Allergy 2006: 61: 27–34

Copyright © Blackwell Munksgaard 2005

ALLERGY
DOI: 10.1111/j.1398-9995.2005.00951.x

Original article

CD4+CD30+ T cells perpetuate IL-5 production in *Dermatophagoides pteronyssinus* allergic patients

Background: Airway allergic diseases are regulated by interleukin (IL)-5, which causes infiltration of eosinophils into the bronchial epithelium, and by IL-4 which increases serum immunoglobulin E (IgE) production and promotes CD30 expression on Th cells. CD30 generates a costimulatory signal involved in apoptosis or cell proliferation, depending on the microenvironment. Our aims were: (i) to analyze if CD4+CD30+ T cells from allergic patients proliferate in response to *Dermatophagoides pteronyssinus*, and (ii) if upon stimulation this cell population produces IL-4 and IL-5.
Methods: Peripheral blood mononuclear cell (PBMC) from 17 allergic rhinitis and mild allergic asthma patients and 12 healthy nonallergic individuals were stimulated with allergen in the presence or absence of anti-IL-4, anti-IL-5 or anti-IL-4Rα monoclonal antibodies (mAbs). TdT-mediated dUTP nick end-labeling (TUNEL) assay, 7-aminoactinomycin-D (7-AAD) intercalation, and flow cytometry were used to determine the CD4+CD30+ blasts percentage, cell proliferation, apoptosis, and intracellular cytokines after 7 culture days.
Results: Cell proliferation induced with allergen showed that 90% of the allergen-stimulated blasts were CD4+, 50% of which were CD30+. Allergen-stimulated PBMC showed a progressive increase (mean: from 7% to 23%) of CD4+CD30+IFN-γ+ and CD4+CD30+IL-4+ blasts which diminished (mean: 6%) after 5 culture days. In contrast, CD4+CD30+IL-5+ blasts showed a continuous progression (from 12% to 24%) that maintained after 7 culture days. The vast majority of CD4+CD30+ blasts were negative to 7-AAD or TUNEL. Additionally, a significant decrease (34%) was observed in the number of CD4+CD30+ blasts when IL-4 was neutralized.
Conclusions: These data suggest that specific allergen stimulation of PBMC isolated from allergic patients generates a nonapoptotic CD4+CD30+ blast subset that produces IL-5.

Y. Garfias[1], B. Ortiz[2], J. Hernández[3], D. Magaña[2], M. Becerril-Angeles[4], E. Zenteno[5], R. Lascurain[5]

[1]Instituto de Oftalmología, 'Fundación Conde de Valenciana'; [2]Departamento de Investigación en Bioquímica, INER, SS; [3]Centro de Investigación en Alimentación y Desarrollo, AC, Hermosillo, Sonora; [4]Centro Medico Nacional 'La Raza', IMSS; [5]Departamento de Bioquímica, Facultad de Medicina, UNAM, Mexico

Key words: allergy; apoptosis; CD4+CD30+ blasts; *Dermatophagoides*; interleukin-5.

Ricardo Lascurain PhD
Laboratorio de Inmunología
Departamento de Bioquímica
Facultad de Medicina
PO Box 70159
Universidad Nacional Autónoma de México, 04510
Mexico

Accepted for publication 1 July 2005

Allergic disease development involves an unbalance between T helper 1 (Th1) and Th2 cell activity (1). Th1 cells drive cellular immunity producing the crucial cytokine interferon (IFN)-γ whereas Th2 cells lead the response toward humoral immunity and produce the defining cytokines interleukin (IL)-4 and IL-5 (2). There is evidence of a Th2-type immune response predominance in airway inflammatory allergic diseases (3–5) that could be secondary to a higher frequency of apoptosis in IFN-γ+ T cells than in IL-4+ T cells infiltrating the airway wall (6), thus suggesting that Th1/Th2 unbalance in asthmatic inflammation may be the result of premature apoptosis in Th1 cells rather than in Th2 cells. Airway allergic disease is characterized by the infiltration of eosinophils into the bronchial mucosa and increased production of serum immunoglobulin E (IgE) (3). Recent reviews (7–9) point to the key role of IL-4, implicated in the switch of B cells to IgE production, and IL-5, shown to cause eosinophil infiltration and mucus production into the bronchial wall, in the development and maintenance of atopic asthma. Likewise, atopic asthmatic patients express CD30 on peripheral blood CD4+ T cells following *in vitro* allergen-specific stimulation (10). Some authors mention that CD30, a costimulatory signal and a member of the tumor necrosis factor receptor (TNFR) family is also expressed on activated Th2-like cells from

Abbreviations: Der p, Dermatophagoides pteronyssinus; PBMC, peripheral blood mononuclear cells; IL-4, interleukin 4; IL-5, interleukin 5; IL-4Rα, interleukin 4 receptor-alpha chain; IFN-γ, interferon gamma; TNFR, tumor necrosis factor receptor; 7-AAD, 7-aminoactinomycin-D; TUNEL, TdT-mediated dUTP nick end-labeling; CFSE, carboxyfluorescein diacetate succinimidyl-ester; mAbs, monoclonal antibodies; PPD, purified protein derivative.

patients with atopic dermatitis (11, 12). Other studies show that CD30 expression on stimulated T cells is induced by IL-4 (13).

The aim of this study was to analyze, in peripheral blood mononuclear cells (PBMC) isolated from allergic patients, cell proliferation, apoptosis, and intracellular cytokines expression on CD4+CD30+ T cell blasts induced by *Dermatophagoides pteronyssinus* allergen.

