

IFN- γ inhibits the proliferation of allergen-activated T lymphocytes from atopic, asthmatic patients by inducing Fas/FasL-mediated apoptosis

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Abstract: The defect in interferon- γ (IFN- γ) production that results in a T helper cell type 2-dominated response may be responsible for a decrease in the apoptosis of allergen-activated T cells in asthma. We investigated the effect of recombinant IFN- γ on proliferation, Fas/Fas ligand (FasL) expression, and apoptosis in allergen-stimulated peripheral blood mononuclear cells obtained from atopic, asthmatic patients and nonatopic, control subjects. The addition of IFN- γ at the start of cultures markedly inhibited the proliferative response to a specific allergen in cells from all asthmatic patients, whereas no change was observed in cells from nonatopic, control subjects. IFN- γ induced an increase in the expression of Fas and FasL by allergen-stimulated CD4⁺ T cells from asthmatic patients and caused the apoptosis of these cells. A Fas-blocking monoclonal antibody prevented the inhibitory effect of IFN- γ on allergen-induced proliferation. These results suggest that IFN- γ inhibits the proliferation of allergen-stimulated CD4⁺ T cells from atopic, asthmatic patients by inducing the surface expression of Fas and FasL, which in turn triggers their apoptotic program. The defect in IFN- γ production involved in the allergic, immune response may therefore be responsible for a decrease in apoptosis of allergen-activated T lymphocytes in the airways of atopic, asthmatic patients. *J. Leukoc. Biol.* 76: 000–000; 2004.

Key Words: asthma · allergy · inflammation · cytokines

INTRODUCTION

Airway inflammation plays a crucial role in the pathogenesis of bronchial asthma [1–3]. T lymphocytes are involved in the inflammatory/immune response in asthma causing tissue infiltration and damage. These cells recognize and respond directly to allergens and by releasing several cytokines, may orchestrate the inflammatory response [4, 5]. Activated CD4⁺ T cells have been detected in the peripheral blood, bronchoalveolar

lavage, and bronchial mucosa of patients with asthma [6–10]. Human CD4⁺ T cells can be divided into two distinct subpopulations based on cytokine production profile: T helper cell type 1 (Th1) cells, which produce large amounts of interleukin (IL)-2 and interferon- γ (IFN- γ), and Th2 cells, which release IL-4 and IL-5 but little or no IFN- γ and IL-2 [11]. CD4⁺ Th2-like cells have been detected in the airways of atopic, asthmatic patients and have been shown to accumulate during the late-phase skin reaction to allergen in atopic individuals [12–14]. Results from in vitro studies of the cytokine production of blood-derived, antigen-specific CD4⁺ T cell clones and lines have suggested that Th2-like cells are preferentially induced following the stimulation by allergen of lymphocytes from atopic donors [15, 16].

Despite the considerable progress toward defining the characteristics of the inflammatory process in allergic asthma, the mechanisms underlying the persistence of inflammation in the airways of asthmatic patients are still poorly understood. Apoptosis plays a critical role in the resolution of inflammation [17, 18]. Stimulation of activated T cells through the T cell receptor/CD3 complex triggers the apoptosis of these cells [19]. The interaction between Fas and Fas ligand (FasL) is the main pathway of activation-induced apoptosis of mature T cells [19–22]. Activated T cells have recently been reported to be resistant to Fas-mediated apoptosis in asthmatics, suggesting that a defect in apoptosis may be involved in the pathogenesis of asthma [23].

IFN- γ plays an important role in regulating the proliferation and apoptosis of T lymphocytes [24, 25]. Lymphocytes from mice with disrupted genes for IFN- γ or IFN- γ receptor-binding chain (IFN- γ R1) display hyperproliferation in response to mitogen and alloantigen [26, 27]. IFN- γ up-regulates the expression of Fas protein on the T cell surface [28]. Furthermore, we have previously shown that activated Th2 clones that do not

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normally express detectable levels of FasL on their surface do so in the presence of IFN- γ [29].

In this context, we speculated that the defect in IFN- γ production that characterizes a Th2 response was responsible for a decrease in the apoptosis of allergen-specific T cells and thus, for their persistence in allergic airway inflammation.

To test this hypothesis, we evaluated the effects of exogenous IFN- γ and a neutralizing antibody directed against IFN- γ on the proliferative response and apoptosis of peripheral blood mononuclear cells (PBMCs) from atopic, asthmatic patients and control subjects stimulated with specific allergen. As Fas/FasL receptors are critical for apoptosis and are modulated by IFN- γ , we also assessed the level of these two receptors on CD4 $^{+}$ T cells stimulated with allergen in the presence and absence of exogenous IFN- γ and an anti-IFN- γ antibody.

MATERIALS AND METHODS

Reagents

Ficoll-Hypaque was obtained from Pharmacia (Uppsala, Sweden). RPMI 1640 was purchased from BioWhittaker (Walkersville, MD). Fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, gentamycin, and trypan blue were obtained from Life Technologies (Grand Island, NY). EDTA, Tween 20, phosphate-buffered saline (PBS), bovine serum albumin (BSA), sodium azide (NaN_3), propidium iodide (PI), and paraformaldehyde were supplied by Sigma Chemical Co. (St. Louis, MO). Mouse immunoglobulin G (IgG) $_1$ - and IgG $_{2a}$ -negative control, biotin-conjugated, rabbit anti-mouse IgG, and streptavidin-phycoerythrin (PE) were obtained from Dako (Denmark). Fluorescein isothiocyanate (FITC)-conjugated mouse IgG $_1$ -negative control, FITC-conjugated mouse anti-human Fas, and biotin-conjugated mouse anti-human FasL were obtained from PharMingen (San Diego, CA). FITC-conjugated mouse anti-human CD4 and PE-conjugated mouse anti-human CD4 were purchased from Becton Dickinson (Mountain View, CA). Mouse anti-human Fas IgM CH11 monoclonal antibody (mAb) was supplied by Upstate Biotechnology (Lake Placid, NY) and mouse anti-human Fas IgG ZB4 mAb, by MBL (Nagoya, Japan).

Patients

Nine atopic asthmatics sensitive to *Dermatophagoides pteronyssinus* (Der.p.; four men and two women), 19–33 years of age, were studied. The patients were selected on the basis of a positive skin-prick test to Der.p. allergen extract (Bayer, Milan, Italy) and an increase in allergen-specific IgE; baseline forced expiratory volume in 1 s (FEV_1) >80% of predicted; increased bronchial responsiveness to inhaled methacholine [i.e., provocative dose required to decrease the FEV_1 by 20% of its baseline value (PD20 methacholine) below 800 μg]; stable, clinical conditions (i.e., no acute asthma attack or respiratory tract infection in the last 2 months); no treatment other than rescue β_2 adrenergic drugs, which were discontinued at least 24 h before the study; and no previous specific immunotherapy. Six nonatopic, healthy volunteers were studied as controls. All subjects were nonsmokers. The study conformed to the Declaration of Helsinki, and informed consent was obtained from all subjects.

Cell separation and cultures

Peripheral venous blood was obtained from each subject. PBMCs were isolated from heparinized venous blood using a standard Ficoll-Hypaque density gradient technique. The mononuclear cell-rich fraction was collected from the plasma/Ficoll interface and washed twice with RPMI 1640. Cells were then cultured in RPMI 1640 and were supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), L-glutamine (2 mM), and 10% heat-inactivated FCS (complete medium).

