

Co-Stimulation-Induced Release of Pro-Inflammatory Cytokine Interleukin-8 by Allergen-Specific T Cells

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Chemokines, which include interleukin (IL)-8, are a family of pro-inflammatory molecules with potent chemoattractant activity on neutrophils, as well as other cell types. IL-8 can be recovered from many inflammatory sites. To test the hypothesis that Th2-type allergen-specific T cells, known to be the main cell type governing the allergic inflammation, are a source of IL-8 and to investigate whether IL-8 release is influenced by the nature of the *in vitro* mitogenic or co-mitogenic stimulation, cypress-specific T-cell clones (TCC) were generated from five allergic subjects during *in vitro* seasonal exposure to the allergen. Purified cypress extract was produced directly from freshly collected pollen and used for *in vitro* stimulation of PBMC bulk cultures. After 5 days priming and a further 7 day period of IL-2-driven cell expansion, monoclonal antibodies to CD3, CD2 and CD28 were adopted for *in vitro* restimulation of allergen-specific cell lines or, subsequently, secondary established TCC. The induction of apoptosis was detected by propidium iodide (PI) cytofluorimetric assay. Basal and co-stimulation-induced IL-8 production was measured by an ELISA method. Both cypress-specific T-cell lines and TCC secreted appreciable amounts of IL-8. By cross-linking T-cell lines or Th2 CD4⁺ TCC with CD3, CD2 or CD28 MoAbs, the authors observed a great stimulation-induced IL-8 secretion, preferentially after CD2 or combined CD2/CD28 stimulation. In addition, CD4⁺ clones released large amounts of IL-8 into culture supernatants after CD2 stimulation while undergoing programmed cell death (30–40% hypodiploid DNA profile of PI-stained cells). In contrast, CD3 crosslinking was unable to determine the release of IL-8 or the induction of apoptosis. Taken together, these results suggest that incomplete TcR engagement by allergen may lead to the secretion of pro-inflammatory cytokines with a contemporary induction of apoptosis in a significant number of target cells. This phenomenon may represent an additional way for local recruitment of neutrophils and basophils.

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INTRODUCTION

Interleukin (IL)-8 belongs to a family of pro-inflammatory molecules called chemokines [1]. The family has been divided into two subfamilies, the CXC and CC chemokines, according to whether the first two cysteine residues in a conserved motif are adjacent to each other or are separated by an intervening residue [1]. In general, CXC chemokines, which include IL-8, melanoma growth-stimulating activity and platelet factor 4, are potent chemoattractants and activators of neutrophils but not monocytes [2]. IL-8 can be recovered from inflammatory sites such as psoriatic scales [3], synovial fluid of rheumatoid joints [4] and bronchial lavage fluids of patients with respiratory distress

syndrome [1]. IL-8, like other chemokines, is produced by a broad spectrum of nucleated cell types, including T lymphocytes, monocytes/macrophages, neutrophils and endothelial cells [1]. Chemoattractants are important for both activating integrin adhesiveness and directing the migration of leucocytes within the inflammatory sites by acting at their site of production via non-covalent interactions with molecules on the vessel wall [5]. Allergic diseases, particularly bronchial asthma, often display the typical pathological findings of inflammation, including the recently described neutrophil and eosinophil infiltrate that follow *in vitro* allergen challenge [6]. Gundel *et al.* have suggested that the neutrophil plays a dominant role in the late asthmatic reaction produced at 6 h by antigen challenge of *Ascaris*-sensitized

monkeys [7], and neutrophil chemotactic activity has been detected in the serum of asthmatics [8]. With these findings in mind, we tested the hypothesis that allergen-specific T cells would be source of IL-8 and showed that its release could be influenced by the nature of the *in vitro* mitogenic or co-mitogenic stimulation.

