

Differential role of CD80 and CD86 on alveolar macrophages in the presentation of allergen to T lymphocytes in asthma

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Summary

In the asthmatic lung the altered expression of costimulatory molecules (CD80, CD86) by alveolar macrophages contributes to T lymphocyte activation and expansion. We hypothesized that CD80 and CD86 on alveolar macrophages could differentially support allergic inflammation in adult asthma. Here we studied 11 subjects with mild allergic asthma and 11 atopic non-asthmatics as controls. *Dermatophagoides*-specific T cell lines were derived from peripheral blood from each subject. Bronchoalveolar lavage with evaluation of lung inflammatory cells was performed in all individuals at baseline and 24 h after allergen challenge. The expression of CD80 and CD86 costimulatory molecules by alveolar macrophages was studied and, in parallel, the efficiency of antigen presentation was measured in terms of IL-4 and IL-5 production by allergen-stimulated autologous T cells. We found that in asthmatic subjects (i) the percent of CD80⁺, but not CD86⁺ alveolar macrophages was increased at baseline and did not change following allergen challenge; (ii) CD86, but not CD80, membrane expression was up-regulated following allergen challenge; (iii) both CD80 and CD86 were required to support Th2 cytokine production by allergen-specific T cells, with a prevalent role of CD86 after allergen challenge. Our data indicate that alveolar macrophages deliver costimulatory signals *via* CD80 and CD86, which support the production of Th2 cytokines by allergen-specific T cells. They also indicate that CD86 *in vivo* is up-regulated in the 24 h following allergen exposure and that this modulation is functionally relevant.

Keywords: allergy, monocytes/macrophages, antigen presentation, costimulatory molecules, lung, asthma

Clinical and Experimental Allergy, Vol. 31, pp. 625–636. Submitted 31 March 2000; revised 30 August 2000; accepted 28 September 2000.

Introduction

The interaction of T lymphocytes with antigen *via* T cell receptor (signal 1) provides the element of specific recognition in the primary and in the memory immune response. Then, the interaction of given membrane receptors on the antigen-presenting cell (APC), such as CD80 and CD86 (previously B7.1 and B7.2) with their respective ligands on T lymphocytes delivers a complex array of signals which direct the progression and the

functional differentiation of the immune response [1–4]. Both CD28 and CTLA-4 act as ligands for the B7 molecules on the membrane of T lymphocytes. While CD28 is expressed by all peripheral T cells, CTLA-4 only appears after T cell activation [5] binds CD80 and CD86 with much higher avidity than CD28 [6] and has a prevalent inhibitory function.

In the lower respiratory tract microenvironment, the priming of the immune response is provided by interstitial dendritic cells that pick up antigens and migrate to regional lymph nodes where they present them to T cells [7]. In contrast, resident alveolar macrophages (AM) are physiologically inefficient as APC [8], probably due to low expression

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of CD80 and CD86. This fact probably prevents chronic activation by inhaled antigens [9]. Recently, we and others reported an increased expression of functionally active costimulatory molecules on AM from allergic asthmatics [10–14]. Correspondingly, in these patients AM could effectively present antigens to memory/effector T cells.

The relative importance of CD80 and CD86 in expanding the critical T cell response to allergens in asthma has not been fully elucidated, although there is accumulating evidence to suggest that both molecules are important in allergen-induced proliferation and cytokine production by T cells from the asthmatic lung [14] or blood [15,16].

Here we measured the expression of CD80 and CD86 on AM from allergic asthmatics in a model of experimental allergen inhalation and studied the relative role of each molecule in the production of Th2 cytokines by autologous allergen-specific T cells. We found that both molecules were involved in the presentation of allergen to T cells and that following *in vivo* allergen challenge, CD86 was up-regulated and became functionally prevalent.

Materials and methods

Patients

We studied 11 atopic non-smoking subjects with mild asthma [17] and 11 atopic non-asthmatic non-smoking control subjects. We compared atopic asthmatic with atopic non-asthmatic volunteers to be certain that the biological parameters we studied were indicative of events of atopic asthma rather than of atopy.

Inclusion criteria were: (i) a positive skin prick test (3×3 mm weal and flare reaction or more) to house dust mite extracts; (ii) a RAST (Radio Allergo Sorbent Test) class III or higher for *Dermatophagoides pteronyssinus*- and *farinae*-specific serum IgE. Specific IgE were measured as Phadebas RAST Units (PRU) per mL serum (Pharmacia, Uppsala, Sweden). RAST class III and IV correspond to the range from 3.5 to 17.5 PRU/mL and above 17.5 PRU/mL, respectively; (iii) a forced expiratory volume in 1 s (FEV₁) > 80% of predicted [18] at the time of study; (iv) no respiratory infections in the 6 weeks before participation in the study. None of the subjects was, at the time of study, under anti-asthmatic treatment, other than short-acting β_2 -stimulants as required (less than four times a day). These were discontinued at least 12 h before challenge. All subjects were informed of the nature and aim of the study and gave written consent. This project was approved by the Ethical Committee of the Institution at which patients were selected.

Atopic asthmatic individuals (four females and seven males; mean age 24.9 ± 3.9 years) had an increased airway responsiveness to methacholine, i.e. accumulative dose producing a fall in FEV₁ of 20% from baseline (PD₂₀) < 16 mg/mL.

The atopic non-asthmatic control volunteers (five females and six males; mean age 29.0 ± 7.5 years) had no history of asthma, a mean methacholine PD₂₀ > 32 mg/mL, and serum IgE of 115.0 ± 73.5 U/mL. The control subjects were asymptomatic at the time of study.

Peripheral venous blood was obtained from the subjects to establish allergen-specific T cell lines. Both asthmatics and non-asthmatic atopic controls underwent the first 'baseline' (i.e. without any allergen challenge) bronchoalveolar lavage (BAL) and the second BAL 3–6 weeks later. The second BAL was performed 24 h after a bronchial allergen challenge.