Allergen-specific proliferation assay

Triplicate cultures of 1×10^5 PBMCs were set up in 96-well tissue-culture plates in complete medium in the presence or absence of purified Der.p. (final

concentration, 10 $\mu\text{g}/\text{ml}$) or Der.p. plus recombinant (r)IFN- γ (final concentration, 1000 U/ml) or anti-IFN- γ $\gamma 123$ mAb [29] (final concentration, 50 $\mu\text{g}/\text{ml}$). PBMCs stimulated with an irrelevant allergen were included in all experiments as a control. After 7 days of culture, 1 μCi [^3H]-thymidine (Amersham, Milan, Italy) was added. Six hours later, cells were harvested, and radioactivity was measured with a Matrix-96 β -counter (Canberra-Packard, Milan, Italy). Results are expressed as mean Δ counts per minute (cpm) $\pm$ SEM of triplicate cultures, calculated by subtracting the cpm of unstimulated cells from that of allergen-stimulated cells.

In parallel experiments, the [^3H]-thymidine uptake of allergen-stimulated PBMCs was evaluated in the presence of blocking anti-Fas IgG ZB4 mAb (final concentration, 1 $\mu\text{g}/\text{ml}$); agonistic anti-Fas IgM CH11 mAb (final concentration, 50 $\mu\text{g}/\text{ml}$); and anti-Fas IgG mAb or anti-Fas IgM mAb plus IFN- γ (final concentration, 1000 U/ml).

Cytokine production assay

IL-4 and IFN- γ production by allergen-stimulated PBMCs was analyzed using an Immunospot assay kit (Bioline Diagnostici, Turin, Italy), according to the manufacturer's instructions. The number of spots/10 ^{6} cells was determined with enzyme-linked immunospot (ELISPOT) Reader (AID, Strassberg, Germany). As a control stimulus, *Mycobacterium tuberculosis* purified protein derivative (PPD; 1 $\mu\text{g}/\text{ml}$, Chiron, Siena, Italy) was used. All patients showed delayed-type, cutaneous hypersensitivity to PPD.

Flow cytometry

Der.p.-stimulated PBMCs ($1 \times 10^6/\text{ml}$) were cultured in 24-well tissue-culture plates for 7 days in the presence or absence of rIFN- γ (final concentration, 1000 U/ml) or neutralizing anti-IFN- γ $\gamma 123$ mAb (final concentration, 50 $\mu\text{g}/\text{ml}$). Nonadherent cells were recovered at various time-points, and the levels of Fas and FasL present on their surfaces were analyzed. Briefly, for Fas, 1×10^5 cells were incubated with a FITC-conjugated, anti-Fas mAb in 0.2% BSA and 0.01% sodium azide in PBS for 30 min at 4°C. FasL was detected by incubation with a biotin-conjugated anti-FasL mAb, followed by streptavidin-PE. Cells were washed twice and incubated for 30 min at 4°C with PE- or FITC-conjugated anti-CD4 mAb. Labeled cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Milan, Italy). Negative controls consisted of isotype-matched, control mAb. Each analysis represented the results from 10,000 events.

IFN- γ R1 was detected with the mouse mAb γ R99, an IgG1 that specifically interacts with the extracellular domain of the human IFN- γ R1 and inhibits the binding of IFN- γ [30]. IFN- γ R2 was detected with the mouse mAb C.11, an IgG2a that specifically interacts with the extracellular domain of the human IFN- γ R2 [29, 31]. Primary antibody binding was detected by incubation with biotin-conjugated rabbit anti-mouse Ig mAb and streptavidin-PE.

DNA staining was performed as described previously [32]. Briefly, 1×10^6 cells were suspended in 0.875 ml cold PBS and 0.125 ml cold 2% paraformaldehyde solution and were incubated for 1 h on ice. The fixed cells were washed and gently resuspended in 1 ml 0.2% Tween 20 in PBS at room temperature. The mixture was incubated for 20 min at 37°C. PBS, supplemented with 2% FCS and 0.1% NaN_3 (1 ml), was added, and the suspension was centrifuged for 5 min at 1300 rpm. The supernatant was decanted, and DNA was stained by incubating the cells in 1 ml PBS-azide containing 10 $\mu\text{g}/\text{ml}$ PI (Boehringer Mannheim, Mannheim, Germany) and 11.25 Kunitz U RNase for at least 30 min in the dark. DNA content was then determined by flow cytometry.

Statistical analysis

Wilcoxon's matched-pairs test was used to compare cell proliferation, apoptosis, and Fas/FasL expression in the various culture conditions. Data are expressed as means $\pm$ SEM.

RESULTS

Effect of IFN- γ on allergen-induced proliferation of T lymphocytes

PBMCs from nonatopic, control subjects showed a weak proliferation in response to allergen, which was not affected by the

addition of rIFN γ ($P=0.058$); cell proliferation, in contrast, was significantly increased in the presence of a neutralizing anti-IFN- γ mAb ($P<0.05$; **Fig. 1**).

PBMCs from Der.p.-sensitive, asthmatic patients proliferated in response to specific allergen stimulation, and responses ranged from 1120 to 28,313 Δ cpm (**Fig. 1**). No proliferation was observed in the presence of an unrelated allergen extract (data not shown). The addition of rIFN- γ (1000 U/ml) at the beginning of culture strongly inhibited proliferation in cells from all patients ($P<0.05$ vs. proliferation in the presence of allergen alone, **Fig. 1**). The simultaneous addition of rIFN- γ and neutralizing anti-IFN- γ mAb completely abolished the inhibition of proliferation of allergen-stimulated PBMCs induced by IFN- γ (data not shown). Addition of a neutralizing anti-IFN- γ mAb (final concentration, 50 μ g/ml) to Der.p.-stimulated PBMCs had no significant effect on allergen-induced proliferation, suggesting that endogenous IFN- γ did not affect the proliferative response to a specific allergen (**Fig. 1**).

To investigate whether exogenous IFN- γ affected the response to Der.p. by inhibiting IL-4 production in allergen-responsive T cells, we added exogenous IL-4 (final concentration, 10 ng/ml) alone or in combination with IFN- γ to allergen-stimulated PBMCs. IL-4 did not reverse the inhibition caused by IFN- γ or induce significant changes in allergen-induced proliferation (**Table 1**). However, we observed that allergen

stimulation induced a preferential expansion of IL-4-producing T cells, as assessed by ELISPOT assay. In allergen-stimulated PBMCs, the ratio between IL-4/IFN- γ -producing cells was consistently more than 10 times that of PBMCs stimulated with PPD (data not shown).

