Chapter 16
PBMC-Derived T Cells

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Abstract T cell cultures are a valuable tool in food research to perform studies within the food allergy field. Their main applications aim to analyze immunological responses towards food protein antigens to gain further insights into the mechanisms responsible for the development of oral tolerance or for the triggering of food allergies. This chapter describes the main applications, isolation techniques, and culture conditions for PBMC-derived T cells. Furthermore, critical parameters of the model, together with the experimental read outs will be discussed.

Keywords T cells cultures • Food allergy • Immunomagnetic cell isolation • PBMCs • CD4+ T cells

16.1 Introduction and Origin

T lymphocytes or T cells are small (<10 μm) resting cells, which are generated in the bone marrow and migrate to the thymus where they become mature. Once matured, they enter the bloodstream and circulate to the secondary lymphoid organs, the sites of lymphocyte activation by the antigens. Actually, T cells are constantly recirculating between these organs until they encounter their specific antigen. Naïve T cells (T cells that have not yet met their specific antigen) bear antigen receptors specific for a single chemical structure, however lymphocytes in the body collectively carry millions of different receptor specificities. Only those that meet an antigen to which their receptors bind will be activated to proliferate and differentiate into effector cells (Janeway et al. 2005).

This chapter deals with T lymphocytes derived from peripheral blood mononuclear cells (PBMCs). T cells comprise, approximately, 45–70 % of PBMCs in human peripheral blood, with a count in healthy subjects in the range of 1 million cells/mL. For many years there was no function ascribed to these cells, until the 1960s, when it gradually became apparent that T lymphocytes were the key mediators

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of adaptive immunity. T cell cultures are a valuable tool in food research to perform studies within the food allergy field. Their main applications aim to analyze immunological responses towards food protein antigens to gain further insights into the mechanisms responsible for the development of oral tolerance or for the triggering of food allergies (Martino et al. 2012).

16.2 Features and Mechanisms

T cells express surface antigen receptors called T cell receptors (TCRs), which are inserted in the T cell surface and never secreted. The most common form is made up of α and β chains and it is found on about 95 % of circulating T cells. The TCR is a clonally distributed receptor, meaning that clones of T cells with different specificities express different TCRs. In the functional TCR, the α and β heterodimers are associated with a complex of four other invariant signaling chains, collectively called CD3, which are required for the cell-surface expression of the antigen binding chains and for signaling (Fig. 16.1) (Farber 2011).

There are two major types of T lymphocytes, classified according to the expression of the cell surface co-receptors CD4 and CD8, which are proteins non-covalently associated with the TCR. Their function is to signal to the T cell that the TCR complex has bound the proper antigen. $CD4^+$ T cells are generally referred as helper T cells or Th cells, because they secrete a multitude of cytokines that help or coordinate cellular and humoral immunity. $CD8^+$ T cells are generally referred as cytotoxic T lymphocytes or Tc cells. Cytotoxic T cells recognize as antigens fragments of viral proteins on the surface of virus-infected cells, which they kill by the activation of a cascade of caspases. In humans, circulating $CD4^+$ T cells outnumber $CD8^+$ T cells by approximately 2:1 (Abbas et al. 2007). This chapter will deal with the $CD4^+$ T lymphocytes, as they are the type of T lymphocytes that become activated during the allergic response.

T cell responses are initiated when a mature $CD4^+$ T cell encounters a properly activated antigen presenting cell (APC), such as a dendritic cell, in a secondary
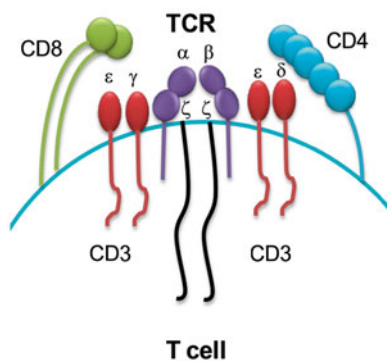

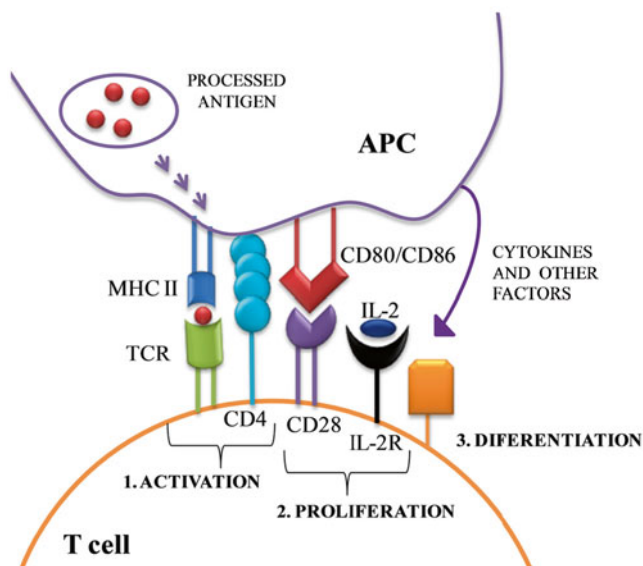**Fig. 16.1** TCR structural components (adapted from Farber 2011)

