg cell levels were sufficiently high, proinflammatory and proapoptotic CD30 levels were high, and proinflammatory antiapoptotic NF-κB levels were low, resulting in mild and self-limiting exacerbations of asthma.

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