

## T-Cell Response to Milk Proteins in Atopic and Nonatopic Donors

P. Parronchi<sup>a</sup>, S. Sampognaro<sup>a</sup>, L. Giannarini<sup>a</sup>, A. Ruffilli<sup>b</sup>, A. Chersi<sup>c</sup>,  
K. Miller<sup>d</sup>, E. Maggi<sup>a</sup>, S. Romagnani<sup>a</sup>

<sup>a</sup> Clinical Immunology Department, University of Florence;

<sup>b</sup> Istituto Internazionale di Genetica e Biofisica, Napoli, and

<sup>c</sup> Center of Experimental Research, Regina Elena Cancer Institute, Rome, Italy;

<sup>d</sup> BIBRA Toxicology International, Carshalton, UK

### Introduction

Atopy is a genetically determined disorder characterized by increased IgE antibodies to certain groups of ubiquitous antigens that can stimulate the immune response after inhalation or ingestion, and perhaps after penetration through the skin (allergens). IgE antibodies are able to bind to high-affinity (type I) Fcε receptors (FcεRI) of mast cells/basophils and allergen-induced FcεRI cross-linking triggers the release of vasoactive mediators, chemotactic factors and cytokines that are responsible for the allergic cascade. In addition, eosinophils also appear to be involved in the pathogenesis of allergic reactions, since they usually accumulate in the sites of allergic inflammation and their toxic products significantly contribute to the induction of tissue damage. The linking of the events leading to the allergic reaction has remained unclear since to the demonstration of existence of distinct subsets of CD4+ helper T (Th) cells, based on their profile of cytokine secretion. At least three different subsets of Th cells have been described in both mouse and man: Th1 cells that produce interleukin (IL)-2, interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-β; Th2 cells that produce IL-4, IL-5 and IL-10; Th0 cells that produce both Th1- and Th2-type cytokines [1, 2]. IL-3, GM-CSF and TNF-α are variably produced by all Th subsets. It is of note that IL-3, IL-4 and IL-10 are growth factors for mast cells and IL-5 is a selective activating and differentiating factor for eosinophils [1, 2]. Finally, it has clearly been shown that IgE synthesis results from the collabora-

tion between Th2 cells and B cells [3]. To this end, Th2 cells provide B cells with at least two signals: the former is delivered by IL-4; the latter is represented by a T-B cell-to-cell physical interaction, occurring between the CD40L expressed on the activated Th cell and the CD40 molecule constitutively expressed on the B cell [4]. Recently, it has been demonstrated that another cytokine, IL-13, is also able to induce germ line  $\epsilon$  expression [5], but its production does not seem to be restricted to the Th2 subset. Based on these findings, Th2 cell is an excellent candidate to explain why the mast cell/eosinophil/IgE-producing B cell triad is involved in the pathogenesis of allergy.

### **Th2 Cells Recognizing Allergen Epitopes Play a Central Role in the Pathogenesis of Allergic Reaction**

The central role of Th2 cells in the pathogenesis of allergic reaction has been recently clarified.

The analysis of lymphokine production by allergen-specific T lymphocytes has shown that, in contrast to T-cell clones specific for bacterial antigens that showed a prevalent Th1/Th0 phenotype, the great majority of allergen-specific CD4<sup>+</sup> T-cell clones derived from peripheral blood lymphocytes (PBL) of atopic donors expressed a Th2/Th0-like phenotype, with high production of IL-4 and IL-5 and no, or low production of IFN- $\gamma$  [6, 7].

On the other hand, evidence that Th2-like cells accumulate in target organs in different allergic disorders has been provided by using either cloning or in situ hybridization techniques (ISHT). The majority of T-cell clones generated from the conjunctival infiltrates of patients with vernal conjunctivitis were found to develop into Th2 clones [8]. Using ISHT, cells showing mRNA for Th2, but not Th1, lymphokines were found at the site of late phase reactions in skin biopsies from atopic patients, in mucosal bronchial biopsies or bronchoalveolar lavage (BAL) from asthma [9–11], and after local allergen challenge in nasal mucosa of allergen-induced rhinitis [12].