Materials and methods

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAbs) to human CD3 and phycoerythrin (PE)-labeled mAbs to human CD30, IL-4 and IL-5 were purchased from BD PharMingen (San Jose, CA, USA). Carboxyfluorescein diacetate succinimidyl (CFS)-labeled mAbs against human CD30 and IL-4, and capture mAb rat antimouse/human IL-5, mouse antihuman IL-4, neutralizing mAb mouse antihuman IL-4Rα (CD124), and isotype-matched negative antibody controls were purchased from R&D Systems Inc. (Minneapolis, MN, USA). FITC- or PE-labeled antihuman IFN-γ mAbs were from Biosource International (Camarillo, CA, USA). Quantum red (QR)-labeled mAb to human CD4, brefeldin-A, propidium iodide, 7-aminoactinomycin-D (7-AAD) dye, bovine serum albumin V (BSA), Triton X-100, saponin, *p*-formaldehyde, fetal bovine serum, L-glutamine, penicillin, streptomycin, β-mercaptoethanol, dimethyl sulfoxide, sodium pyruvate, RPMI-1640 culture medium, trypan blue dye, and salt reagents were from Sigma (St Louis, MO, USA). TdT-mediated dUTP nick end-labeling (TUNEL) assay, an *in situ* cell death detection kit was from Roche Molecular Biochemicals (Mannheim, Germany). Lymphoprep was purchased from Axis-Shield PoC AS (Oslo, Norway). Carboxyfluorescein diacetate succinimidyl-ester (CFSE) was obtained from Molecular Probes (Eugene, OR, USA). QCL-1000 quantitative chromogenic *Limulus* amebocyte lysate (LAL) end point test was from Bio-Whittaker Inc. (Walkersville, MD, USA). Scintillation fluid was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and [^{3}H]-thymidine was from Amersham Pharmacia Biotech (NSW, Australia).

Patients

Seventeen patients from the Hospital 'La Raza', IMSS, Mexico City, diagnosed with allergic rhinitis and mild allergic asthma (nine males and eight females), 27 ± 9 years of age, were studied. Allergic condition was confirmed by clinical evaluation, physical examination, skin prick test positive only to *Der p* (9 ± 3 mm), elevated serum IgE (735 ± 195 IU/ml) and eosinophilia (7 ± 1%). Twelve nonallergic subjects (five females and seven males); age 34 ± 10 years with no history of atopy and <100 IU/ml serum IgE, <5% blood eosinophils, and skin prick test negative to *Der p* served as controls. About 15 ml sample of peripheral blood was obtained from nonallergic control subjects or allergic patients (before specific treatment was initiated). The protocol was approved by the hospital ethics committee and all patients and controls were informed.

Allergen preparation

Phenol, albumin, and glycerol-free *Der p* allergen extract was generously provided by Allerstand Co. (Mexico City, Mexico). The allergen protein concentration was determined by the Bradford method (14) and analyzed by 10% polyacrylamide gel electrophoresis (PAGE) according to Laemmli (15). The endotoxin concentration in allergen extract was measured by QCL-1000 quantitative LAL test according to the manufacturer's instructions. The content of endotoxin in the test samples was <0.1 EU/ml, hence it was used for cell cultures.

Cell proliferation assay

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Lymphoprep density-gradient centrifugation for 30 min at 500 ***g*** at 16°C (16). Isolated PBMC were washed twice in RPMI-1640 culture medium and cell viability was determined by trypan blue dye exclusion method.

For analysis of cell proliferation, PBMC were stained with CFSE (17). Briefly, 1 ml of PBMC at a density of 1×10^7/ml of RPMI medium, were incubated with 15 μl of 0.5 mM CFSE (prepared from a 5 mM stock solution dissolved in dimethyl sulfoxide) in culture medium for 10 min at room temperature in the dark. After incubation, 8 ml of culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 M β-mercaptoethanol, 0.1 M sodium pyruvate, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin were added to the cells. Subsequently, cells were centrifuged, and suspended in 1 ml of supplemented culture medium; cell viability was determined by trypan blue dye exclusion. The CFSE-treated PBMC were seeded into 96-well culture plates (Nalgene Nunc International, Roskilde, Denmark) at 2×10^5 cells/well in 200 μl supplemented RPMI-1640 culture medium at 37°C in a humid atmosphere containing 5% CO_2. The CFSE-treated PBMC proliferation assays, were performed with optimal protein concentrations of *Der p* allergen at the beginning, and after 3 and 5 days of cell culture, when half of the culture medium supernatant was removed and the well was replenished with fresh supplemented RPMI-1640 medium each time. Previously, in order to establish the optimal dose of antigenic stimuli a cell proliferation kinetic was carried out over a 7-day time period using different protein concentrations of *Der p* allergen. At the end of the culture period the cells were analyzed on the FACSCalibur equipped with CELLQUEST software (Becton-Dickinson, San Jose, CA, USA) and the blast cells were gated by forward scatter (FSC) and side scatter (SSC) characteristics. An histogram based on the fluorescence intensity of unstimulated CFSE-stained cells, which lay within the blast scatter gate, was made in order to differentiate the divided cells of lower intensity. The initial gate (X_0) included the undivided cell population and the subsequent gates (X_{1-7}) enclose populations with progressive twofold decreases in fluorescence intensity. Cell proliferation was evaluated by the division index (DI), which was calculated as follows: $(100 - Y)/Y$, where $Y\ (\%) = X_0 + X_1/2 + X_2/4 + X_3/8 + X_4/16 + X_5/32 + X_6/64 + X_7/128$ and X_0 represents the blasts that have not divided, and X_1–X_7 represents those with progressive CFSE division gates, according to Angulo and Fulcher (18).