Media, monoclonal antibodies, antigens

Complete medium used for cell cultures was prepared as follows. RPMI 1640 (PBI, Milan, Italy) was supplemented with gentamycin 5 µg/mL, glutamine 2 mM (PBI) and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, The Netherlands). FITC-conjugated and unlabelled purified monoclonal antibodies to human CD80 and CD86 were used for flow cytometry and for inhibition experiments, respectively. These antibodies were: clone BB1 (mouse IgM, κ , anti-human CD80), clone IT2.2 (mouse IgG2b, κ , anti-human CD86) (PharMingen, San Diego, CA, USA). Purified unlabelled and FITC-conjugated mouse IgM and IgG2b monoclonal antibodies with irrelevant specificity from the same manufacturer were used as controls.

The allergen used for *in vivo* challenge consisted of a *D. pteronyssinus* and *farinae* dry mix (50/50) predosed in arbitrary units (AU) by RAST inhibition technique with an internal reference extract to which a value of 1000 AU had been assigned (Lofarma Allergeni, Milan, Italy). One AU corresponded to 7.5 U of the WHO International Union of Immunological Societies *Dermatophagoides pteronyssinus* International Standard (National Institute for Biological Standards and Control code 82/518). For *in vitro* use, the major allergen Der p 1 was immunopurified with a specific monoclonal antibody [19]. The preparation contained > 95% specific protein at standard SDS-PAGE followed by comassie blue staining. The *Lymlus* amoebocyte gelification test (PBI) was used to assess the presence of bacterial endotoxins in the allergen extracts [20]. Briefly, the assay was performed, upon reconstitution in culture medium of the allergen extract at 1 mg/mL, according to the manufacturer's instructions. It measured 0.01 Endotoxin Units/mL. These levels were not different from those found in saline solution and in complete medium.

Establishment of allergen-specific T cell lines

Gradient-separated peripheral blood mononuclear cells (PBMC) were isolated with standard procedures (Lymphoprep; Sigma Italia, Milan, Italy) and cultured (2×10^6 /mL,

12-well plates) in complete medium for 7 days in the presence of 20 µg/mL of Der p 1 allergen. Human recombinant IL-2 (Roche, Nutley, NJ, USA) was added at low concentration (20 U/mL) in the last 3 days of culture. These single primary T cell lines were maintained in different wells and separately frozen (four to nine aliquots per individual) in liquid nitrogen until autologous AM became available. This approach was more profitable than pooling T cells for the isolation of T cell lines strongly reacting to allergen. The cultures most responsive to subsequent *in vitro* stimulation with allergen presented by blood monocytes were retained for further analysis (see below).

Allergen challenge

A powder allergen method was used [21]. The mean particle size of the allergen powder determined by a laser particle sizer (Analysette 22; Fritsch GmbH, Idar, Oberstain, Germany), with ethanol as dispersing fluid, was 2.5 µm, with 99.9% particles < 18.2 µm, 70% < 3.9 µm, 50% < 2.5 µm, 30% < 1.5 µm, and 10% < 0.7 µm. Micronized allergen in the form of inert lactose powder was contained in rigid gelatine capsules (40 + 2 mg of powder each). The following doses were made available by the manufacturing firm: 5, 10, 25, 50, 100, 200 and 400 AU. The powder was administered through a turbo-inhaler device (Schiapparelli-Searle, Torino, Italy) with a series of slow inspiratory capacity manoeuvres repeated until the capsule was empty. A control measurement of FEV₁ was obtained 15 min after inhalation of lactose. The challenge was then started from the lowest dose of allergen (5 AU) and stopped when the FEV₁ measured 15 min after the dose was decreased below 80% of control value or at the maximum cumulative dose of 1190 AU. This dose was obtained by repeating twice the maximum dose of 400 AU. FEV₁ was measured hourly for the following 24 h, except when the subject was asleep, by means of a portable microspirometer (Micromedical Ltd, Rochester, UK). From the dose-response curve (cumulative allergen dose vs. FEV₁ fall) the allergen PD₂₀ was calculated. The Late Asthmatic Reaction (LAR) value was calculated as the maximum percent FEV₁ fall compared with the pre-challenge value.

Fibreoptic bronchoscopy and BAL

Bronchoscopy with BAL were performed as follows. After premedication with atropine (0.5 mg i.m.) and diazepam (10 mg i.m.) local anaesthesia of the nostrils was obtained with lidocaine 2% and adrenaline 0.1/1000 (1 mL each side). A fibreoptic bronchoscope (Olympus BF, type PIO; Osaka, Japan) was then passed through the nose. Pharynx

and large airways were anaesthetized by instillation of lidocaine 2% and the tip of the bronchoscope was wedged into a subsegmental branch of the right middle lobe and of the lingula. Five aliquots of 20 mL each of sterile, warm (37°C) saline were infused through the channel of the bronchoscope and recovered at a negative pressure of 50–120 mmHg.

BAL sample processing

The BAL fluid was filtered through two layers of sterile gauze and centrifuged at 250 g for 5 min to separate the cells from the supernatants. The cell pellet was resuspended in ice-cold PBS without Ca²⁺ and Mg²⁺, at a concentration of 10⁶ cells/mL. An aliquot of the cell suspension was centrifuged by using a cytocentrifuge (Cytospin; Shandon Southern Instruments, Sewickley, PA, USA) spinning approximately 100 000 cells at 50 g for 5 min onto a glass slide. BAL cells were air-dried and stained with DiffQuick staining (Merz & Dade A.G., Duding, Switzerland). Cell differentials were determined by counting at least 300 cells per slide, using a light microscope (Carl Zeiss, Oberkochen, Germany). Macrophages, lymphocytes, neutrophils, and eosinophils were expressed as percent of total cells recovered.