MATERIALS AND METHODS

Reagents. *Cupressus sempervirens* pollen extract was prepared as previously described [9]. Phytohaemagglutinin (PHA) was purchased from Sigma (St. Louis, MO, USA) and recombinant human IL-2 (rIL-2) from Biogen (Geneva, Switzerland). Anti-CD3 MoAb was obtained from ATCC (Rockville, MA, USA), anti-CD28 (9.3) MoAb was a gift from Dr Claudio Anasetti (Fred Hutchinson Cancer Center, Seattle, USA), anti-CD2 (T11₂ and T11₃) MoAb a gift from Dr Stuart F. Schlossman (Division of Tumor Immunology, Dana Farber Cancer Center, Boston, MA, USA).

Patients. Peripheral blood mononuclear cells (PBMC) were obtained from five adult (two male and three female, age range 18–35 years) monosensitive patients with winter (from January to March) oculorhinitis due to hypersensitivity to cypress tree pollens, as demonstrated by positive (wheal and flare lesion always > 25 mm²) cutaneous prick tests (Lofarma, Allergeni, Milan, Italy) and elevated circulating specific IgE (DPC Corp., Los Angeles, CA, USA) to cypress extracts. A polyatopic status was ruled out by clinical history, physical examination and negative prick tests to other common allergens. Blood specimens were collected during seasonal exposure to cypress pollen but before starting any symptomatic medical treatment.

Generation of allergen-specific T-cell clones. Allergen-specific T-cell clones (TCC) were obtained by a technique described previously [10]. Briefly, 0.5×10^6 PBMC per ml from the five donors were stimulated with allergen (purified cypress pollen extract, 10 µg/ml) in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 20 µM 2-mercaptoethanol, 100 U/ml penicillin, 50 µg/ml streptomycin (complete medium), and 5% human AB serum in 24-well flat-bottomed culture plates (Costar, Denmark) for 5 days. Recombinant human IL-2 (rIL-2) (25 U/ml, Biogen, Geneva, Switzerland) was then added and culturing continued for a further 7 days. Viable T-cell blasts were separated by Ficoll–Hypaque density gradient centrifugation and allergen specificity of T-cell lines assessed before cloning procedure. For this purpose, 2×10^4 T-cell blasts were incubated in triplicate cultures in the presence of 10^5 irradiated (5000 rads) autologous feeder (non-T PBMC) cells plus 10 µg/ml cypress pollen extract in 96-well round-bottomed microtitre plates (Nunc, Nunc, Denmark) for 48 h at 37°C in a humidified atmosphere of 5% CO₂. After pulsing for 16 h with 0.5 µCi ³H-thymidine (Amersham, UK) radionuclide uptake was measured by scintillation counting. Responses were considered positive when the stimulation index (ratio between the mean cpm

obtained in triplicate cultures with autologous irradiated PBMC plus allergen and the mean cpm obtained in triplicate cultures containing PBMC alone) was >10. For cloning procedure only viable T-cell blasts from allergen-specific T-cell lines were seeded at 0.5 cells per well in 96-well round-bottomed plates (Nunc) with 10^5 irradiated autologous feeder cells, 1% PHA and rIL-2 (20 U/ml) in complete medium supplemented with 10% heat-inactivated human AB serum. Growing microcultures were then expanded at weekly intervals with 10^5 irradiated feeder cells and rIL-2. To expand established clones, T-cell blasts were restimulated every 3 weeks with 0.1% PHA and irradiated autologous feeder cells. The TCC phenotype was examined on a FACScan (Becton Dickinson, Mountain View, CA, USA) using fluorescein-isothiocyanate or phycoerythrin-conjugated anti-CD3, anti-CD4 and anti-CD8 (Immunothec, Marseille, France) MoAbs.