Effect of IFN- γ on Fas/FasL expression by allergen-activated T lymphocytes

We evaluated Fas/FasL expression on resting CD4+ T cells and at various times after allergen-stimulation, in the presence and absence of IFN- γ or an anti-IFN- γ mAb. Fas was detected on freshly prepared CD4+ T cells from asthmatic patients (percentage of positive cells, $23.1 \pm 4.9\%$), whereas FasL was barely detectable (percentage of positive cells, $0.3 \pm 0.2\%$). Allergen stimulation induced a slight increase in Fas expression (percentage of positive cells on the fifth day of culture, $29.1 \pm 8\%$), whereas FasL levels remained very low (percentage of positive cells on the fourth day of culture, $4.9 \pm 3\%$). Fas was up-regulated by IFN- γ after 3 days of culture, and this up-regulation was maximal at 5 days. FasL levels increased slightly after 3 days of culture, became maximal at 4 days, and decreased thereafter (**Fig. 2A**). On the basis of the time-course study, the effects of exogenous IFN- γ and an anti-IFN- γ mAb were therefore studied using cells from all patients on the fifth day of culture for Fas expression and on the fourth day for FasL. The addition of IFN- γ induced a significant increase in Fas expression on the surface of allergen-stimulated CD4+ T cells in all cases; the percentage of positive cells (mean $\pm$ SEM) was $29.1 \pm 8\%$ in the absence of IFN- γ and $51.2 \pm 7.9\%$ in the presence of IFN- γ ($P<0.05$). In contrast, the addition of an anti-IFN- γ mAb alone to allergen-stimulated cells did not significantly affect Fas expression ($29.1 \pm 8\%$ vs. $30 \pm 7.9\%$ in the absence and presence of the mAb, respectively; **Figs. 2B and 3**). The percentage of cells expressing FasL was significantly higher in the presence of IFN- γ ($21 \pm 6.7\%$) than in cells stimulated with the allergen alone ($4.9 \pm 3\%$, $P<0.05$), whereas no change was induced by the addition of an anti-IFN- γ antibody. IFN- γ induced a slight increase in Fas and FasL levels in unstimulated cells, whereas an anti-IFN- γ mAb had no effect (**Figs. 2B and 4**). The increase in expression of Fas and FasL on Der.p.-stimulated T cells was completely abolished if IFN- γ and an anti-IFN- γ antibody were added simultaneously to the cultures (data not shown).

In cells from control subjects, allergen stimulation did not induce significant changes in Fas expression (percentage of positive cells, 29.8 ± 3.5 vs. 27.3 ± 1.8 in unstimulated cells). The addition of rIFN- γ to allergen-stimulated cells induced a slight increase of Fas expression (percentage of positive cells, 37.1 ± 6 vs. 29.8 ± 3.5 in cells stimulated with allergen alone), whereas no significant changes were observed in the presence of an anti-IFN- γ mAb (percentage of positive cells, 28.8 ± 5.2). Similar results were observed as far as FasL is concerned (data not shown).

As IFN- γ -dependent Fas/FasL induction has also been reported on CD8+ T cells, which might contribute to the induction of apoptosis of CD4+ T cells, we also evaluated

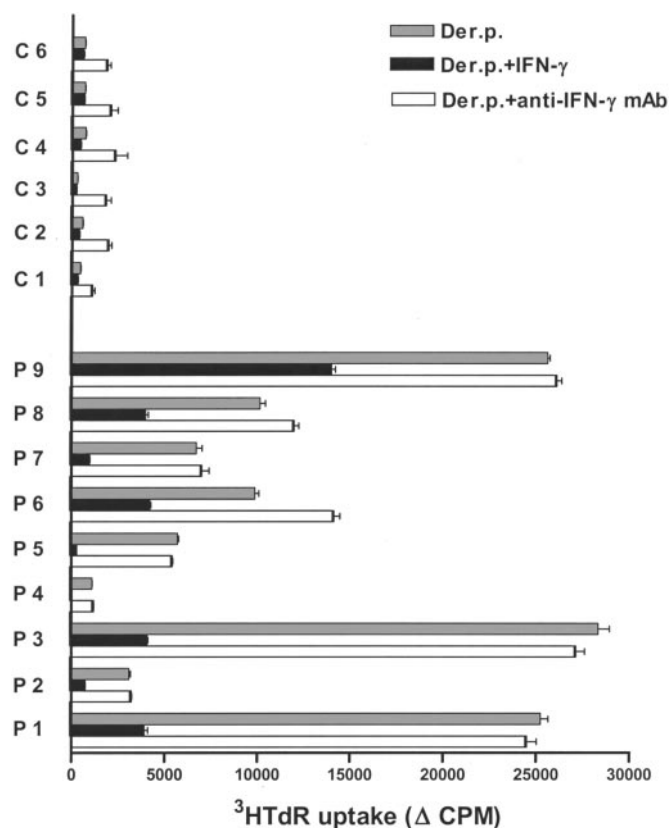


Fig. 1. Effect of IFN- γ and an anti-IFN- γ mAb on allergen-induced proliferation of T lymphocytes from nonatopic, control subjects (C, $n=6$) and atopic, asthmatic patients (P, $n=9$). PBMCs were cultured in the presence of 10 μ g/ml purified Der.p. or Der.p. plus 1000 U/ml IFN- γ or 50 μ g/ml anti-IFN- γ mAb. After 7 days, proliferation was assessed by ^{3} H-thymidine (^{3} HTdR) incorporation. Data are mean $\pm$ SEM Δ cpm for triplicate cultures.

TABLE 1. Effect of IL-4 and IFN- γ on Allergen-Induced Proliferation of T Lymphocytes from Atopic, Asthmatic Patients

Culture conditions ^a	³ H-Thymidine incorporation ^b					
	Patient n. 1	Patient n. 3	Patient n. 4	Patient n. 7	Patient n. 8	Patient n. 9
Der.p.	26,287 $\pm$ 225	29,947 $\pm$ 420	1120 $\pm$ 16	6710 $\pm$ 312	10,128 $\pm$ 298	25,596 $\pm$ 130
Der.p./IFN- γ	7046 $\pm$ 158	7217 $\pm$ 180	0	980 $\pm$ 27	3980 $\pm$ 155	13,991 $\pm$ 220
Der.p./IFN- γ /IL-4	8194 $\pm$ 185	8126 $\pm$ 230	0	1663 $\pm$ 316	3787 $\pm$ 412	16,772 $\pm$ 190
Der.p./IL-4	24,980 $\pm$ 210	33,142 $\pm$ 510	970 $\pm$ 9	4062 $\pm$ 419	8712 $\pm$ 480	23,482 $\pm$ 190

^a PBMCs from atopic, asthmatic patients were cultured in the presence of Der.p. alone (10 μ g/ml), Der.p. plus IFN- γ (1000 U/ml) or IL-4 (10 ng/ml), or both. After 7 days, proliferation was assessed by ³H-thymidine incorporation. ^b Data are mean $\pm$ SEM Δ cpm for triplicate cultures and represent results from different experiments from those shown in Figure 1, for patients n. 1 and n. 3. ^c Patient number.

the expression of these receptors on CD8+ T cells from asthmatic patients at various times (3–6 days) after allergen-stimulation in the presence and absence of IFN- γ or an anti-IFN- γ mAb. IFN- γ induced a very slight increase of Fas expression on CD8+ T cells throughout the culture period (Fig. 3); FasL was barely detectable on allergen-stimulated CD8+ T cells in the absence or presence of IFN- γ (Fig. 4), excluding that the enhanced expression of this receptor on CD8+ T cells may be responsible for the induction of CD4+ T cell apoptosis.