Lymphocyte-depleted AM were prepared from total BAL cells by reacting them with anti-CD3-coated magnetic beads in cold RPMI medium without serum (Dynal, Oslo, Norway) according to the manufacturer's instructions. We used this approach to avoid the modulation of CD80 and CD86 that could be induced *in vitro* by culturing AM at 37°C in tissue culture plastic. The lymphocyte-depleted AM preparation was used for flow cytometry and for *in vitro* culture.

Flow cytometry

Aliquots of lymphocyte-depleted AM (2.5 × 10⁵ cells in 100 µL PBS containing 1% bovine serum albumin (BSA)) were incubated with FITC-conjugated monoclonals to human CD80 or CD86, or with the corresponding isotype-matched FITC-conjugated control to a final concentration of 2 µg/mL in ice for 10 min, washed twice in PBS with 1% BSA and 0.1% sodium azide, fixed in 2% formaldehyde and then analysed with a FACScan (Becton Dickinson, Mountain View, CA, USA). AM were identified on the basis of forward and light scatter characteristics, and the expression of CD80 and CD86 was analysed in terms of specific mean fluorescence relative to isotype control antibody. Exclusion of cell debris was done by applying a proper threshold on the forward scatter. Dead cells were not excluded by propidium iodine since in our experience AM start to die *in vitro*

following a few days of culture. In each experiment 10 000 events per sample were collected.

Cytokine generation by allergen-specific T cell lines

T cells from primary T cell lines were thawed, washed, counted and added (10^5 /well in 96-well flat-bottomed plates, in duplicates) to lymphocyte-depleted AM (2.5×10^4 /well) or monocytes (5.0×10^4 /well, as positive control for antigen presentation) in the presence (or absence) of 20 µg/mL Der p 1 allergen. The 5–1 ratio of responder T cells vs. AM as APC was chosen on the basis of preliminary experiments and of published data [10,21]. In separate samples blocking monoclonal antibodies, singularly or mixed together, were added (2 µg/mL, previously determined as a saturating concentration in terms of binding) specific to human CD80 and CD86, respectively. Two isotype-matched monoclonals with irrelevant specificities were added together as controls in a separate sample. As a further control for the production of lymphokines from residual BAL lymphocytes, lymphocyte-depleted AM were incubated with allergen in the same culture volume, without adding T cells. After 48 h, supernatants were collected and kept frozen for the determination of IL-4 and IL-5 content. For each individual, this set of experimental conditions was simultaneously established with AM taken at 'baseline' (i.e. without any *in vivo* stimulation) and with AM taken after allergen challenge, while the same lymphocytes served as responder T cells. Moreover, since different allergen-stimulated T cell lines were previously separately frozen and subsequently separately thawed from each individual, this experiment was repeated two to four times for each subject. The experiment where the higher production of IL-4 and IL-5 was observed in the lymphocyte-monocyte co-culture (positive control) was retained for analysis of results.

Cytokine assays

IL-4 and IL-5 concentrations in culture supernatants were assayed by specific sandwich ELISA. For each cytokine, a monoclonal was used for coating which was paired with a biotinylated monoclonal with a different fine specificity for probing, according to the manufacturer's instructions (PharMingen). IL-4 and IL-5 ELISA sensitivities were 3.5 and 5.0 pg/mL, respectively.

Statistical analysis

Unpaired *t*-test and Mann–Whitney test were used for comparisons between groups depending on whether data were normally distributed or not, respectively.

Paired *t*-test and Wilcoxon paired test were used for comparison within group and between times. $P < 0.05$ was considered statistically significant.

Results

Functional data on specific allergen challenge

Bronchial responsiveness to *D. pteronyssinus* ('PD₂₀ allergen') was calculated or extrapolated in all experimental subjects as defined in Materials and Methods. PD₂₀ allergen log values were 2.307 ± 0.605 and 2.924 ± 0.157 (mean $\pm$ SD) in asthmatics and controls, respectively ($P < 0.05$). In asthmatics, the maximum percent FEV₁ fall, compared with the pre-challenge value, in the 24 h following allergen challenge (maximum LAR) was $29.7 \pm 8.7\%$ (mean $\pm$ SD). In control subjects the maximum LAR was in all cases $< 10\%$. These results indicate that in asthmatic individuals the experimental inhalation of allergen was capable of inducing a functionally measurable early broncho-spastic reaction. Moreover, they show that in asthmatics a relevant late broncho-spastic reaction took place in the 24 h following the challenge, when the second BAL was performed.

BAL cell data

BAL cell data are shown in Table 1 as grouped results.

Inter-group comparison BAL cell numbers and cell percentages were not different in asthmatics vs. controls, except for eosinophils which were increased (as percentages and as absolute numbers) both 'at baseline' and after allergen challenge in asthmatics vs. controls.

Intra-group comparison BAL cell numbers were higher post-challenge vs. 'baseline' both in asthmatics ($P = 0.091$) and in non-asthmatic controls ($P = 0.024$). Cell percentages were not changed following allergen challenge, except for macrophages ($P = 0.076$), which decreased, and eosinophils, which increased ($P = 0.091$) in asthmatics after allergen challenge. Absolute cell numbers were not changed after allergen challenge, except for lymphocytes and eosinophils, which both increased in asthmatics ($P = 0.026$ and $P = 0.007$, respectively).

The increased absolute numbers of BAL cells in asthma is probably due to the expected recruitment of inflammatory cells (lymphocytes and eosinophils) to the lower respiratory tract following allergen challenge.