Quantitation of cytokine production in T-cell supernatants. T helper (Th) phenotype of allergen specific TCC was assessed by measuring IL-4 and IFN-γ secretion in supernatants of anti-CD3 (25 ng/ml) plus PMA (5 ng/ml)-stimulated cells. Th0 clones were defined by the contemporary production of both IL-4 and IFN-γ, whereas Th2 or Th1 TCC were recognized by the specific production of one of the two major cytokines. For this purpose, IL-4 kit (Advanced Magnetics Inc, Cambridge, MA, USA) and IFN-γ kit (Gibco BRL, Eggenstein, Germany), respectively, were used according to the manufacturer's instructions. For IL-8 production experiments, viable T-cell blasts from allergen-specific bulk cultures and CD4⁺ Th2-type TCC were extensively washed, incubated at 10^6 cells per ml in complete medium and then stimulated for 24 h with plastic-coated anti-CD3 (25 ng/ml) and anti-CD2 (1:400 final dilution) MoAb alone or in combination with anti-CD28 (1:200 final dilution). For the quantitation of the cytokine, a commercially available kit (Innogenetics, Haven, Belgium) was employed. Control experiments were run in parallel with unstimulated TCC. Values are expressed in pg/ml.

Evaluation of cell death by flow-cytometry with propidium iodide (PI) staining. Activation-induced death of CD4⁺ Th2-type allergen-specific TCC was analysed by the method of Nicoletti *et al.* [11] In these experiments cells were cultured with plastic-coated anti-CD3 MoAb (25 ng/ml), anti-CD2 (T11₂ and T11₃, 1:400 final dilution) alone or co-immobilized with anti-CD28 MoAb (9.3, 1:200 final dilution) in complete medium for 24 h. The forward scatter (FSC) and side scatter (SSC) of particles were simultaneously measured and all the data recorded in a Hewlett-Packard (HP 9000, model 310) computer using specific FACScan research software (Lysis II, Becton Dickinson). Cell debris were excluded from analysis by appropriately raising the FSC threshold. Control experiments were run in parallel with unstimulated (i.e. cultured in medium alone) or anti-CD28-triggered cells.

Statistical analysis. The Mann–Whitney *U*-test was adopted to evaluate mean IL-8 secretion during different experimental settings. Values of *P* below 0.05 were considered as significant.

RESULTS

Long-term bulk cultures of PBMC primed basally with cypress pollen were tested for ongoing IL-8 secretion at various time intervals (every 3 days) during culture periods (12 days). The cytokine production ranged from 76.5 to 95.2 pg/ml. However, when cells were washed and deaths eliminated by density gradient centrifugation at the end of the 21 day culture, anti-CD3 re-stimulation of viable PBMC with or without co-stimulatory molecules gave different results (Fig. 1). Co-engagement of CD3 and CD28 surface molecules enhanced significantly the IL-8 production above the basal levels (135 ± 25 pg/ml versus 78 ± 15 pg/ml, $P < 0.05$). In addition, CD2 cross-linking in combination with CD28 significantly increased the production of this chemokine about threefold above the basal levels ($P < 0.05$) and twofold those reached by stimulation with CD2 or CD28 alone ($P < 0.05$). However, because the PBMC cultures are contaminated with some percentage of monocytes and/or other non-T cells, there is a possibility that IL-8 detected was derived not only from T cells, but also from such contaminating cells. Previous reports established that monocytes are the major source of IL-8 produced by peripheral blood mononuclear cells [3]. To rule out this possibility, experiments with TCC were performed.