Effect of IFN- γ on apoptosis of allergen-activated T lymphocytes

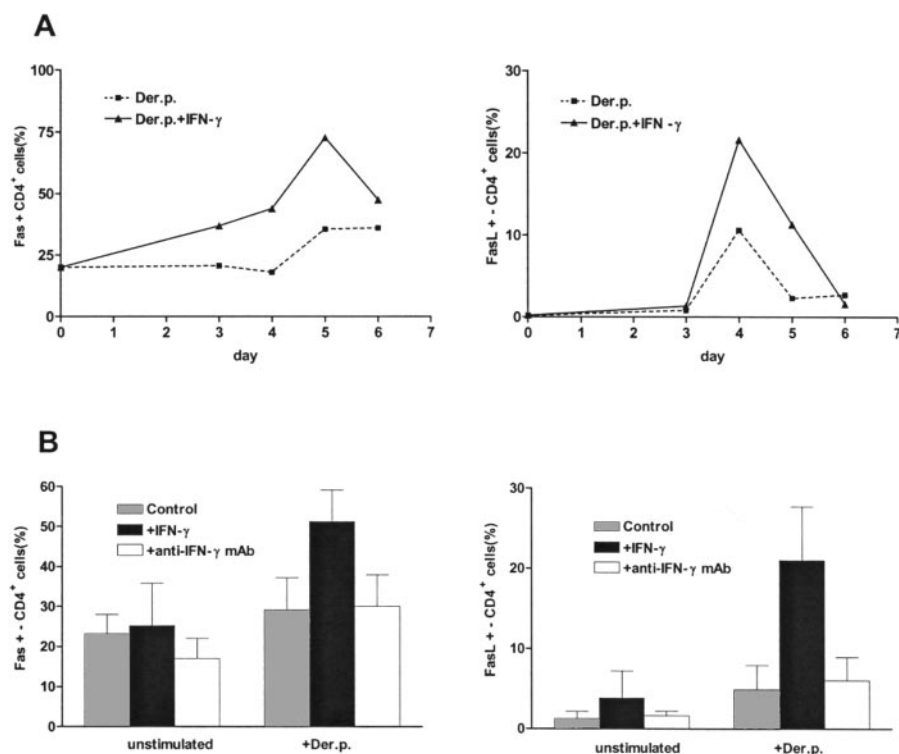
After 7 days of culture in the absence or presence of IFN- γ or an anti-IFN- γ mAb, the percentage of apoptotic cells was higher in the presence of the cytokine (mean $\pm$ SEM: 42.8 $\pm$ 5.9%) than in cells stimulated with allergen alone (mean $\pm$ SEM: 12.7 $\pm$ 6.8%, $P < 0.05$). Consistent with its lack of effect on Fas/FasL expression, the addition of an anti-IFN- γ

mAb to the cultures did not significantly affect the percentage of apoptotic cells (mean $\pm$ SEM: 16.5 $\pm$ 5%; Fig. 5).

To confirm that IFN- γ inhibited allergen-induced proliferation via Fas-dependent apoptosis, we also evaluated the effect of Fas blockade in the presence and absence of exogenous IFN- γ . The addition of an anti-Fas IgG mAb prevented the inhibition of proliferation by IFN- γ , indicating that this effect was indeed mediated by IFN- γ -induced up-regulation of Fas (Fig. 6). In contrast, the anti-Fas IgG mAb had no significant effect on the proliferation of allergen-activated cells if added alone to the cultures.

It has recently been reported that mitogen-stimulated peripheral blood T cells from asthmatic patients express Fas at the cell surface but do not undergo apoptosis after Fas receptor ligation [23]. To determine whether this defect could also occur after allergen-specific stimulation, we tested the effect of an agonistic anti-Fas IgM mAb (a specific antibody to the Fas receptor that induces apoptosis of Fas+ target cells) on cell proliferation. Consistent with previous findings in mitogen-

Fig. 2. (A) Kinetics of Fas (left panel) and FasL (right panel) expression on allergen-stimulated CD4+ T cells from atopic, asthmatic patients in the presence or absence of IFN- γ . PBMCs were cultured in the presence of 10 μ g/ml Der.p. or Der.p. plus 1000 U/ml IFN- γ for 6 days. At various time-points, nonadherent cells were recovered and washed twice, and the expression of Fas and FasL on the surface of CD4+ T lymphocytes was analyzed by flow cytometry. The results of one of three independent experiments are shown and are expressed as a percentage of positive cells. (B) Expression of Fas (left panel) and FasL (right panel) on allergen-stimulated CD4+ T cells from atopic, asthmatic patients (n=9). PBMCs were cultured in the presence of 10 μ g/ml Der.p. or Der.p. plus 1000 U/ml IFN- γ or 50 μ g/ml anti-IFN- γ mAb. Nonadherent cells were recovered and used to assess Fas and FasL levels after 5 and 4 days of culture, respectively. Fas and FasL levels on the surface of CD4+ T lymphocytes were analyzed by flow cytometry. Data are means $\pm$ SEM.



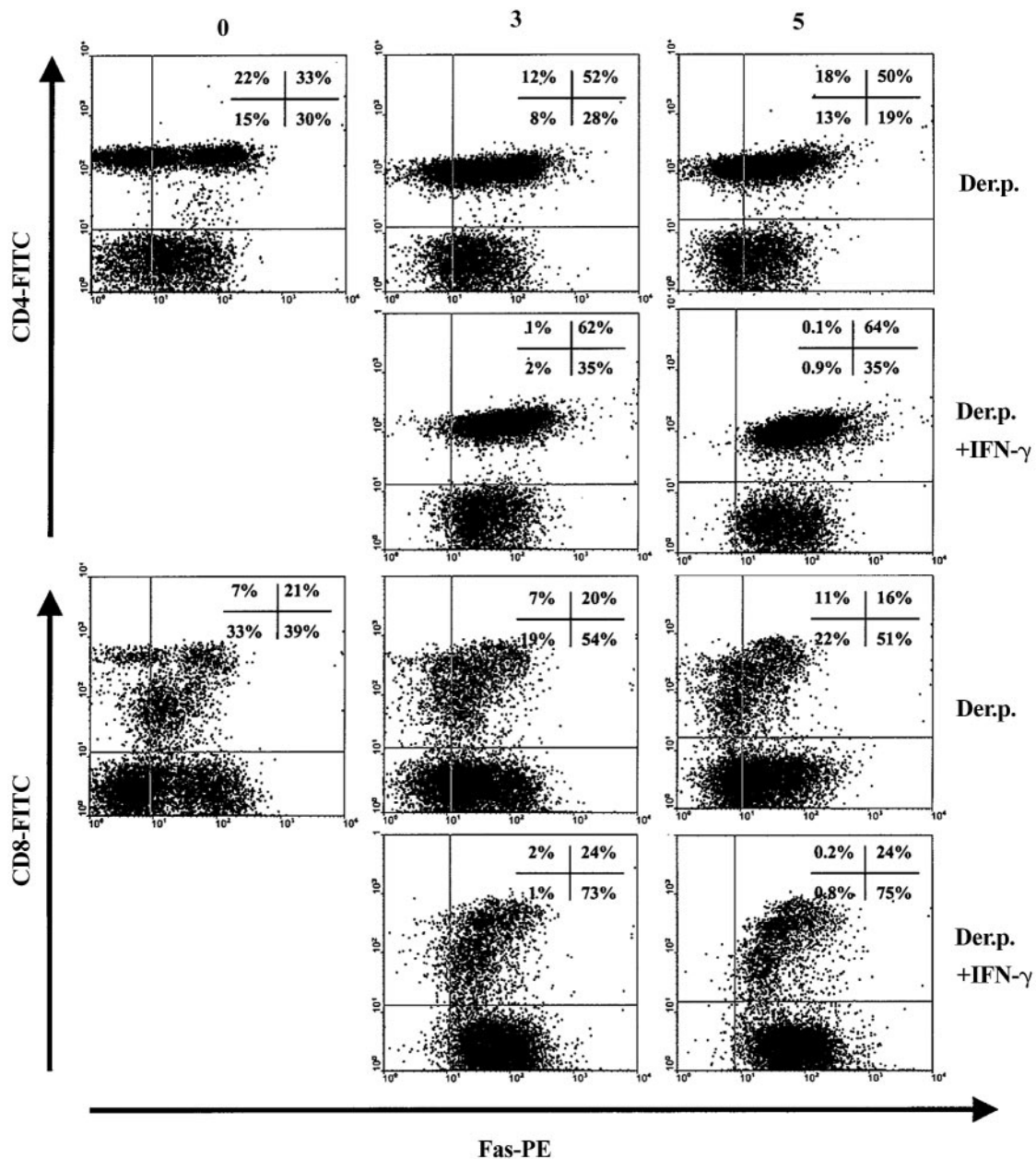


Fig. 3. Kinetics of Fas expression (at time 0 and after 3 and 5 days of culture) on allergen-stimulated CD4⁺ and CD8⁺ T cells from an atopic, asthmatic patient in the absence or presence of IFN- γ . Results of one representative experiment are shown.

stimulated cells, the anti-Fas IgM mAb had no effect on the proliferation of allergen-stimulated PBMCs (**Fig. 7**).