Taken together, these results indicate that allergic inflammation was present in the lower respiratory tract in the asthmatic individuals before experimental allergen

Table 1. Data on lung inflammatory cells at baseline ('bas') and 24 h after allergen challenge ('post-ch')

Group	Macr., %		Lymph., %		Neutr., %		Eosin., %	
	bas	post-ch	bas	post-ch	bas	post-ch	bas	post-ch
Atopic asthmatics	89.4 ± 4.0	79.7 ± 11.3	5.2 ± 3.2	10.6 ± 10.4	0.7 ± 0.8	1.4 ± 1.7	4.2 ± 2.3	8.3 ± 4.8
Atopic non-asthmatics	90.1 ± 2.4	89.7 ± 1.8	8.6 ± 1.8	9.2 ± 1.8	1.1 ± 0.9	0.9 ± 0.3	0.1 ± 0.2	0.2 ± 0.3

	Cells × 10 ⁶		Macr.		Lymph.		Neutr.		Eosin.	
	bas	post-ch	bas	post-ch	bas	post-ch	bas	post-ch	bas	post-ch
Atopic asthmatics	7.4 ± 2.6	10.8 ± 2.9	6.61 ± 0.104	8.6 ± 0.327	0.38 ± 0.083	1.14 ± 0.301	0.05 ± 0.02	0.15 ± 0.049	0.31 ± 0.059	0.89 ± 0.139
Atopic non-asthmatics	8.2 ± 1.7	10.1 ± 1.3	7.38 ± 0.408	9.59 ± 0.023	0.7 ± 0.03	0.91 ± 0.003	0.09 ± 0.004	0.09 ± 0.002	0.01 ± 0.004	0.01 ± 0.004

Macr., Macrophages; Lymph., lymphocytes; Neutr., neutrophils; Eosin., eosinophils. Results are expressed as mean ± SD.

Inter-group comparison. BAL cell numbers and cell percentages not different in asthmatics vs. controls, except for eosinophils which were higher (as percentages and as absolute numbers) in asthmatics both a baseline and after allergen challenge.

Intra-group comparison. BAL cell number higher post-challenge vs. baseline both in asthmatics ($P = 0.091$) and in non-asthmatic controls ($P = 0.024$). Cell percentages not changed following allergen challenge, except for macrophages ($P = 0.076$), which decreased, and eosinophils, which increased ($P = 0.091$) in asthmatics. Absolute cell numbers not changed after allergen challenge, except for lymphocytes and eosinophils, which both increased in asthmatics ($P = 0.026$ and $P = 0.007$, respectively).

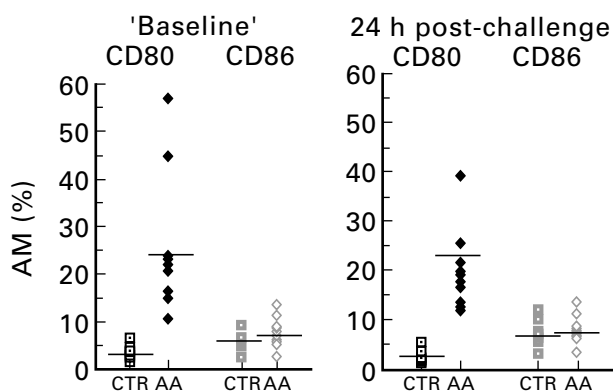


Fig. 1. Percent of alveolar macrophages (AM) expressing CD80 and CD86 in atopic asthmatics (AA) and in atopic control individuals (CTR). Each dot represents an individual subject.

inhalation and that inflammatory cells increased in the 24 h following the challenge.

There was no correlation between PD₂₀ allergen or LAR values and the percentages or absolute numbers of eosinophils in the lavage fluid.

Proportion of AM expressing the CD80 and CD86 costimulatory molecules

Inter-group comparison The percent of AM expressing CD80 was increased in asthmatics compared with controls (at baseline 24.3 ± 14.1 vs. 1.8 ± 0.9 ; after challenge 19.8 ± 7.7 vs. 1.9 ± 0.6) ($P < 0.001$ in both cases). The proportion of AM expressing membrane CD86 was comparable between asthmatics and controls both at baseline (7.3 ± 2.2 and 6.5 ± 2.5 , respectively) and after challenge (7.3 ± 2.9 and 6.8 ± 2.7 , respectively) (Fig. 1).

Intra-group comparison The percentages of AM expressing CD80 or CD86 were not significantly changed following allergen challenge (Fig. 1).

These results indicate the subset of CD80⁺ AM is expanded in asthmatics while the subset of CD86⁺ AM is not. Moreover, they show that the inflammatory phenomena that take place in the 24 h following experimental inhalation of allergen are not associated with the expansion of either AM population.

Levels of membrane expression of the CD80 and CD86 costimulatory molecules on AM

The expression of CD80 and CD86 was analysed in terms of specific mean fluorescence intensity (MFI) of the monoclonal relative to isotype control antibody (relative MFI) (Fig. 2).

Inter-group comparison The expression of CD80 was not different in asthmatics vs. controls, both 'at baseline' (6.3 ± 2.4 and 5.5 ± 1.7 , respectively) and after allergen challenge (6.9 ± 1.4 and 6.2 ± 2.7 , respectively). The expression of CD86 was similar in asthmatics and in controls at baseline (6.4 ± 2.7 and 5.4 ± 2.4 , respectively). Following allergen challenge CD86 expression was significantly higher in asthmatics than in controls (10.8 ± 3.6 vs. 5.1 ± 2.9 , respectively; $P < 0.01$) (Fig. 3).

Intra-group comparison Allergen challenge was not associated with a modulation in the expression of CD80 on AM either in asthmatics or in controls. In contrast, CD86 expression was increased in asthmatics following allergen challenge, while it remained unchanged in controls (Fig. 3). Taken together, these results indicate that in the asthmatic patients included in this study the inflammatory changes which take place in the 24 h following a functionally relevant allergen inhalation are associated with an increased expression of CD86 on AM.