We have also provided evidence that allergen-challenge results in the activation and recruitment of allergen-reactive Th2 cells in target organs. In fact, biopsy specimens were obtained from the bronchial or nasal mucosa of patients with grass pollen-induced asthma or rhinitis 48 h after positive bronchial or nasal provocation test with the relevant allergen. Proportions ranging from 14 to 22% of T-cell clones derived from stimulated airway mucosae of grass-allergic patients appeared to be specific for grass allergens and most of them exhibited a definite Th2 profile [13]. Likewise, high proportions of *Dermatophagoides pteronyssinus* (DP)-specific Th2-like CD4<sup>+</sup> T-cell clones were generated from the intact skin of patients with atopic dermatitis (AD) taken after contact chal-

lenge with DP, suggesting that percutaneous sensitization to aeroallergens may play a triggering role in the induction of skin lesions in patients with AD [14, 15].

Very recently, a marker for Th cells inducible to the production of Th2-type cytokines was identified in our laboratory by assessing large panels of human CD4+ T-cell clones with established Th1, Th2 or Th0 profiles [16]. Th1 clones expressed poor or no CD30 mRNA and showed low or undetectable expression of surface CD30, whereas Th2 clones consistently expressed both CD30 mRNA and surface CD30 [17]. CD30 expression was then evaluated in circulating CD4+ T cells from 6 atopic grass pollen-sensitive patients and 6 nonatopic controls before and during the seasonal exposure to grass pollens. No CD4+CD30+ cells were detected in any of the nonatopic donors or the atopic patients examined before the grass pollination season, whereas 4 out of the 6 grass-sensitive donors examined during the pollination period showed small proportions of circulating CD4+CD30+ cells. CD4+ T cells from these patients were fractionated into CD30+ and CD30- cells by sorting with anti-CD30 antibody and cell fractions were then assessed for their ability to produce type 1 (IFN- $\gamma$  and TNF- $\beta$ ) or type 2 (IL-4 and IL-5) cytokines and to proliferate in response to *Lolium perenne* group 1 (Lol p 1) allergen. Only CD30+ cells proliferated in response to Lol p 1 and exhibited the ability to produce IL-4 and IL-5, whereas production of both IFN- $\gamma$  and TNF- $\beta$  was prevalent in the CD30- cell fraction [17]. These findings clearly demonstrate that grass allergen-reactive CD4+CD30+ T cells inducible to the production of Th2 cytokines can circulate in the peripheral blood of grass-sensitive patients during the in vivo natural exposure to allergen.

### Signals Regulating the Th2 Cell Development

The preferential development of allergen-reactive Th2 cells in atopic subjects have not yet been completely clarified. Accounting with a bulk of evidence a possible role on Th2 development of antigen-presenting cells (APC), of the T-cell repertoire and of soluble factors present in the microenvironment at the time of allergen presentation has been proposed.

It is likely that Langerhans' cell (LC) present in the skin, as well as dendritic cells (DC) in the respiratory mucosa, represent the primary site of contact between the immune system and allergens. LC and DC are probably involved in driving allergen to draining lymph nodes where allergen presentation to allergen-specific CD4+ T cells occurs. It has been recently shown that B7.1 and B7.2 molecules present on APC can give co-stimulatory signals able to induce a Th1 or a Th2 switch, respectively. Some data suggest that atopic patients with asthma may have higher numbers of intraepithelial DC than nonasthmatic subjects and

that these cells pulsed with allergen can induce T-cell activation and release of IL-4 and IL-5 [18].

The role of T-cell repertoire in determining the development of Th1 or Th2-type responses is controversial. In mice infected with *Leishmania major* both Th1 and Th2 cells possessing the same repertoire and recognizing the same peptide have been demonstrated [19]. However, evidence for the pivotal role of specific V $\beta$ -expressing T-cell subsets in the stimulation of IgE production and increased airways responsiveness induced by ragweed allergen has been reported [20]. Thus, it cannot be excluded that the recognition of allergen provides a signal(s) that driving T cells towards the production of Th1- or Th2-type cytokines.

The role of hormones in promoting the differentiation of Th cells or in favoring the shifting of already differentiated Th cells from one to another cytokine profile has also been suggested. Glucocorticoids enhance Th2 activity, and synergizes with IL-4, whereas dehydroepiandrosterone sulfate (one of several steroids that circulate as inactive prohormones, becoming activated in target organs) enhance Th1 activity [21]. Another major prohormone, 25-hydroxy-cholecalciferol (25(OH)vitamin D<sub>3</sub>) may have a reverse effect on the Th1/Th2 balance. There is intense conversion of 25(OH)vitamin D<sub>3</sub> to 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> (calcitriol) which is able to decrease output of IL-2 and IFN- $\gamma$  and when administered peripherally with antigen, evokes a Th2 pattern of response localized to mucosal surfaces [21]. Finally, progesterone favors the in vitro development of human Th cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established human Th1 clones [22]. Thus, increasing evidence is accumulating to suggest that hormones and peripherally activated prohormones may regulate the Th1/Th2 balance.