Expression of IFN- γ R on allergen-activated T lymphocytes

In a previous study, we observed a linear relationship between susceptibility to IFN- γ -induced apoptosis and increased expression of the IFN- γ R-transducing chain (IFN- γ R2) [31]. We therefore evaluated IFN- γ R expression on CD4⁺ T cells from asthmatic patients, at various times after allergen stimulation. IFN- γ R-binding chain (IFN- γ R1) was expressed on allergen-activated T cells. In contrast, IFN- γ R2 expression was barely detectable during the first 3 days of culture (percentage of positive cells from 0.5% to 3.8%). Between 4 and 6 days of

culture, IFN- γ R2 levels on the surface of activated T cells increased markedly (percentage of positive cells, 40.7 ± 6.5 ; **Fig. 8**). IFN- γ R2 was barely detectable on unstimulated T cells throughout the culture period (data not shown). These results are consistent with our previous observation that the level of expression of this receptor is correlated with the susceptibility of activated T cells to IFN- γ -induced apoptosis.

DISCUSSION

In this study, we found that exogenous IFN- γ strongly inhibited the proliferative response to specific allergen in PBMCs from atopic, asthmatic patients by up-regulation of Fas and FasL on

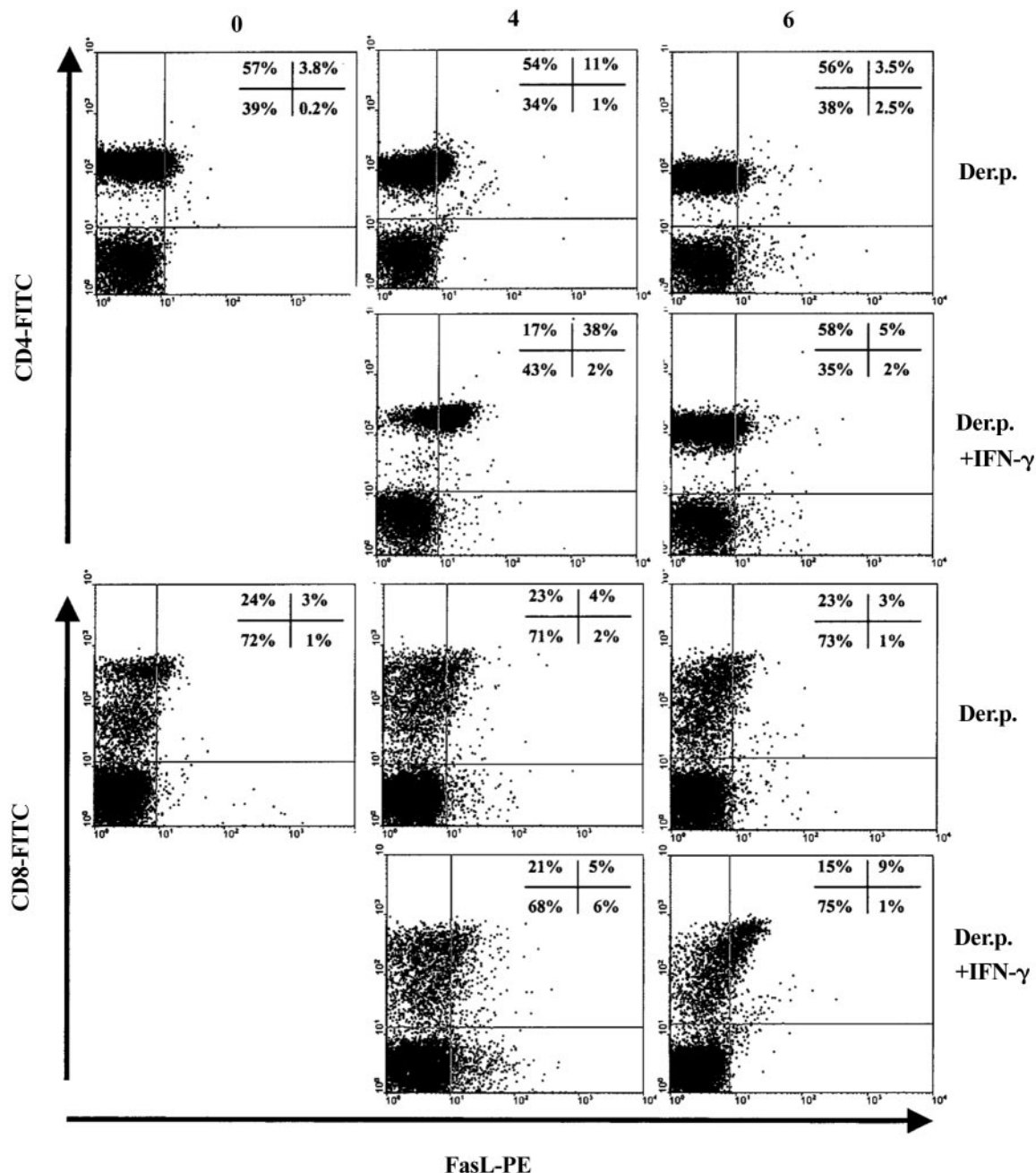


Fig. 4. Kinetics of FasL expression (at time 0 and after 4 and 6 days of culture) on allergen-stimulated CD4⁺ and CD8⁺ T cells from an atopic, asthmatic patient in the absence or presence of IFN- γ . Results of one representative experiment are shown.

the surface of activated CD4⁺ T cells and associated apoptosis of these cells. We also found that the neutralization of IFN- γ had no effect on the proliferation and apoptosis of allergen-stimulated cells or on Fas/FasL levels, suggesting that endogenous IFN- γ does not modulate allergen-activated cell death in these patients.

Evidence has accumulated that IFN- γ regulates the apoptosis of human T lymphocytes by modulating Fas and FasL expression on the surface of activated T cells. Oyaizu and co-workers [28] have shown that the cross-linking of CD4 molecules results in Fas up-regulation and lymphocyte apoptosis, both of which are inhibited by neutralizing anti-IFN- γ antibodies. In addition, lymphocytes from mice with disrupted

genes for IFN- γ or IFN- γ R1 have been reported to display hyperproliferation in response to mitogen or alloantigen, consistent with the involvement of this cytokine in regulation of the death of activated T lymphocytes [26, 27].