There was no correlation between CD86 expression following allergen challenge and PD₂₀ allergen or LAR values (not shown).

Th2 cytokine production by specific T cells stimulated with allergen presented by AM

Allergen-specific T cell lines were used as responder cells in assays where IL-4 and IL-5 production was measured after 2 days' *in vitro* culture with allergen in the presence of AM as APC. As positive controls, microcultures where blood monocytes served as APC were used. In these cultures, IL-4 and IL-5 production was similar in asthmatics and in controls (IL-4, 382.8 ± 128.7 pg/mL and 431.5 ± 70.3 pg/mL; IL-5, 328.5 ± 89.4 pg/mL and 375.3 ± 86.9 pg/mL, respectively) (Fig. 4).

IL-4 and IL-5 production by T cells stimulated with allergen presented by AM from asthmatics was lower (180.4 ± 46.4 pg/mL, $P < 0.01$, and 202.8 ± 74.4 pg/mL, $P < 0.01$, respectively) in 'baseline' AM samples compared with positive controls where monocytes were used. In contrast, AM taken 24 h post-challenge supported a production of IL-4 and IL-5 comparable to positive control cultures (324.5 ± 70.0 and 460.8 ± 132.9 , respectively) (Fig. 4, top panel).

AM from controls were poor APC, since IL-4 and IL-5 production 'at baseline' (49.5 ± 22.8 pg/mL and 68.3 ± 28.5 pg/mL, respectively) was lower compared with (positive control) monocytes, with 'baseline' and with 'post-challenge' AM from asthmatics. Following allergen challenge, production of IL-4 and IL-5 supported by AM from controls remained unchanged compared with

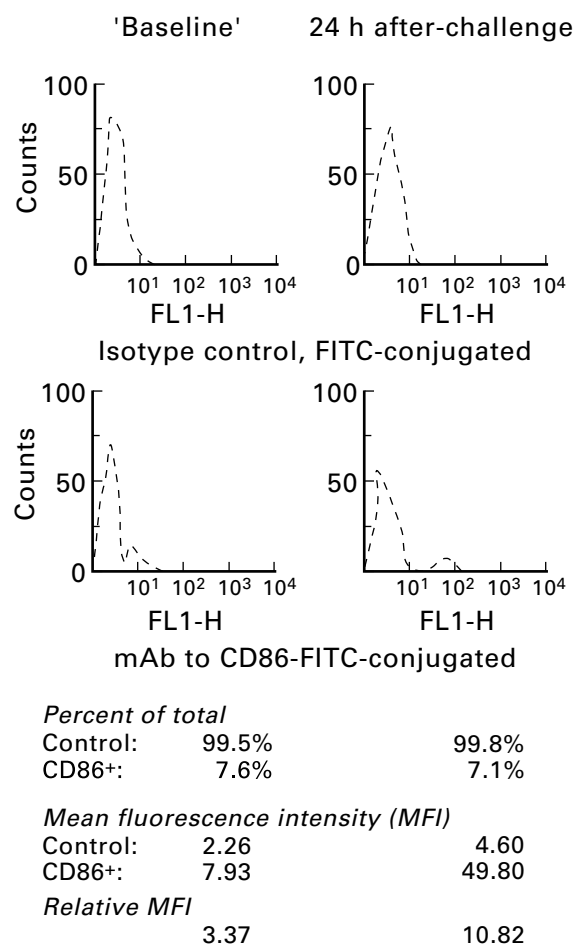


Fig. 2. Cytofluorimetric analysis of CD86 expression by lymphocyte-depleted alveolar macrophages (AM) (representative profiles). Profiles of lymphocyte-depleted AM taken at 'baseline' (left panels) and 24 h after allergen challenge (right panels) are shown as histograms where green fluorescence (on the abscissa) is plotted against the number of acquired events. In the bottom panels, cells were reacted with a monoclonal to human CD86, conjugated with FITC. In the top panels, cells were reacted with equal amounts of an FITC-conjugated, isotype-matched monoclonal with irrelevant specificity. The subset of AM that could be discriminated by the monoclonal antibody to CD86 was quantified as percent of cells (vs. total) and the relative mean fluorescence intensity (MFI) was calculated (ratio between the MFI of CD86⁺ cells to the MFI of cells reacted with the control antibody). Virtually all cells in the top panels (control) and CD86⁺ cells in the bottom panels were used for statistical analysis of results by using the CellQuest[®] software (Becton Dickinson).

'baseline' (54.5 ± 23.9 pg/mL and 71.9 ± 26.7 pg/mL, respectively) (Fig. 4, bottom panel).

IL-4 and IL-5 production in cultures where allergen was added to AM in the absence of T cells was always < 5 pg/mL and < 10 pg/mL, respectively. Similarly, when T cells were co-cultured with AM but the allergen was not added,