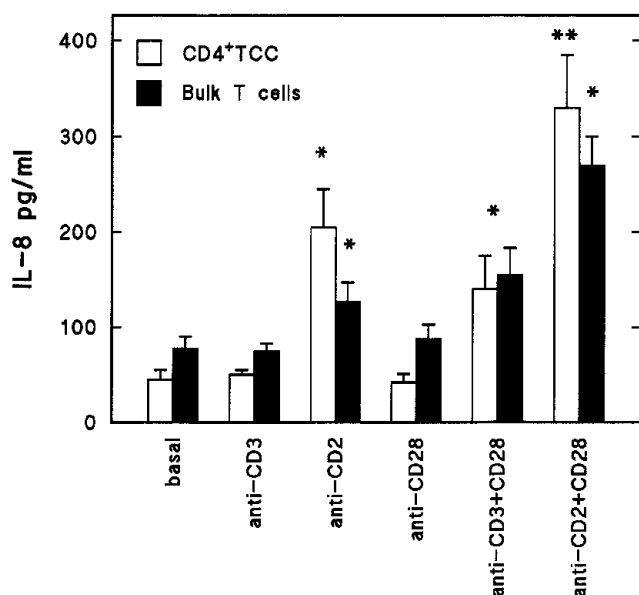


Fig. 1. The IL-8 secretion pattern of both cypress-specific T-cell lines and clones can be modified by MoAb stimulation. Culture plates were coated with goat anti-mouse IgG and subsequently with MoAb directed against TcR or other co-stimulatory molecules. Significant rises, with respect of basal values, in IL-8 secretion were noted for bulk T-cell co-stimulated with anti-CD3 and anti-CD2 plus anti-CD28 ($*P < 0.05$) and for TCC stimulated with either anti-CD2 alone ($*P < 0.01$) or co-stimulated with anti-CD3 and anti-CD2 in combination with anti-CD28 ($*P < 0.05$ and $**P < 0.005$, respectively). Values are represented as means $\pm$ SD of all experiments performed on all bulk cultures and 120 allergen-specific CD4⁺ TCC.

Clonal efficiency was always greater than 65% among all subjects tested. A total amount of 205 CD4⁺ and 55 CD8⁺ clones were recovered. The vast majority of CD4⁺ ones were of the Th2-type cytokine profile, i.e. their IFN- γ secretion was below 10 U/ml. Cypress-specific TCC produced varying amounts of IL-8 (ranging from 55 to 68 pg/ml), irrespective of their phenotype (CD4⁺ or CD8⁺) and functional activity (180 CD4⁺ Th2, 9 CD4⁺ Th1, 16 CD4⁺ Th0, 5 CD8⁺ Th2, 10 CD8⁺ Th1 and 40 CD8⁺ Th0). A total number of 120 Th2 CD4⁺ TCC, randomly selected from all cultures, were employed for testing stimulation-induced IL-8 production. As shown in Fig. 1, CD4⁺ TCC could be induced to secrete large amounts of IL-8, in the absence of APC, by co-engagement of both CD3 ($P < 0.05$) and CD2 ($P < 0.005$) with CD28. In addition, cross-linking of CD2 alone was able to enhance cytokine production ($P < 0.01$). PI staining of CD4⁺ TCC during the same experiments showed a progressive loss of cell viability with cytofluorimetric pattern of apoptotic cell death after CD2 and CD2+CD28 stimulation (Fig. 2). In contrast, triggering of TCC with anti-CD3 MoAb gave the same percentages of hypodiploid DNA contents as unstimulated cells.

DISCUSSION

The mechanism through which T lymphocytes, and particularly CD4⁺ cells, secrete IL-8 is poorly understood. Peripheral blood T lymphocytes secrete this cytokine both spontaneously and after mitogen stimulation irrespective of their phenotype (CD4⁺ or CD8⁺) [12]. Many other cytokines are involved in the activation of the IL-8 gene and IL-1 and TNF rapidly and dramatically induce IL-8 mRNA in various cell types [13]. However, the activation of T lymphocytes leading to proliferation and cytokine gene expression and secretion requires two independent signals. The first one involves stimulation of the TcR and associated CD3 complex molecules in an MHC-restricted fashion. A second signal essential to T-cell activation, the co-stimulatory signal, is delivered when the CD28 cell surface glycoprotein interacts with any of the B7 family protein expressed on APCs [14]. Co-stimulated cells react both by increasing the levels of lymphokine gene transcription and by stabilizing selected mRNA [15]. However, proper antigen stimulation performed on already activated T-cell lines or TCC has led to different patterns of IL-8 secretion, as reported by Schall *et al.* on TCC during the induction phase of T-cell energy [16]. It is worth noting that CD3 cross-linking, in our *in vitro* system, was less effective than other accessory molecules, like CD2, in promoting IL-8 secretion, unless used in combination with CD28. Similar results had been reported recently by Wechsler and co-workers on *in vitro* activated normal T cells and Jurkat T-cell line [17]. However, in these experiments the maximal IL-8 production was achieved by simultaneous treatment of T cells with PMA in combination with CD3 or CD28 MoAbs. These data suggest that PKC activation and raised levels of cytosolic free calcium are important co-factors in the activation of IL-8 mRNA and IL-8 secretion. The strong secretory effect exerted by anti-CD2