T lymphocytes play a crucial role in the inflammatory/immune response in asthma [4, 5]. Allergen-driven T cell recruitment and activation are well-recognized features of bronchial inflammation in asthma [4, 5, 33–35], and lymphocytes with the cytokine profile of Th2 cells have been shown to accumulate in the airways of atopic, asthmatic patients [12, 13]. However, the mechanisms underlying the persistence of activated T cells are unclear. The number of lymphocytes in the lung depends on the balance among their entry, exit,

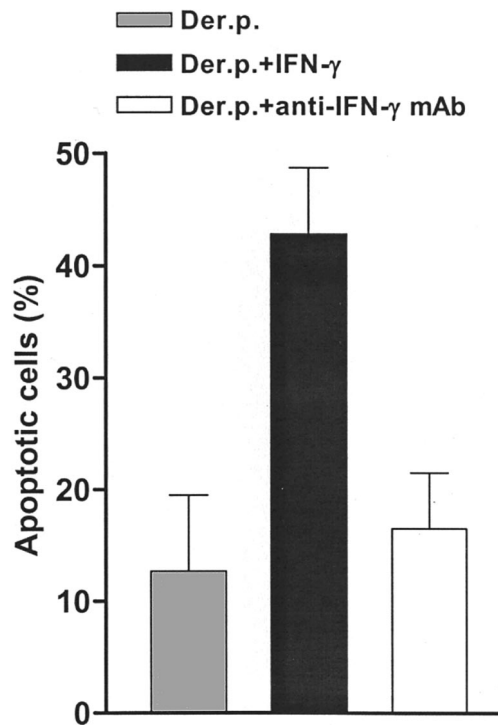


Fig. 5. Effect of IFN- γ and an anti-IFN- γ mAb on the apoptosis of allergen-stimulated T cells from atopic, asthmatic patients (n=9). PBMCs were cultured as indicated in Figure 1. After 7 days of culture, nonadherent cells were recovered, and the percentage of apoptotic cells was determined by PI staining. Data are means $\pm$ SEM.

proliferation, and cell death [35]. A delay or reduction in apoptosis may therefore contribute to the chronic persistence of these cells in the inflamed airways. Chronically stimulated T cells are eliminated by activation-induced cell death, which is controlled mainly by Fas/FasL interactions. This process limits the expansion of populations of lymphocytes chronically exposed to the antigen, preventing the accumulation of antigen-reactive T cells [19, 20].

Whereas Fas has been reported to be present on Th2 and Th1 subsets, only Th1 cells seems to express FasL upon activation [36, 37]. This difference in FasL expression affects susceptibility to activation-induced apoptosis, as Th1 clones are highly sensitive to activation-induced apoptosis, whereas Th2 clones are resistant [20]. The results of this study are consistent with those of a previous study in which we showed that Th2 cells express FasL in the presence of IFN- γ [29]. In this study, the addition of IFN- γ to allergen-stimulated T cells caused a significant increase in the level of Fas on the surface of activated CD4 $^{+}$ T cells, consistent with previous data showing that IFN- γ up-regulates Fas mRNA [38]. Our data show that IFN- γ -dependent induction of Fas and FasL is restricted to CD4 $^{+}$ T cells, as we did not observe a similar effect on CD8 $^{+}$ T cells. However, as IFN- γ has been reported to induce FasL on macrophages [39, 40], we cannot rule out the possibility that these cells contribute to induce the apoptosis of Fas-expressing T cells. To the best of our knowledge, this is the first study showing that blocking Fas inhibits the antiproliferative effect of IFN- γ ; our finding that a Fas-blocking mAb abolished the inhibitory effect of IFN- γ on allergen-induced

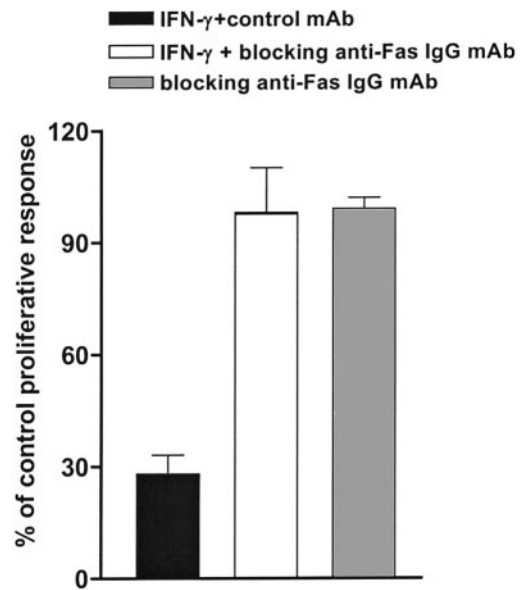


Fig. 6. Effect of blocking anti-Fas IgG mAb on the proliferation of allergen-stimulated T cells from atopic, asthmatic patients (n=5). T cells were stimulated with 10 μ g/ml Der.p. in the absence or presence of IFN- γ (1000 U/ml) and an anti-Fas IgG mAb (final concentration, 1 μ g/ml) or isotype-matched, control mAb. After 7 days, proliferation was assessed by ^{3} H-thymidine incorporation. Results are expressed as a percentage of control proliferation (proliferation in the presence of allergen alone).

proliferation indicates that this effect is indeed related to the capability of this cytokine in inducing cell apoptosis through Fas/FasL up-regulation. This is further endorsed by our observation that cell-surface expression of IFN- γ R2, conferring susceptibility to IFN- γ induced apoptosis [29, 31], was up-regulated along with Fas and FasL on allergen-activated T

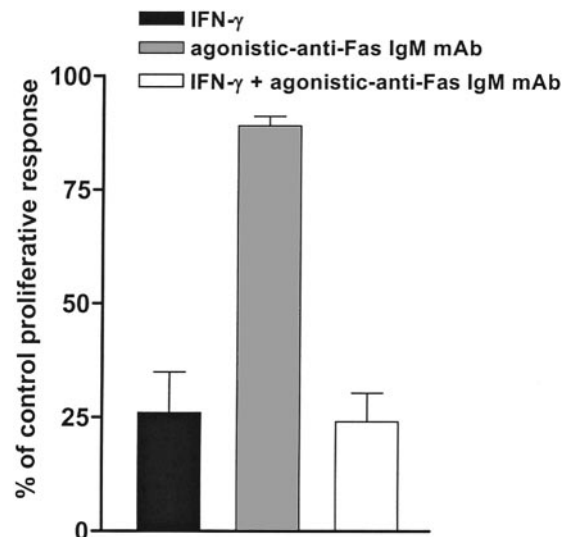


Fig. 7. Effect of agonistic anti-Fas IgM mAb on the proliferation of allergen-stimulated T cells from atopic, asthmatic patients (n=5). T cells were stimulated with 10 μ g/ml Der.p. in the absence or presence of IFN- γ (1000 U/ml), an anti-Fas IgM mAb (final concentration, 50 μ g/ml), or both. After 7 days, proliferation was assessed by ^{3} H-thymidine incorporation. Results are expressed as a percentage of control proliferation (proliferation in the presence of allergen alone).