Cell proliferation was also corroborated by [^{3}H]-thymidine uptake. The PBMC were placed into 96-well plates (2×10^5 cells/well) in a final volume of 200 μl of supplemented RPMI-1640 culture medium. Cells were cultured for 3, 5 and 7 days with the optimal concentration of *Der p* extract at 37°C in a 5% CO_2 humidified atmosphere. Eighteen hours before harvesting, cells were pulsed with 1 μCi of [^{3}H]-thymidine, and then harvested with a Cell Harvester Brandel (Gaithersburg, MD, USA) onto glass microfibre filter paper (Whatman, Maidstone, UK). The paper was dried, immersed in vials containing scintillation fluid, and the incorporated radioactivity was measured in a Beckman LS-6000 SE-Counter (Beckman Coulter, Fullerton, CA, USA).

Flow cytometry

For the detection of intracellular cytokine production by allergen-stimulated PBMC, a three-color immunofluorescence approach was used following the method described by Lecoeur et al. (19). Cell cultures were established at different time periods as indicated above. Four hours before cell harvesting, 10 μg/ml brefeldin-A was added to inhibit new cytokine release. Cells were washed with phosphate-buffered saline (PBS; 0.15 M), with 0.2% BSA, 0.2% NaN_3 and labeled with mouse antihuman cell surface markers mAbs: FITC-CD3 or QR-CD4, and PE-CD30 or CFS-CD30. After staining, cells were washed, fixed with 4% *p*-formaldehyde for 10 min at 4°C, washed, and permeabilized with 0.1% saponin in PBS with 10% BSA and 1% NaN_3, gently shaking in the dark for 15 min at room temperature. Cells were stained with PE-, or FITC-labeled mAbs against human IL-4, IL-5, or IFN-γ. Finally, cells were analyzed by flow cytometry using CELLQUEST software, and 10 000 events were counted. To analyze the staining of cell surface markers and intracellular cytokines, the blasts were first gated by their physical properties (FSC and SSC), then a second gate was drawn based on the fluorescence characteristics of the gated cells. Background staining was assessed using fluorochrome-conjugated isotype-matched antibodies.

Blockade of cytokines

Capture mAbs of either rat antihuman IL-5 (1 μg/ml), mouse antihuman IL-4 (10 μg/ml) or neutralizing mouse antihuman IL-4Rα (CD124) mAbs (10 μg/ml) were added at the beginning of the cell culture according to the manufacturer's protocol (R&D System). Then, 0.5 μg/ml of anti-IL-5 or 5 μg/ml of anti-IL-4 and/or anti-IL-4Rα mAbs were added daily as a maintenance dose the subsequent culture days. Isotype-matched negative antibody controls were used at the same concentrations.

Cell apoptosis

Two methods were used to determine apoptosis, one detects the loss of cell membrane integrity by 7-AAD dye, which is also an intercalating DNA molecule (19). The second, a TUNEL assay, identifies the DNA degradation resulting in cleavage of nuclear DNA into oligonucleosomesized fragments (20). Both assays were performed in PBMC cultures stimulated continuously with *Der p* for 7 days.

7-AAD. To evaluate the membrane integrity of the CD4+CD30+ blast subset, PBMC were incubated for 20 min at 4°C in the dark with 7-AAD. Afterwards cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD30 mAbs for 30 min. After washing, cells were analyzed by flow cytometry according to size and 7-AAD staining; 7-AAD-negative blasts were considered nonapoptotic cells and were selected for further cytofluorometric analysis.

TUNEL assay. Briefly, after labeling cell surface markers with fluorochrome-conjugated mAbs, cells (1×10^6) were fixed with 100 μl 1% *p*-formaldehyde for 15 min, then washed twice with the kit's staining buffer and incubated with 1 ml of 70% ethanol for 30 min at 4°C. Afterward, cells were washed and incubated with 1 ml of permeabilizing buffer (0.6 M NaCl, 60 mM sodium citrate, 0.2% Triton X-100, 1% BSA) for 5 min at room temperature. Cells were washed twice and incubated with 45 μl of mixture buffer (kit's TdT reaction buffer which also contains FITC-dNTPs) and 5 μl of TdT enzyme for 1 h at 37°C. Finally, cells were analyzed by flow cytometry using negative controls incubated only with 45 μl of the kit's mixture buffer without the enzyme.

Statistical analysis

The data obtained were evaluated with the SIGMASTAT software using the Mann–Whitney *U*-test to compare two independent groups; correlation coefficient was calculated with Pearson product. A *P*-value of <0.05 was considered as statistically significant.

Results

Allergen-specific cell proliferation

The evaluation and analysis reported in the present study were performed on the blast population generated by gating (Fig. 1A). The analysis made on this gated population according to the method described by Angulo and Fulcher (18) allowed us to identify at least seven mitosis after 7 days of continuous allergen stimulation (Fig. 1B). About 20 μg/ml was the optimal allergen protein concentration needed to induce maximal blast transformation in CFSE-treated PBMC obtained from allergic patients (Fig. 1C). The PBMC from nonallergic control individuals proliferated weakly in the presence of the allergen (Fig. 1C,D). The reproducibility of the assays in terms of culture time using an optimal allergen dose showed that the number of blasts increased dramatically after 7-day of culture (Fig. 1D).