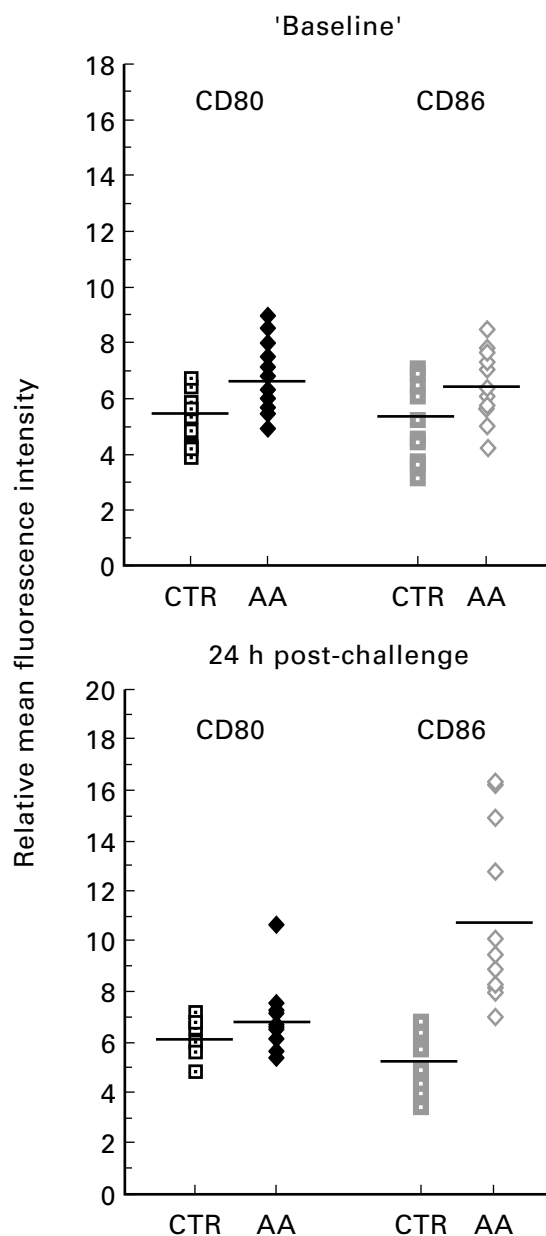
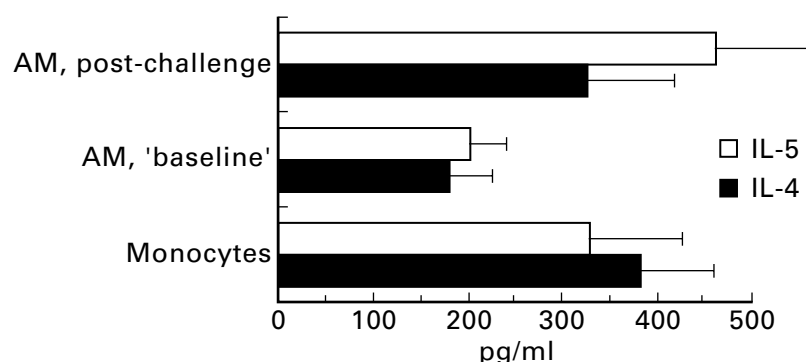


Fig. 3. Relative mean fluorescence intensity of CD80 and CD86 on alveolar macrophages (AM) from atopic asthmatics (AA) and atopic control individuals (CTR). Each dot represents an individual subject.

IL-4 and IL-5 production was always < 15 pg/mL and < 10 pg/mL, respectively.

Taken together, these results indicate that the expansion of a CD80⁺ AM subset in asthmatics is associated with an increased capability of allergen presentation to Th2 lymphocytes. Moreover, they also indicate that following allergen challenge, there is a further increase in this activity in the absence of a further increase of CD80-expressing AM.

Asthmatics



Controls

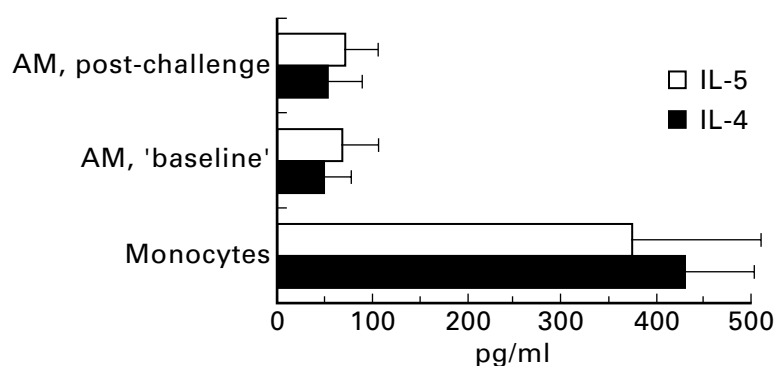


Fig. 4. Production of IL-4 and IL-5 (pg/mL) by T cell lines stimulated with allergen (Der p I) presented by autologous alveolar macrophages (AM) or by autologous blood monocytes. Results obtained from atopic asthmatics and atopic non-asthmatic control individuals are shown.

Inhibition of Th2 cytokine production by anti-CD80 and/or anti-CD86 monoclonals

In cultures where AM taken 'at baseline' were used, there was a significant inhibition of IL-4 and IL-5 production following incubation of AM with blocking monoclonal antibodies to CD80 (83.3 ± 23.5 pg/mL and 94.7 ± 28.4 pg/mL, respectively; in both cases $P < 0.01$ vs. positive controls) and CD86 (79.0 ± 24.0 pg/mL and 91.2 ± 42.6 pg/mL, respectively; in both cases $P < 0.01$ vs. positive controls). The extent of inhibition obtained with anti-CD80 was comparable to that obtained with anti-CD86 for each cytokine. The usage of both antibodies yielded a synergistic effect and further inhibited IL-4 and IL-5 production (12.9 ± 5.5 pg/mL and 13.0 ± 4.7 pg/mL, respectively) (Fig. 5, two top panels).