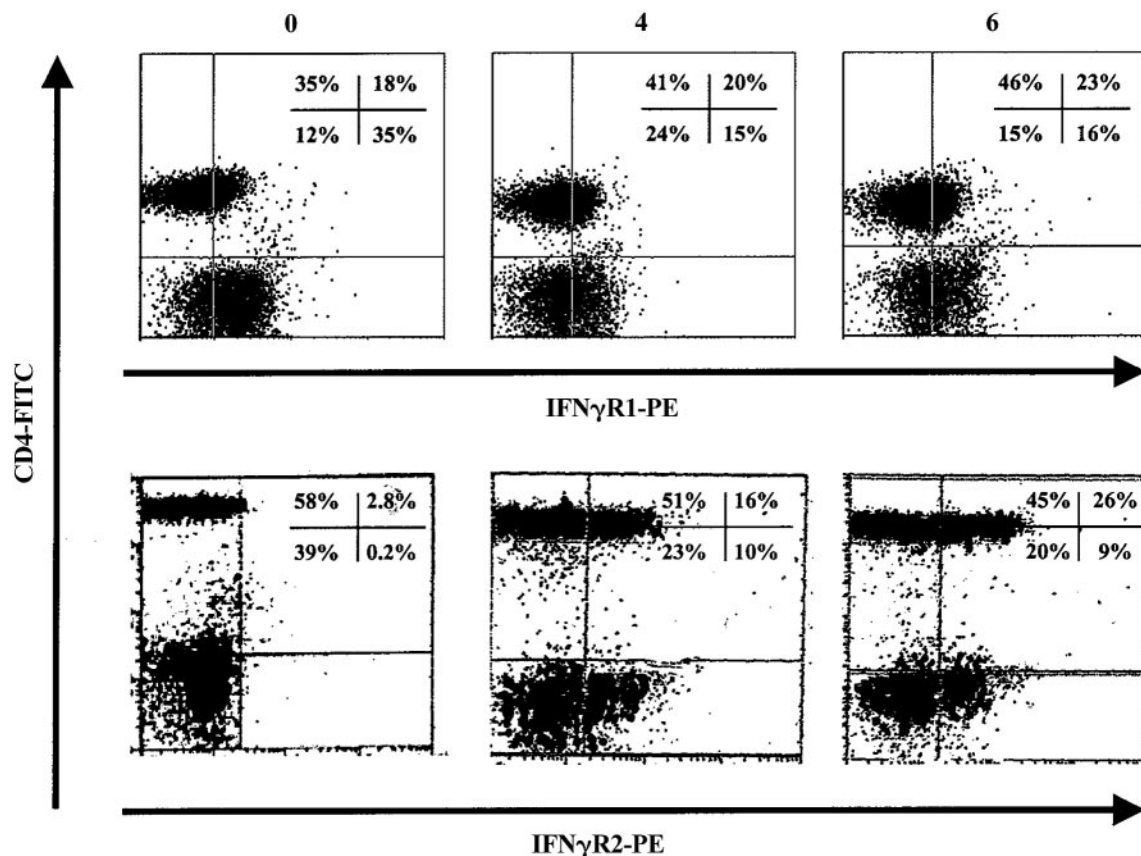


Fig. 8. Kinetics of IFN- γ R1 and IFN- γ R2 chain expression (at time 0 and after 4 and 6 days of culture) on CD4+ T cells from an atopic, asthmatic patient. Results of one representative experiment are shown.

cells. Thus, the results of this study suggest that activation-induced apoptosis of T lymphocytes may be reduced in the airways of allergic, asthmatic patients, as a result of the IFN- γ defect typical of the allergic, immune response. Consistent with this hypothesis, recent studies have shown that there are very few activated apoptotic T lymphocytes in the bronchial mucosa of asthmatic subjects [41] and that mitogen-stimulated peripheral blood T cells of asthmatic subjects express Fas on their surface but do not undergo apoptosis after its ligation [23]. In line with this latter observation, in this study, we found that a Fas-agonistic mAb did not inhibit allergen-induced T cell proliferation. Together, these results suggest that the resistance of activated T cells from asthmatic patients to Fas-induced apoptosis reflects impaired transduction of the Fas signal and that IFN- γ restores the Fas/FasL apoptotic pathway, up-regulating Fas and FasL on the T cell surface.

The hypothesis that the defective down-regulation of the allergen-specific, immune response in atopic, asthmatic patients is a result of a low level of IFN- γ production is further supported by the observation that the neutralization of endogenous IFN- γ did not significantly affect the proliferative response, apoptosis, or Fas/FasL expression of allergen-activated Th2 cells. This is consistent with our previous results in T cell clones showing that anti-IFN- γ mAb did not affect the low percentage of apoptosis displayed by Th2 clones [29]. This hypothesis is also endorsed by a recent study in a mouse model of asthma [42], showing that the administration of IL-12 re-

duced ovalbumin-induced, pulmonary eosinophilia and CD4+ T cell infiltration and resulted in increased IFN- γ production and enhanced apoptosis of CD4+ T cells in allergic airway infiltrates.

IL-4 inhibits the apoptosis of cytokine-deprived, activated T cells in vitro by up-regulating Bcl-2 and Bcl-xL [43]. Therefore, the effects of IL-4 and IFN- γ on T cell apoptosis appear to be dependent on different mechanisms. In the present study, IL-4 did not abolish the inhibitory effect of IFN- γ on the proliferation of allergen-stimulated PBMCs. However, we cannot exclude the possibility that the decrease in IFN- γ levels and the release of IL-4 by allergen-responsive T cells are involved in defective apoptosis.

In conclusion, this study shows that exogenous IFN- γ inhibits the allergen-induced proliferation of PBMCs from atopic, asthmatic patients by inducing Fas/FasL and the apoptosis of activated CD4+ T cells. The IFN- γ defect associated with the allergic, immune response may thus reduce the apoptosis of allergen-activated lymphocytes in the airways of allergic, asthmatic patients and contribute to the persistence of airway inflammation in asthma.