The clearest examples of factors affecting the differentiation pathways of human Th cells appear to be cytokines released by APC and/or other cell types at the time of antigen presentation [23]. Thus, IFN- $\alpha$ , IL-12 and TGF- $\beta$  produced by macrophages and B cells particularly in response to intracellular bacteria play an important role in the induction of Th1 expansion, as well as IFN- $\gamma$ , produced mainly by T cells and NK cells, also promotes the differentiation of Th1 cells, both in vitro and in vivo. Thus IL-12, which is a powerful IFN- $\gamma$  inducer, appears to be the most important natural initiator of Th1 responses [23].

IL-1 is a selective co-factor for the growth of some murine Th2 clones [24] and can favor the in vitro development of human Th2-like clones [25]. However, most striking is the requirement for IL-4 for maturation of Th cells into Th2 cells. In both murine and human systems, IL-4 appears to be the most dominant factor in determining the likelihood for Th2 polarization in cultured cells [23]. Accordingly, IL-4-gene-targeted mice fail to generate mature Th2 cells in vivo and to produce IgE antibodies [26], suggesting that early IL-4 production by

another cell type must be involved. Possible candidates include a still uncharacterized T-cell subset [27], a double negative (CD4-CD8-)  $\alpha\beta$ TCR early thymic emigrant T-cell subset [28], as well as mast cells and basophils, which have been shown to release stored IL-4 in response to Fc $\epsilon$ R triggering [29]. However, mast-cell-deficient mice develop normal Th2 responses [30]. Thus, IL-4 production by mast cells triggered by antigen-IgE antibody immune complexes may play a role in amplifying secondary responses to parasites, but cannot account for the Th2 development in primary immune responses.

However, the fact that allergens induce Th2-type responses only in selected people suggests that atopic individuals would have genetic dysregulation in the production of IL-4 and/or of cytokines exerting regulatory effects on the development and/or function of Th2 cells.

### **Evaluation of T-Cell Response to Milk Allergens**

Milk allergy is a peculiar form of allergic disease in which IgE antibodies are directed to proteins which are introduced daily. The most important question arising on the pathogenesis of this disease is which molecular and cellular events lead to oral tolerance towards milk proteins in nonatopic individuals during childhood and which impairment of the immune system is able to modify such mechanism in allergic patients.

This study has been devoted to explore the immunodominant T-cell epitopes of milk proteins in atopic and in nonatopic individuals and to evaluate the possible role of allergen-specific CD8 cells in the induction and maintenance of oral tolerance to milk proteins. We first evaluated the capacity of peripheral blood mononuclear cells (PBMC) derived from healthy and atopic donors to proliferate in response to purified proteins derived from bovine milk. To this end,  $\alpha$ -lactalbumin (LA),  $\beta$ -lactoglobulin (variant A and B, LGA and LGB) and  $\alpha$ - $\beta$ -casein ( $\alpha$ C and  $\beta$ C) were used. PBMC from 5 normal donors (negative history for allergy, normal values of total IgE and absence of specific IgE for milk), 4 allergic patients (positive history for adverse reactions after milk ingestion, increased levels of total IgE and presence of specific IgE towards milk proteins) and 2 atopic patients (negative history for food allergy, presence of reactions towards inhaled allergens, increased levels of total IgE but absence of specific IgE towards milk) were tested against a large panel of concentrations of different allergens. A consistent proliferation was obtained from all the patients and the response was irrespective to the allergic or normal status of donors, to the nature of the allergen and the doses of allergen used. However, the T-cell response to LA was consistently higher than the one obtained with the other milk allergens. A proliferative response similar to the one obtained from adult donors

was also achieved when PBMC from cord blood of newborns were tested. These data suggest that a high proportion of Th memory cells towards milk proteins are present in the circulation of both adult and newborn subjects and that an in utero immunization to these antigens can occur. In order to define immunodominant T-cell epitopes of milk proteins and to characterize milk-reactive T cells, T-cell lines (TCL) specific LA, LGA and LGB were then generated stimulating PBMC from 8 normal donors and 5 allergic subjects for 5 days with antigens and subsequently adding rIL-2 to the cultures in order to amplify the number of specific T-cell blasts. TCL were then evaluated for their phenotype, their ability to produce IL-4 and IFN- $\gamma$  and their specificity. The analysis of the phenotype showed similar percentages of CD4+, CD8+ and CD4-/CD8- cells indicating that no preferential expression of a particular subpopulation could be seen in relation to the different antigen used and the different status (atopic or nonatopic) of the donors. After stimulation with PMA plus anti-CD3 mAb a poor or any IL-4 production was seen, while a consistent IFN- $\gamma$  production is obtained in all groups of TCL. No statistical difference in the levels of cytokine production between normal and atopic subjects was seen. Similar results were obtained when TCL were stimulated with the specific antigen in MHC-restricted conditions, suggesting that immunodominant T-cell epitopes selectively expand Th1-like cells.