Also in cultures where AM taken 24 h after challenge were used, there was a significant inhibition of IL-4 and IL-5 production following incubation of AM with blocking monoclonal antibodies to CD80 (211.5 ± 74.8 pg/mL and 277.2 ± 101.4 pg/mL, respectively; for each cytokine $P < 0.01$ vs. positive controls) or to CD86 (86.7 ± 37.8 pg/mL and 112.9 ± 48.2 pg/mL, respectively; for each cytokine $P < 0.01$ vs. positive controls). In this case, the

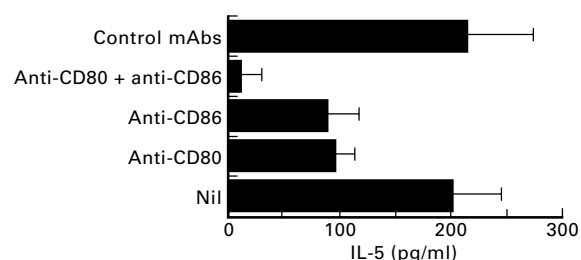
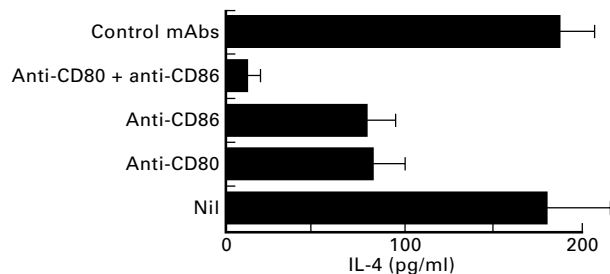
inhibition obtained with anti-CD86 was significantly higher than that obtained with the anti-CD80 monoclonal (for each cytokine $P < 0.01$). The use of both antibodies yielded a synergistic effect and further inhibited IL-4 and IL-5 production (17.8 ± 8.4 pg/mL and 14.9 ± 5.7 pg/mL, respectively) (Fig. 5, two bottom panels).

In cultures where AM taken from control individuals were used as APC there was a comparable inhibition of Th2 cytokine production by anti-CD80 and anti-CD86 monoclonal antibodies, but the levels of cytokines were close to or below the lower limit of sensitivity of the assay (not shown). The use of both monoclonals did not produce any further measurable inhibition (not shown).

The usage of isotype-matched monoclonals in negative control cultures did not significantly change IL-4 and IL-5 production ($\pm 4\%$ of the lymphokine content in the corresponding culture without allergen, in each experimental set) (not shown).

Taken together, these results indicate that in asthmatics: (i) both CD80 and CD86 are involved in the presentation of allergen to Th2 lymphocytes by AM; (ii) the increased expression of CD86 on AM following allergen challenge is associated with an increased

Asthmatics, 'Baseline'



Asthmatics, 24 h after challenge

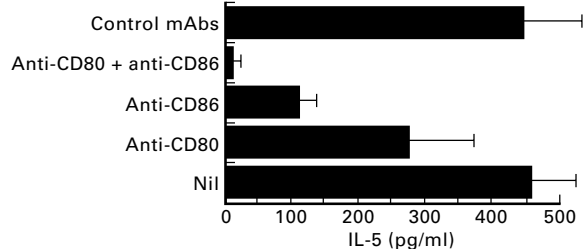
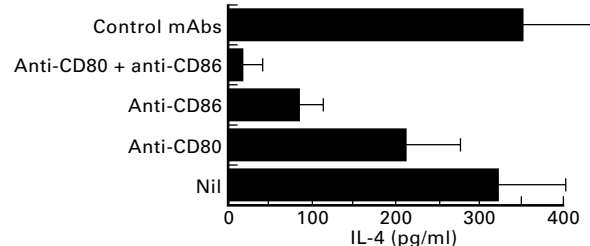


Fig. 5. Selective inhibition of IL-4 and IL-5 production (in pg/mL) by T cell lines stimulated with allergen presented by autologous alveolar macrophages (AM) from asthmatic individuals in the presence of blocking monoclonal antibodies to CD80 (anti-CD80) and/or CD86 (anti-CD86) or in the presence of medium only (nil). Control microcultures were prepared by adding a mixture of two isotype-matched monoclonals with irrelevant specificity (control mAbs).

functional dependence on CD86 as a co-stimulator molecule in antigen presentation.

Discussion

In the present work we have demonstrated that AM

efficiently present allergen to autologous Th2-type cells via CD80 and CD86 in sensitized asthmatic individuals. We have also shown that *in vivo* allergen challenge is associated with a further increase in the capability to present antigen by AM and that this can be correlated with an up-regulation of CD86 expression on a per cell basis. These findings have important implications in supporting the design of anti-asthmatic therapies based on interference with costimulation.

CD4 T lymphocytes play a central role in the induction and in the maintenance of airway inflammation in allergic asthma. Several studies in asthmatic volunteers with the use of BAL and bronchial biopsy via the fiberoptic bronchoscope have shown that exposure to allergen, both natural and experimental [21–26], results in an influx of T cells into the airways where they become activated and produce Th2-type cytokines [21]. The cell-mediated amplification of memory allergic responses thus generated is important for the perpetuation of chronic airway inflammation that underlies the pathophysiological and clinical abnormalities of asthma. The molecular basis for specificity of this mechanism is provided by the T cell receptor on a CD4 cell which is engaged by a properly processed and presented antigen, which in this case is an allergen (signal 1). The subsequent functional outcome (anergy, immunity) of this recognition event depends on the concurrent signal 2 or 'co-stimulus' [21]. A major co-stimulus is provided by CD80 and/or CD86 on the APC which interacts on the membrane of a T lymphocyte with CD28 (constitutively expressed) or its homologous CTLA-4 (which is up-regulated upon T cell activation and sends negative feedback signals).

AM taken by BAL are not the most important cell type involved in antigen presentation in the lower respiratory tract in physiological conditions. Indeed, lung dendritic cells are the crucial player in picking up the antigen and carrying it to local lymph nodes, where a specific immune response can be initiated [7,22]. On the other hand, AM physiologically act as silencers of any immune response in the lung [8,9]. However, in pathological conditions such as asthma, hypersensitivity pneumonitis, sarcoidosis [23–30], AM become involved in the maintenance and in the further expansion of the immune response at the target organ.