CD4+CD30+ blast cells

Dermatophagoides pteronyssinus induced proliferation of CD4+ T cells on CFSE-treated PBMC. About 90% of the CFSE-negative proliferating cells were CD4+ (Fig. 2A). After 3 days of culture 1% of the CD4+ cells expressed CD30, this figure increased to 8.2% after 5 days and reached 50% after 7 days of culture in comparison with CD4+CD30− cells that showed a progressive diminution from 90% to 30% (Fig. 2B).

Production of IL-4, IL-5, and IFN-γ

The existence of intracellular cytokines was determined after 3, 5 and 7 days of allergen stimulation. The percentage of CD4+CD30+IFN-γ+ cells was 7.5% after 3 days, reaching a 25% peak after 5 days and decreasing to 5% after 7 days of culture (Fig. 3A). CD4+CD30+IL-4+ cells maintained similar percentages than IFN-γ+ cells, but the percentage of CD4+CD30+IL-5+ cells showed a progressive increase that reached a 24.2% after 7 days of culture. It was worth noticing that IL-4+ and IFN-γ+ blasts showed an statistically significant decrease ($P < 0.05$) after 7 days of culture when compared with the percentage obtained by these cells at day 5. The cytokine pattern of the CD4+CD30− cells showed a statistically significant

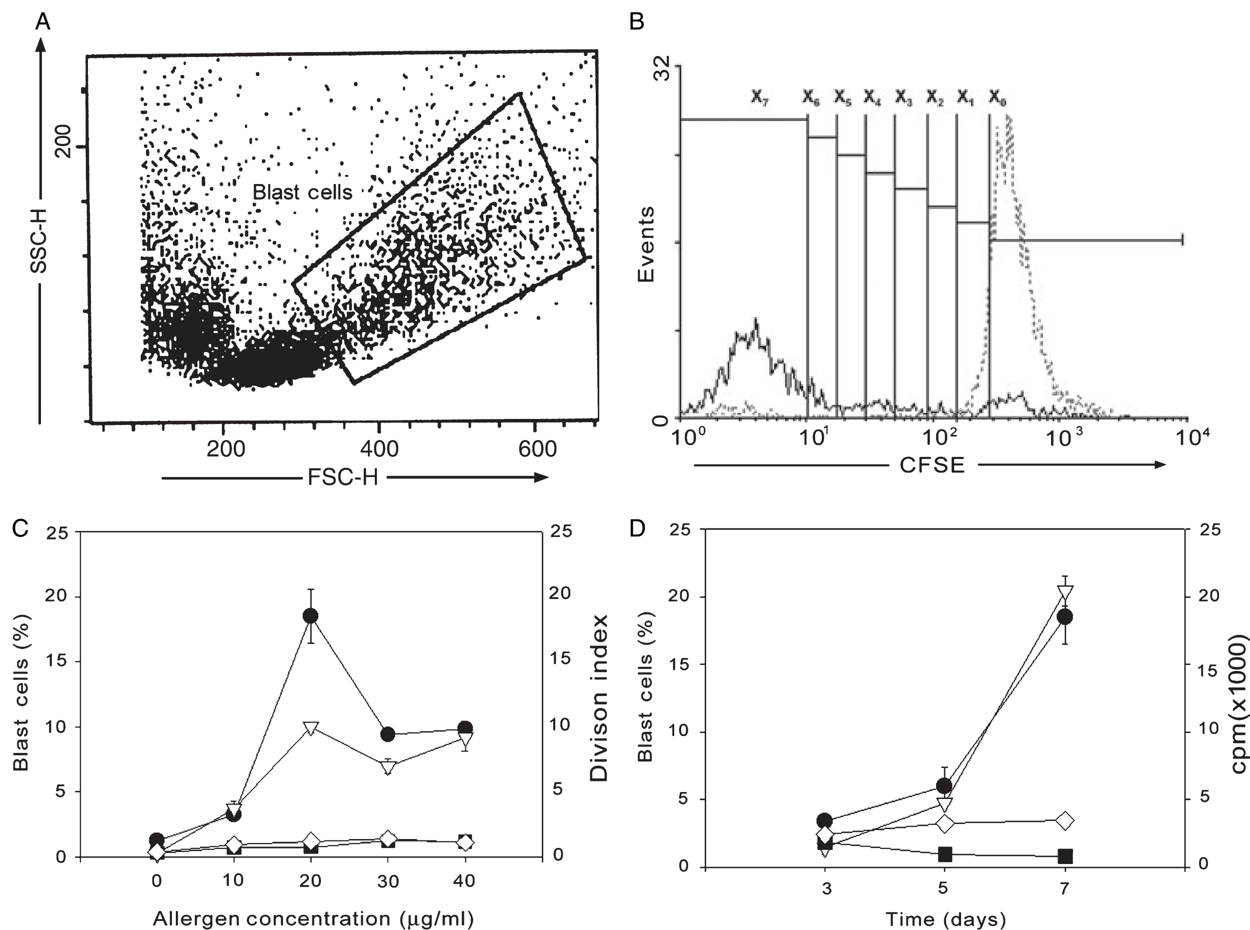

Figure 1. Effect of *Dermatophagoides pteronyssinus* on peripheral blood mononuclear cell (PBMC) proliferation after 7 days of culture. (A) Dot plot of blast cells gated according to size and granularity (inner frame). (B) Analysis on gated blast cells in a proliferating culture of allergen-stimulated and carboxyfluorescein diacetate succinimidyl-ester (CFSE)-stained PBMC show a sequential halving of fluorescence intensity (X_{1-7}) that correspond to cell divisions (continuous line). X_0 denote the population without cell division (dotted line). (C) Blast cells [allergic patients (●) and nonallergic subjects (■)] and division index [allergic patients (▽) and nonallergic subjects (◇)] generated by different allergen protein concentrations. (D) Kinetics of blast cell percentages [allergic patients (●) and nonallergic subjects (■)] and [^{3}H]-thymidine incorporation [allergic patients (▽) and nonallergic subjects (◇)] measured at different days. PBMC were pulsed with *Der p* (20 µg/ml) at 0, 3 and 5 days. Results from (C) and (D) are expressed as mean ± SE ($n = 10$), and as mean counts per minute (cpm) ± SE ($n = 3$) per triplicate.