TCL specific for LGB were also screened for their specificity towards four different peptides (a kind gift from Dr. M. Morgan, Norwich, UK) just defined for their ability to be recognized by LG-specific IgG or IgE antibodies, but none of the TCL showed any reactivity. Since also LG-specific CD4 T-cell clones (TCC) derived from the same TCL were unable to proliferate to the same peptides in a MHC-restricted fashion, we can hypothesize that LG-T and B-cell epitopes have not overlapping sequences.

TCC specific for milk allergens were then generated from LA, LGA and LGB-specific TCL from normal and atopic donors. TCC were screened for their specificity and subsequently analyzed for their phenotype and ability to produce cytokines. An unusual high specific clonal efficiency was obtained from normal and atopic donors when LA was used (normal subjects  $27 \pm 5\%$ , atopic donors  $31 \pm 7\%$ ). However, a lower specific clonal efficiency was seen when LGA and LGB were used to stimulate T cells, thus confirming that Th memory cells towards LA are more expanded in vivo than T cell specific for other milk proteins.

No statistical difference in the number of IL-4, IL-5 and IFN- $\gamma$  producing TCC was seen in atopic and normal subjects. On the other hand, when TCC were divided in the three main subsets of T cells, a statistically reduced number of IFN- $\gamma$  producing TCC (Th1 and Th0) was seen in atopic patients ( $< 0.05$ ). However, the great majority of LA- and LG-specific TCC showed a clear-cut Th1-like profile; no Th2 cells were derived from atopic or nonatopic subjects, thus

suggesting that in cow's milk allergenic epitopes are a minority in comparison with other antigens. Furthermore, LA-specific TCC deriving from T cells infiltrating the enteric mucosa of biopsies from two milk-sensitive patients showed a similar pattern of cytokine secretion, suggesting that no difference exists between circulating and local milk-specific T-cell response in atopic individuals.

When we analyzed the phenotype of specific TCC, we found that a consistent number of LA-specific CD8+ TCC were present. More importantly, normal donors showed a significantly higher number of LA-specific CD8+ TCC (in comparison with those derived from allergic donors,  $p < 0.001$ ) and a parallel decrease in CD4+ specific TCC ( $p < 0.0005$ ).

The presence in peripheral blood of CD8+ T cells specific for allergens is not limited to milk proteins. In fact, we have recently studied the specific response of T cells towards the extract of *Parietaria officinalis* pollen and we were able to derive a high number of specific TCC in normal and allergic donors. The analysis of TCC revealed that the majority of them were able to recognize a nonapeptide (p92) from the sequence of the allergen, showing a high homology with a sequence defined in LA protein. More importantly, the p92-specific TCC showed a clear-cut Th1-type profile of cytokine secretion. We derived TCC specific for p92 and for another non related nonapeptide from *Parietaria* sequence (defined p96) from healthy and allergic donors. P92- and p96-specific TCC showed peculiar characteristics. While p96-specific TCC were able to produce high levels of IL-4 and low (or any) concentrations of IFN- $\gamma$  both in normal and in allergic donors, different amounts of IL-4 production could be seen when we analyzed p92-specific TCC deriving from the two groups of subjects. P92-specific TCC from normal donors or subjects with mild atopy produced significantly lower amounts of IL-4 than allergic subjects affected by severe atopy ( $p < 0.001$ ). Accounting with our results on milk-specific TCC, a different phenotypic distribution of peptide-specific TCC was seen. A higher proportion of p92-specific CD8+ TCC was derived from normal donors or mild atopic subjects than from patients with severe atopy (in these cases a prevalent CD4+ response was seen;  $p < 0.01$ ).

These findings strongly suggest that a reduction of specific CD8 response towards allergens could play a crucial role in the dysregulation of IL-4 production in atopic subjects and, probably, in the breakthrough of oral tolerance towards milk proteins.

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P. Parronchi, University of Florence, Clinical Immunology Department, I-50134 Florence (Italy)