We previously demonstrated the anomalous expression of the CD80 costimulatory molecule on a subset of AM from allergic asthmatics, and that this correlated with a higher antigen-presenting function [10]. However, the relative role of CD80 vs. CD86 in providing the prerequisites for the efficient presentation of allergen to T cells in the asthmatic lung is still controversial. In fact, in some experimental settings there is evidence favouring CD86 [14,31], in others favouring CD80 [12,32], while several reports do not discriminate between these

possibilities and test the importance of costimulation blockade through a high-affinity ligand binding to both molecules [13,33,34]. To the best of our knowledge, the direct measurement of CD80 and CD86 expression after allergen challenge in humans has never been reported before.

In order to address this issue we used an experimental approach which allowed us to study in humans CD80 and CD86 expression on AM from mild asthmatics in conditions of 'natural' exposure to environmental allergen (at 'baseline', i.e. without asthmatic symptoms) and following the experimental induction of broncho-constriction through the controlled inhalation of allergen. In this fashion, we could measure *ex vivo* the modulation of each costimulatory molecule in two functionally different contexts. Notably, all patients included in this study experienced a late asthmatic reaction to allergen inhalation in the hours following allergen challenge. This functionally relevant event indicates that the chronic inflammation observed at 'baseline', expressed by the increased number of eosinophils, was substantially affected by the experimental inhalation of allergen.

We used allergen-specific T cells as prototypic Th2 cells in order to mimic *in vitro* the *in vivo* situation whereby T lymphocytes are activated and expanded outside the organized lymphoid tissues. Lymphocytes with this phenotype, although derived from peripheral blood, are representative of memory-resident lung T cells [35–37]. It is likely that the mechanisms that underlie the sensitization (priming) to allergen could be substantially different from those reported here, where a typical memory response is considered. However, in animal models a central role for CD28/B7s interactions has been identified for both the sensitization and the secondary Th2-type immune response to allergen [34], although less stringent requirements for the activation of memory T cells have been reported [38].

We found that the subset of AM expressing CD80 is expanded in atopic asthmatics compared with atopic non-asthmatic individuals. In contrast, the proportion of CD86⁺ AM was similar in asthmatics and in controls. Following allergen challenge, no significant changes in the proportion of CD80- or CD86-expressing AM were observed. However, when the MFI (which is an indicator of the number of membrane molecules) was compared, CD86 expression was found up-regulated following allergen challenge, while CD80 expression was not. Taken together, these results indicate that: (i) the expansion of a CD80⁺ AM subset in asthmatics is associated with an increased capability of antigen presentation to Th2 lymphocytes; (ii) following allergen challenge, there is a further increase in this activity in the absence of a corresponding increase of CD80-expressing AM and in the presence of an up-regulation of CD86 expression on a per cell basis.

In functional assays, where the inhibition obtained with

single costimulation blockers was evaluated, CD80 and CD86 appeared to contribute comparably to antigen presentation and Th2 cytokine production in 'baseline' conditions. In contrast, following allergen challenge a blocking antibody to CD86 inhibited Th2 cytokine production by allergen-stimulated T cells more efficiently than a blocking antibody to CD80. When added together, the two antibodies provided a synergistic inhibitory effect. Taken together, these results indicate that both CD80 and CD86 are crucial to antigen presentation in the lower respiratory tract in asthmatics and that CD86 is positively modulated and becomes functionally prevalent in antigen presentation in the hours that correspond to the late reaction to allergen. This post-challenge situation is similar to peripheral blood, where CD86 had a prevalent functional role in presentation of allergens by blood monocytes (not shown), in agreement with previous reports [12,15,16]. The addition of anti-CD80 or anti-CD86 blocking antibodies was done at a single concentration for a fixed amount of time. This is obviously an oversimplification, since it is possible that blocking each single molecule can affect the expression of the other molecule over time in a different fashion depending on factors such as the concentration, the affinity and the fine specificity of the monoclonals used. However, since CD86 increased expression after allergen challenge correlates with an increased loss of function following its blockade *in vitro*, it is very likely that our experimental system is revealing a functional difference of CD80 vs. CD86.

Recently, a bronchial explant model was developed by Holgate's group, that enables mucosal tissue obtained by bronchial biopsy to be challenged with allergen *ex vivo* and the supernatants to be analysed for cytokine and chemokine levels and for other functionally relevant activities [12,13,39]. This approach allowed measurement of α/β T cell-dependent production of Th2 cytokines directly from challenged tissues and demonstrated the prevalent role of CD80 and CD86 (vs. CD40/CD40 ligand) costimulation in this process. These authors also reported synergistic inhibitory effects by blocking both costimuli and observed that the up-regulation of the expression of CD86, compared with CD80, was more affected by allergen challenge. Moreover, the seminal paper by Tsuyuki *et al.* in a mouse model of asthma [40], as well as more recent evidence obtained by Mark *et al.* with CD80 and CD86 (single or double) knockout mice [41] support the concept that B7-1 and B7-2 have complementary roles in the allergic response. Taken together, those results are in agreement with the present data, which were obtained *in vivo* in humans in conditions of functionally relevant allergen exposure.

Complex ligand–receptor interactions take place in the asthmatic airways, involving both CD80 and CD86, that

could be sequentially distributed over time. These interactions are probably relevant not only to support the secretion of Th2 cytokine, but also of interferon-gamma (IFN- γ). In fact, in another set of experiments we could demonstrate that by blocking *in vitro* CD80 and CD86 on alveolar macrophages from asthmatic individuals we could differently interfere with IFN- γ secretion and with the subsequent production of eotaxin, which is a well-known chemokine involved in eosinophil recruitment (Burastero *et al.*, in preparation).

In conclusion, we found that CD80 and CD86 on AM are costimulatory molecules which contribute to expanding Th2 lymphocytes in the lower respiratory tract in asthma. Moreover, we found that CD86 is dynamically regulated in the context of the inflammatory changes which take place in the 24 h following an acute asthmatic attack and which functionally correspond to the late broncho-spastic reaction to allergen.

Acknowledgement

This work was supported by a grant from ALK Abellò to S.E.B.

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