increase in IFN-γ ($P < 0.05$) after 5 days of culture when compared with the cell percentages from days 3 to 7 (Fig. 3B), which did not reach the higher values observed in the CD4+CD30+ blasts in the same culture time. The intracellular expression of IL-5, IL-4, and IFN-γ showed an impressive diminution in the CD4+CD30− cells after 7 days of culture (Fig. 3B).

Apoptosis assays

Less than 1% of the allergen-specific CD4+CD30+ blasts, isolated after 7 days of culture under *Der p*-stimulated conditions, were apoptotic according to the TUNEL assay (Fig. 4A) and had an abnormal cell membrane according to the 7-AAD-positive staining (Fig. 4B). This result confirm that the diminution in intracellular cytokine expression (Fig. 3A) is not secondary to cell death.

Effect of IL-4 and IL-5 on CD30 expression

Figure 5 illustrates the decrease in the CD4+CD30+ blast subset when cells were cultured with *Der p* in the presence of anti-IL-4Rα mAb, isotype-matched mAb served as control. Blocking the IL-4Rα resulted in a 28% decrease in CD4+CD30+ cells ($P = 0.03$); a significant decrease of 34% ($P = 0.01$) was also observed in CD4+CD30+ cells when IL-4 was neutralized (Fig. 5). Additional experiments showed that the combination of anti-IL-4Rα and anti-IL-4 mAb had no synergic effect.

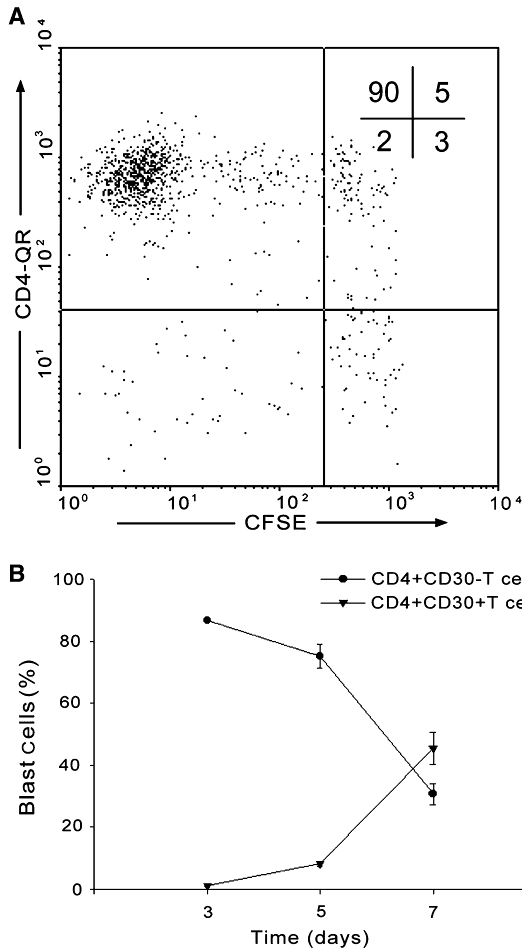

Figure 2. Analysis of blast cells generated by *Dermatophagoides pteronyssinus*-stimulated peripheral blood mononuclear cells. (A) Dot plot of surface CD4-labeled cells, shown in the *y*-axis, and carboxyfluorescein diacetate succinimidyl-ester (CFSE)-stained cells losing sequentially the fluorescence intensity by cell division, shown in the *x*-axis. Numbers in the upper right corner denote the cell percentages in each quadrant. (B) Kinetic of the progressive decrease of CD4+CD30− blast cells (●) and increase of CD4+CD30+ blast cells (▼) under *Der p* stimulation at different times. Results are shown as mean ± SE ($n = 5$).