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REFERENCES

- Howarth, P. H., Bradding, P., Montefort, S., Peroni, D., Djukanovic, R., Carroll, M. P., Holgate, S. T. (1994) Mucosal inflammation and asthma. *Am. J. Respir. Crit. Care Med.* **150**, S18–S22.
- Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., Godard, P., Michel, F. B. (1990) Eosinophilic inflammation in asthma. *N. Engl. J. Med.* **323**, 1033–1039.
- Vignola, A. M., Campbell, A. M., Chanez, P., Bousquet, J., Paul-Lacoste, P., Michel, F. B., Godard, P. (1993) HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. *Am. Rev. Respir. Dis.* **148**, 689–694.
- Kay, A. B. (1992) "Helper" (CD4+) T cells and eosinophils in allergy and asthma. *Am. Rev. Respir. Dis.* **145**, S22–S26.
- Busse, W. W., Coffmann, R. L., Gelfand, E. W., Kay, A. B., Rosenwasser, L. Y. (1995) Mechanisms of persistent airway inflammation in asthma: a role of T-cells and T-cell products. *Am. J. Respir. Crit. Care Med.* **152**, 388–393.
- Corrigan, C. J., Kay, A. B. (1990) CD4 T lymphocyte activation in acute severe asthma: relationship to disease severity and atopic status. *Am. Rev. Respir. Dis.* **141**, 970–977.
- De Rose, V., Rolla, G., Bucca, C., Ghio, P., Bertoletti, M., Baderna, P., Pozzi, E. (1994) Intercellular adhesion molecule-1 is upregulated on peripheral blood T lymphocyte subsets in dual asthmatic responders. *J. Clin. Invest.* **94**, 1840–1845.
- Wilson, J. W., Djukanovic, R., Howarth, P. H., Holgate, S. T. (1992) Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. *Am. Rev. Respir. Dis.* **145**, 958–960.
- Robinson, D. S., Bentley, A. M., Hartnell, A., Durham, S. R., Kay, A. B. (1993) Activated memory T helper cells in bronchoalveolar lavage from atopic asthmatics. Relationship to asthma symptoms, lung function and bronchial responsiveness. *Thorax* **48**, 26–32.
- Azzawi, M., Bradley, B., Jeffery, P. K., Frew, A. J., Wardlaw, A. J., Knowles, G., Assoufi, B., Collins, J. V., Durham, S., Kay, A. B. (1990) Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis.* **142**, 1407–1413.
- Romagnani, S. (1994) Lymphokine production by human T cells in disease states. *Annu. Rev. Immunol.* **12**, 227–257.
- Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R., Kay, A. B. (1992) Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* **326**, 298–304.
- Del Prete, G. F., De Carli, M., D'Elis, M. M., Maestrelli, P., Ricci, M., Fabbri, L., Romagnani, S. (1993) Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur. J. Immunol.* **23**, 1445–1449.
- Kay, A. B., Ying, S., Varney, V., Caga, M., Durham, S. R., Moqbel, R., Wardlaw, A. J., Hamid, Q. (1991) Messenger RNA expression of the cytokine gene cluster interleukin 3 (IL-3), IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor in allergen-induced late-phase cutaneous reactions in atopic subjects. *J. Exp. Med.* **173**, 775–778.
- Yssel, H., Johnson, K. E., Schneider, P. V., Videman, J., Terr, A., Kasteleien, R., De Vries, J. E. (1992) T cell activation-inducing epitopes of the house dust mite allergen Der p1. Proliferation and lymphokine production patterns by Der p1-specific CD4⁺ T cell clones. *J. Immunol.* **148**, 738–745.
- Parronchi, P., Macchia, D., Piccini, M. P., Biswas, P., Simonelli, C., Maggi, E., Ricci, M., Ansari, A. A., Romagnani, S. (1991) Allergen and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA* **88**, 4538–4542.
- Haslett, C. (1992) Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clin. Sci.* **83**, 639–648.
- White, E. (1996) Life, death and pursuit of apoptosis. *Genes Dev.* **10**, 1–15.
- Kabelitz, D., Pohl, T., Pechhold, K. (1993) Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today* **14**, 338–339.
- Lynch, D., Ramsdell, F., Alderson, M. L. (1995) Fas and FasL in the homeostatic regulation of immune responses. *Immunol. Today* **16**, 569–574.
- Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W. R., Lynch, D. H., Ware, C. F., Green, D. (1995) Cell autonomous Fas (CD95)/Fas-ligand interaction mediated activation-induced apoptosis in T-cell hybridomas. *Nature* **373**, 441–444.
- Ju, S., Panka, D. J., Cui, G., Ettinger, R., El-Khatib, M., Sherr, D. H., Stanger, B. Z., Marshak-Rothstein, A. M. (1995) Fas (CD95)/FasL interaction required for programmed cell death after T-cell activation. *Nature* **373**, 444–448.
- Jayaraman, S., Castro, M., O'Sullivan, M., Bragdon, M. J., Holtzman, M. J. (1999) Resistance to Fas-mediated T cell apoptosis in asthma. *J. Immunol.* **162**, 1717–1722.
- Novelli, F., Di Pierro, F., Francia di Celle, F., Bestini, S., Affaticati, P., Garotta, G., Forni, G. (1994) Environmental signals influencing expression of IFN- γ receptor on human T cells control whether IFN- γ promotes proliferation or apoptosis. *J. Immunol.* **152**, 496–504.
- Dalton, D. K., Haynes, L., Chu, C. Q., Swain, S. L., Wittmer, S. (2000) Interferon γ eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. *J. Exp. Med.* **192**, 117–122.
- Dalton, D. K., Pitts-Meek, S., Keshav, S., Figari, I. S., Bradley, A., Steward, T. A. (1993) Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* **259**, 1739–1742.
- Schijns, V. E., Haagmans, B. L., Rijke, E. O., Huang, S., Aguet, M., Horzinek, M. (1994) IFN- γ receptor-deficient mice generate antiviral Th1-characteristic cytokine profiles but altered antibody responses. *J. Immunol.* **153**, 2029–2037.
- Oyaizu, N., McCloskey, T. W., Than, S., Hu, R., Kalyanaraman, S. V., Pahwa, S. (1994) Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon- γ and tumor necrosis factor- α secretion. *Blood* **84**, 2622–2631.
- Novelli, F., D'Elis, M. M., Bernabei, P., Ozmen, L., Rigamonti, L., Almerigogna, F., Forni, G., Del Prete, G. (1997) Expression and role in apoptosis of the α - and β -chains of the IFN- γ receptor on human Th1 and Th2 clones. *J. Immunol.* **159**, 206–213.
- Garotta, G., Ozmen, L., Fountoulakis, M., Dmbic, Z., Van Loon, A. P. G., Stuber, D. (1990) Human interferon- γ receptor: mapping of epitopes recognized by neutralizing antibodies using native and recombinant receptor proteins. *J. Biol. Chem.* **265**, 6908–6915.
- Novelli, F., Bernabei, B., Ozmen, L., Rigamonti, L., Allione, A., Pesta, S., Garotta, G., Forni, G. (1996) Switching on the proliferation or apoptosis of activated human T lymphocytes by IFN- γ is correlated with the differential expression of the α - and β -chains of its receptor. *J. Immunol.* **157**, 1935–1943.
- Schmid, I., Uittenbogaart, C. H., Giorgi, J. V. (1991) A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry* **12**, 279–285.
- Weersink, E. J. M., Potsma, D. S., Aalbers, R., De Monchy, J. G. R. (1994) Early and late asthmatic reaction after allergen challenge. *Respir. Med.* **88**, 103–114.
- Graziou, C., Carroll, M., Montefort, S., Teran, L., Howarth, P. H., Holgate, S. T. (1996) Inflammatory and T-cell profile of asthmatic airways 6 hours after local allergen provocation. *Am. J. Respir. Crit. Care Med.* **153**, 515–520.
- Krug, N., Tschermig, T., Holgate, S. T., Pabst, R. (1998) How do lymphocytes get into the asthmatic airways? Lymphocyte traffic into and within the lung in asthma. *Clin. Exp. Allergy* **28**, 10–18.
- Ramsdell, F., Seaman, M. F., Miller, R. E., Picha, K. S., Kennedy, M. K., Lynch, D. H. (1994) Differential ability of Th1 and Th2 cell to express Fas ligand and to undergo activation-induced apoptosis. *Int. Immunol.* **6**, 1545–1553.
- Suda, T., Okazaki, Y., Naito, Y., Noyota, T., Arai, N., Ozaki, S., Nakao, K., Nagata, S. (1995) Expression of the Fas ligand in cells of T cell lineage. *J. Immunol.* **154**, 3806–3813.
- Watanabe-Fukunaga, R., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A., Nagata, S. (1992) The cDNA structure, expression and chromosomal assignment of the mouse Fas antigen. *J. Immunol.* **148**, 1274–1279.
- Badley, A. D., Dockrell, D., Simpson, M., Schut, R., Lynch, P., Leibson, P., Paya, C. V. (1997) Macrophage-dependent apoptosis of CD4⁺ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. *J. Exp. Med.* **185**, 55–64.

40. Dockrell, D. H., Badley, A. D., Villacian, J. S., Heppekmann, C. J., Algeciras, A., Ziesmen, S., Yagita, H., Lynch, D. H., Roche, P. C., Leibson, P. J., Paya, C. V. (1998) The expression of Fas ligand by macrophages and its upregulation by human immunodeficiency virus infection. *J. Clin. Invest.* **101**, 2394–2405.
41. Vignola, M. A., Chanez, P., Chiappara, G., Siena, L., Merendino, A., Reina, C., Gagliardo, R., Profita, M., Bousquet, J., Bonsignore, G. (1999) Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in the mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J. Allergy Clin. Immunol.* **103**, 563–573.
42. Kodama, T., Kuribayashi, K., Nakamura, H., Fujita, T., Takeda, K., Dakhama, A., Gelfand, E. W., Matsuyama, T., Kitada, O. (2003) Role of interleukin-12 in the regulation of CD4⁺ T cell apoptosis in a mouse model of asthma. *Clin. Exp. Immunol.* **131**, 199–205.
43. Akbar, A. N., Borthwick, N. J., Wickermasinghe, R. G., Panayiotidis, P., Pilling, D., Bofill, M., Krajewski, S., Reed, J. C., Salmon, M. (1996) Interleukin-2 receptor common γ -chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *Eur. J. Immunol.* **26**, 294–299.