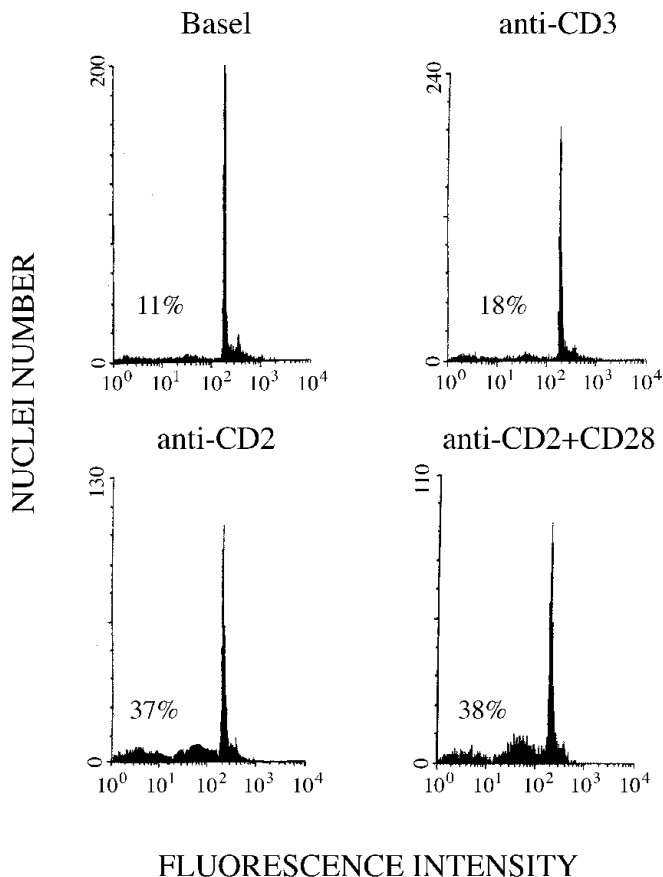


Fig. 2. The propidium iodide staining profile of CD⁺ TCC shows that CD2 stimulation is able to induce programmed cell death in target cells. Control cultures stimulated with anti-CD3 displayed a percentage of apoptotic cells similar to that of unstimulated cultures. Co-engagement of CD28 in CD2-triggered TCC cannot further modify the propidium iodide profiles. The experiment is representative of 25 performed in duplicate.

stimulation on IL-8 production by allergen-specific CD4⁺ TCC is also of considerable interest. The CD2 antigen is known to be a co-stimulatory molecule that, together with cell-adhesion properties, acts as an amplifier of Ca²⁺ signals that, if properly delivered in the context of other molecular systems (e.g. CD3 or MHC class II antigens), is essentially devoted to the polyclonal co-stimulatory recruitment of a large cellular repertoire [18].

However, in certain lymphocyte subpopulations [19] or antigen-specific TCC [20] the CD2 stimulation is able to induce apoptosis. Similarly, significant IL-8 secretion by peripheral blood eosinophils was detected after stimulation with calcium ionophore, a substance known to be a potent inducer of endonuclease activation [21]. However, the possibility exists that these phenomena (apoptosis and IL-8 secretion) cannot involve the same cell [22]. In other words, perhaps depending on the avidity and signal-transducing properties of engaged CD2 receptor either alone or in combination with CD28, among the same clonal population a proportion of cells could be induced to secrete IL-8 while the others can be triggered to die. The overall

secreted IL-8 could then stimulate neutrophil infiltration, basophil chemotaxis and degranulation. Such a mechanism may shed some light on the complex way by which allergens interact with the responding cells and trigger the inflammatory response.

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