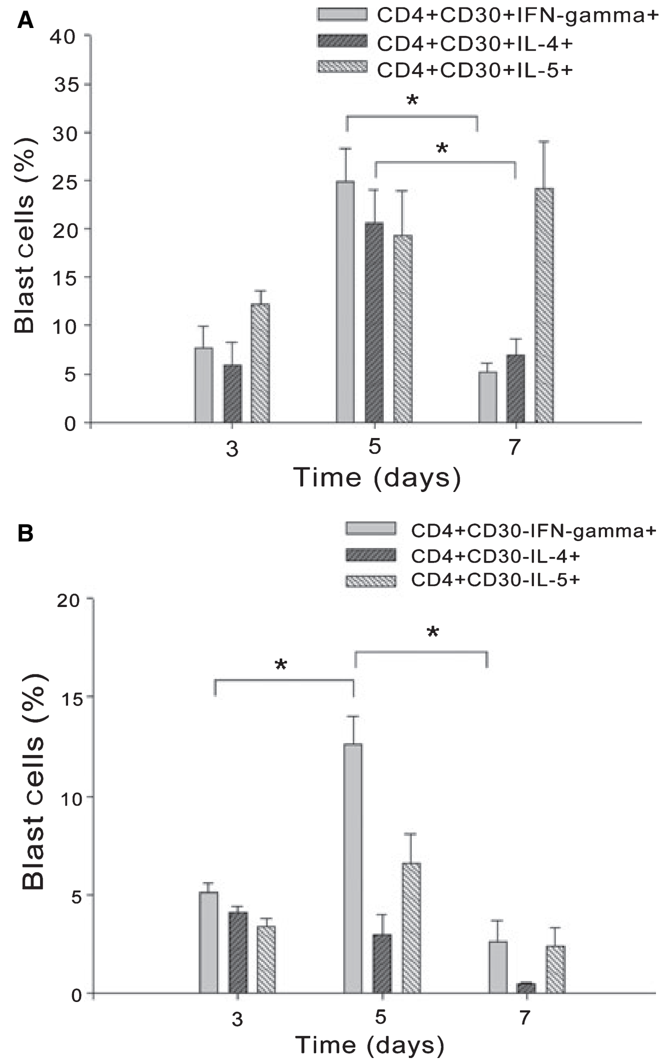

Figure 3. Intracellular cytokine expression in *Dermatophagoides pteronyssinus*-stimulated peripheral blood mononuclear cells obtained from patients. (A) CD4+CD30+ blast cells, (B) CD4+CD30− blast cells. Interleukin (IL)-5+ (light dashed), IL-4+ (dark), and interferon (IFN)-γ+ (gray) cells, measured at different days. Results are shown as mean ± SE ($n = 5$). $*P < 0.05$.

Anti-IL-5 mAb alone, or in combination with anti-IL-4 mAb, did not affect the number of CD4+CD30+ cells (data not shown).

Discussion

The association of Th2-type cytokine profile with responses to allergens has been well established. It is well known that IL-4 increases serum IgE, a pivotal element in airway allergic diseases (8) and promotes CD30 expression on Th cells (13). We focused our interest in trying to determine the presence of CD4+CD30+ T cells in *Der p* allergic patients and to evaluate the cytokines produced by this cell population, because CD30 has been proposed as a marker of human-activated CD4+ T-cell clones producing Th2-type cytokines (21). Subsequent reports have shown this to hold true in atopic dermatitis (11, 12), but other authors have suggested that CD30 is not related with Th2 cytokine production in peripheral blood (22) or in bronchoalveolar lavage fluid (23) but rather it has a more costimulator-specific function (24). A recent report shows that CD30, a member of the TNFR family, is predominantly expressed on activated Th2 cells although there are differences in the regulatory pathways controlling its expression (13). Our initial results showed that PBMC from *Der p* allergic patients do not contain a

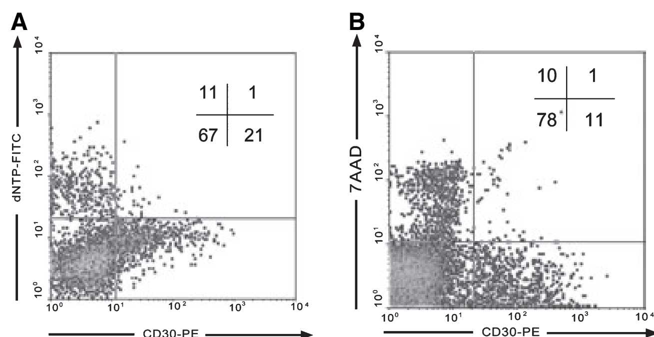

Figure 4. Apoptotic blast cells determined by phycoerythrin (PE)-labeled anti-CD30 monoclonal antibodies and either fluorescein isothiocyanate (FITC)-TdT-mediated dUTP nick end-labeling (A) or 7-aminoactinomycin-D (7-AAD) dye (B). The vast majority of CD30+ cells were not apoptotic. Right upper corner numbers represent the percentage of cells per quadrant.

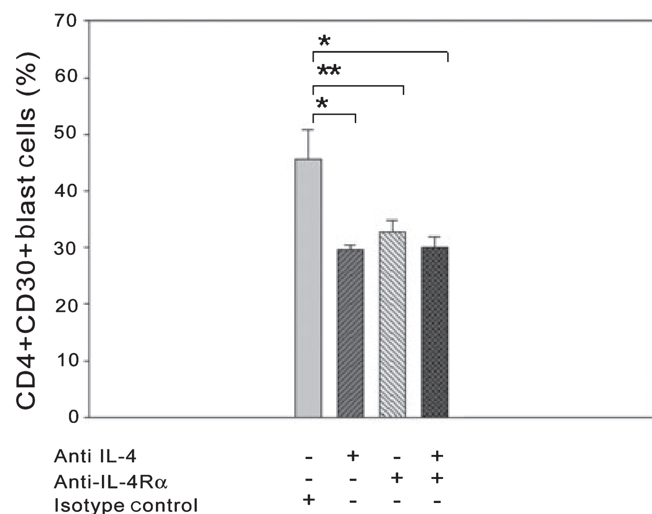

Figure 5. Effect of anti-interleukin (IL)-4 or IL-4Rα monoclonal antibodies (mAbs) on CD4+CD30+ gated peripheral blood mononuclear cells. Cells were stimulated with *Dermatophagoides pteronyssinus* allergen in the presence of either mAbs or isotype-matched control mAb for 7 days. Results are shown as mean ± SE ($n = 4$). $^{*}P = 0.01$, $^{**}P = 0.03$.

CD4+CD30+ cell subpopulation; based on this observation we decide to evaluate if the continuous stimulation of PBMC with the allergen generated such a cell population. Our results showed that *Der p*-activated PBMC contained CD4+ CD30+ T blasts which were positive to IFN-γ, IL-4, and IL-5 after 5 days of culture suggesting that CD30 is preferentially expressed on early antigen-activated Th cells from allergic patients independently of their cytokine profile. Minimal CD4+CD30+ T blasts percentage was originated after *Der p* stimulation on PBMC from nonallergic individuals, which is in accordance with Till et al. (25). Cell subpopulation analysis after 7 days of culture demonstrated that IL-5 was mainly detected in CD4+CD30+ T cells. CD4+CD30− T cells produced less amounts of IL-4, IL-5, and IFN-γ in comparison with those produced by CD4+CD30+ cells. Previously, Till et al. (25) found a relationship between IL-5 secretion by *Der p*-stimulated PBMC and clinical status of the allergic disease. Also, they found that IL-5 secretion was originated from CD4+ T cells but not from CD8+ T cells. Our results are in agreement with those of Leonard et al. (10) whom reported that *Der p* induces CD30 expression on CD4+ T cells from allergic asthma patients; and with those of Till et al. (25) regarding that IL-5 is produced by *Der p*-stimulated CD4+ T cells from allergic patients; the difference in the IFN-γ results could be secondary to the fact that we measured intracellular cytokines whereas Till et al. (25) measured secreted cytokines. In the present study, the IFN-γ+ cells reached their maximum percentage on day 5, and then drastically decreased in both CD4+CD30+ and CD4+CD30− T-cell subsets. This was not the case with the IL-5+ cells, which progressively increased and persisted during 7 days of culture. Probably the prolonged presence of allergen in *Der p*-specific cultures might select clones, because IL-4 abrogation had a partial effect on the number of CD4+CD30+ T cells, possibly there is a CD30+ population which is a stable expressor of CD30 that derives from the original antigen-specific population. The persistence of the CD4+CD30+ T cells producing IL-5 eventually will maintain the allergic status in a Th2-type manner. It has been observed in murine models that IL-5 induces airway damage and bronchial hyperreactivity because of eosinophil infiltration and mucus production (26–28). The latter observation enhances the importance of the CD4+CD30+ T-cell population in the pathogenesis of some allergic diseases.

Our results also showed that the CD4+CD30+ cell subpopulation is partially dependent on the continuous presence of IL-4 in the milieu, as Breit et al. (29) have suggested, but as shown in Fig. 5 the lack of IL-4 does not diminished completely the percentage of CD4+CD30+ cells suggesting that other factors, possibly liberated by cells of the monocyte/macrophage lineage, which were present in our cell cultures, are also important for the full development of this cell subpopulation. Murine models have shown that IL-4 is necessary for the induction of CD30 expression on antigen-activated CD4+ T cells (13, 30, 31), although in human PBMC IL-12 and IL-18 seem to be the relevant cytokines (32).

The reason behind the disappearance of IFN-γ and IL-4-producing cells after 7 days of culture remains unknown. This is not secondary to genes kinetic expression, as one study showed that PBMC from asthmatic patients produced high IFN-γ levels on stimulation with purified protein derivative (PPD) (*Mycobacterium tuberculosis*) after 6 days of culture (25). We are currently performing

experiments addressing this issue. Nonetheless, as our results showed (Fig. 4), the absence of CD30 was associated with a higher apoptosis rate, an observation that despite being interesting does not explain if apoptosis is the result of being CD30− or that non-IL-5-producing T-cell blasts are somehow initiated into the apoptotic process. In conclusion, our data suggest that *in vitro*-specific allergen stimulation of PBMC isolated from allergic patients generates a nonapoptotic CD4+CD30+ blast cell subset producer of IL-5 after 7 days of culture, an observation that could explain the long-term clinical manifestations of some allergic diseases and is in accordance with the observation made by Rawle et al. (33) which showed that patients with symptoms have greater numbers of circulating allergen-specific T cells.

Acknowledgments

Authors thank Misael Gonzalez, Allerstand Labs (Mexico), who provided the *Dermatophagoides pteronyssinus* allergen extract. Also wish to thank Mrs Rosa Nieto (INER) for her technical assistance. This work was partially financed by DGAPA-UNAM (Project PAPIIT IX235304-1), Mexico.

References

1. Rojas-Ramos E, Martinez-Jimenez N, Reyes-Salinas A. The Th2 theory in allergy: present and future directions. Rev Alerg Mex 2003;**50**:64–70.
2. Abbas AK, Lichtman AH. Cellular and molecular immunology, 5th edn. Philadelphia, PA, USA: Saunders Elsevier Science, 2003.
3. Li XM, Schofield BH, Wang QF, Kim KH, Huang SK. Induction of pulmonary allergic responses by antigen-specific Th2 cells. J Immunol 1998;**160**:1378–1384.
4. Settipane RJ, Settipane GA. IgE and the allergy-asthma connection in the 23-year follow-up of Brown University students. Allergy Asthma Proc 2000;**21**:221–225.
5. Bottcher MF, Bjurstrom J, Mai XM, Nilsson L, Jenmalm MC. Allergen-induced cytokine secretion in atopic and non-atopic asthmatic children. Pediatr Allergy Immunol 2003;**14**:345–350.
6. Cormican L, O'Sullivan S, Burke CM, Poulter LW. IFN-gamma but not IL-4 T cells of the asthmatic bronchial wall show increased incidence of apoptosis. Clin Exp Allergy 2001;**31**:731–739.
7. Riffo-Vasquez Y, Spina D. Role of cytokines and chemokines in bronchial hyperresponsiveness and airway inflammation. Pharmacol Ther 2002;**94**:185–211.
8. Foster PS, Martinez-Moczygemba M, Huston DP, Corry DB. Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease. Pharmacol Ther 2002;**94**:253–264.
9. Fireman P. Understanding asthma pathophysiology. Allergy Asthma Proc 2003;**24**:79–83.
10. Leonard C, Tormey V, Faul J, Burke CM, Poulter LW. Allergen-induced CD30 expression on T cells of atopic asthmatics. Clin Exp Allergy 1997;**27**:780–786.
11. Caproni M, Bianchi B, D'Elios MM, De Carli M, Amedei A, Fabbri P. In vivo relevance of CD30 in atopic dermatitis. Allergy 1997;**52**:1063–1070.
12. Yamamoto J, Adachi Y, Onoue Y, Kanegane H, Miyawaki T, Toyoda M et al. CD30 expression on circulating memory CD4+ T cells as a Th2-dominated situation in patients with atopic dermatitis. Allergy 2000;**55**:1011–1018.
13. Toennies HM, Green JM, Arch RH. Expression of CD30 and Ox40 on T lymphocyte subsets is controlled by distinct regulatory mechanisms. J Leukoc Biol 2004;**75**:350–357.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;**72**:248–254.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;**227**:680–685.
16. Boyum A. Isolation of lymphocytes, granulocytes and macrophages. Scand J Immunol Suppl 1976;**5**:9–15.
17. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods 1994;**171**:131–137.
18. Angulo R, Fulcher DA. Measurement of *Candida*-specific blastogenesis: comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. Cytometry 1998;**34**:143–151.
19. Lecoeur H, Ledru E, Gougeon ML. A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. J Immunol Methods 1998;**217**:11–26.
20. Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends Cell Biol 1995;**5**:21–26.
21. Del Prete G, de Carli M, Almerigogna F, Daniel CK, D'Elios MM, Zancuoghi G et al. Preferential expression of CD30 by human CD4+ T cells producing Th2-type cytokines. FASEB J 1995;**9**:81–86.
22. Hamann D, Hilkens CM, Grogan JL, Lens SM, Kapsenberg ML, Yazdanbakhsh M et al. CD30 expression does not discriminate between human Th1- and Th2-type T cells. J Immunol 1996;**156**:1387–1391.
23. Petkova D, Xaubet A, Picado C, Filella X, Agusti C, Luburich P et al. Evaluation of CD30 as a marker for th2 lymphocytes in bronchoalveolar lavage in interstitial lung diseases. Respir Med 2000;**94**:345–349.
24. Pellegrini P, Berghella AM, Contasta I, Adorno D. CD30 antigen: not a physiological marker for TH2 cells but an important costimulator molecule in the regulation of the balance between TH1/TH2 response. Transpl Immunol 2003;**12**:49–61.
25. Till S, Dickason R, Huston D, Humbert M, Robinson D, Larche M et al. IL-5 secretion by allergen-stimulated CD4+ T cells in primary culture: relationship to expression of allergic disease. J Allergy Clin Immunol 1997;**99**:563–569.
26. Trifilieff A, Fujitani Y, Coyle AJ, Kopf M, Bertrand C. IL-5 deficiency abolishes aspects of airway remodeling in a murine model of lung inflammation. Clin Exp Allergy 2001;**31**:934–942.
27. Crosby JR, Shen HH, Borchers MT, Justice JP, Ansay T, Lee JJ et al. Ectopic expression of IL-5 identifies an additional CD4(+) T cell mechanism of airway eosinophil recruitment. Am J Physiol Lung Cell Mol Physiol 2002;**282**:L99–108.

28. Justice JP, Crosby J, Borchers MT, Tomkinson A, Lee JJ, Lee NA. CD4(+) T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. Am J Physiol Lung Cell Mol Physiol 2002;**282**:L1066–1074.
29. Breit S, Steinhoff M, Blaser K, Heusser CH, Sebald W, Levine AD et al. A strict requirement of interleukin-4 for interleukin-4 induction in antigen-stimulated human memory T cells. Eur J Immunol 1996;**26**:1860–1865.
30. Gilfillan MC, Noel PJ, Podack ER, Reiner SL, Thompson CB. Expression of the costimulatory receptor CD30 is regulated by both CD28 and cytokines. J Immunol 1998;**160**:2180–2187.
31. Nakamura T, Lee RK, Nam SY, Al-Ramadi BK, Koni PA, Bottomly K et al. Reciprocal regulation of CD30 expression on CD4+ T cells by IL-4 and IFN-gamma. J Immunol 1997;**158**:2090–2098.
32. Tarkowski M, Chrul S, Bodalski J. The effect of IL-18 on IL-12 induced CD30 expression and IL-4 and IFN-gamma production by allergen and PPD specific T cells. Clin Exp Immunol 2002;**127**:78–84.
33. Rawle FC, Mitchell EB, Platts-Mills TA. T cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*, antigen P1: comparison of patients with asthma, atopic dermatitis, and perennial rhinitis. J Immunol 1984;**133**:195–201.