Fig. 16.2 Signals involved in the activation of naïve T cells by APC

lymphoid organ. If the APC displays the appropriate peptide ligand through the major histocompatibility complex (MHC) class II molecule, it is recognized by the TCR. This is essential for activating a naïve T cell, but even if the co-receptor CD4 is also ligated, for the T cell to proliferate and differentiate, two other stimulatory signals delivered by the APC are required. These signals are, on the one hand, the binding of two different ligands on the APC surface, designated as CD80 and CD86, to a surface molecule on the T cell, called CD28, and on the other, those involved in directing T cell differentiation into different subsets of effector T cells, the latter being mainly, but not exclusively, driven by cytokines, such as IL-6, IL-12 and TGF-β (Fig. 16.2). The CD28-dependent co-stimulation of activated T cells leads to production of IL-2 by the activated T cell themselves. Following expression of IL-2, there is also an upregulation of the third component (called α-chain) of the IL-2 receptor, also known as CD25, in addition to other regulatory molecules such as ICOS and CD40L (Farber 2011). Binding of IL-2 to its high affinity receptor promotes cell growth, whilst APCs, mainly dendritic cells generate various cytokines or express surface proteins that induce the differentiation of CD4+ T lymphocytes into cytokine producing effector cells, depending on environmental conditions.

Originally, two main types of effector CD4+ T cells, called T-helper 1 (*Th1*) and 2 (*Th2*) cells, were distinguished by the pattern of their cytokine secretion. Th1 cells secrete mainly IL-2, IFNγ and TNFα, and Th2 cells secrete IL-4, IL-13 and IL-5 (Romagnani 2000). Recently, this view has been challenged by the discovery of a new lineage of T cells characterized by their ability to secrete a proinflammatory cytokine, IL-17, and thus designated Th17 cells. This new T cell type has been related with autoimmune diseases (Jing and Dong 2013). Another subset of antigen-driven CD4+ T cells, named regulatory T cells (*Treg*), acts by inhibiting T cell

responses by the production of cytokines, such as IL10 and TGF-β and/or via cell–cell interactions (Jutel and Akdis 2011). Treg are CD4$^+$ cells that also express the α-chain of the IL-2 receptor (CD25$^+$) and the transcription factor FoxP3.

The immune response leading to food allergy is driven by two main groups of signals. The first signal favors the differentiation of naïve T cells into a Th2 phenotype, and the second comprises the Th2 induced activation of B cells (mediated by cytokines such as IL-4 and IL-13 and co-stimulatory signals) to generate allergen-specific IgE antibodies (Mayorga et al. 2013).

16.3   Applications of T Cell Cultures

The main application for T cell cultures is the *characterization of CD4+ T cell epitopes from food allergens*. The mapping of CD4$^+$ T cell epitopes contributes to a better understanding of the pathophysiology of food allergy by enabling the identification of the major peptides that target allergen-specific T cells (Prickett et al. 2011, 2013). This requires the comparison of the epitope recognition patterns of a diverse and representative sample of allergic individuals, since a given T cell epitope elicits a significant response only in subjects expressing MHC-class II molecules able to recognize and bind that particular epitope. In addition, the knowledge of the sequence of T cell epitopes that stimulate CD4$^+$ T lymphocyte responses allows the development of new forms of immunotherapy, which are safer and more effective than those using whole allergens. In this respect, peptide-based vaccines corresponding to T cell epitopes of the allergen of interest retain immunogenicity, but they are of insufficient length to cross-link allergen specific IgE on the surface of effector cells and elicit an allergic response (Worm et al. 2011).

Another important application related with the use of T cell cultures is the *study of the allergen-specific T cell responses during or after immunotherapy*. In this context, evidence has accumulated that immunotherapy treatments shift a patient's immune reaction to a specific allergen from a predominately allergic Th2 response to a Th1 response, while inducing Treg that downregulate Th2 and Th1-cells activity. The characterization of the frequency and phenotype of allergen-specific T cells is useful to understand the immunological changes subjacent to therapeutical interventions in allergic diseases (DeLong et al. 2011; Foster et al. 2011). In this context, the role of Treg producing IL-10 in allergen specific T cell tolerance and immune deviation has been studied by comparing the response of PBMCs and PBMCs depleted of CD25$^+$ in patients undergoing immunotherapy, as well as in tolerant and allergic patients, allowing the identification of potential markers that might be indicators of a favorable prognosis (Shreffler et al. 2009; Bohle et al. 2007). Similarly, allergen-specific signaling can be assessed in activated and purified CD4$^+$ T cells to test whether there is a differential expression in the neonates who subsequently develop allergic diseases (Martino et al. 2012).

In the field of food allergy, T cell gene and protein expression assays are very important for the understanding of the functional characteristics of allergen

reactive T cells. The use of PBMCs as a source of circulating T cells, that mimic effects occurring in remote target tissues of antigen exposure, presents the advantage that they can easily obtained by blood extraction and allow the study of parameters that otherwise would require more invasive methods, such a biopsy (de Mello et al. 2012). However, certain applications based on the exclusive employment of PBMC-derived T cells are limited because of the lack of APCs that are keys to the regulation of the complicated set of mechanisms that determine the immune responses, as well as of other environmental influences that play an important role in shaping the APC functions. To overcome this limitation, there are other options, such as the use of co-cultures with APCs (Frischmeyer-Guerrerio et al. 2011; Hofmann et al. 2012) or complex culture systems with other cell types, such as APCs and intestinal epithelial cells (Mileti et al. 2009). Another alternative are cell cultures from tissues of animal models of food allergy (spleen, lymph nodes, Peyer's patches) (López-Expósito et al. 2011). In any case, and because of extension limits, this chapter will only deal with T cells that do not require APCs in the culture (more information available in Chap. 17).

Because of the low frequency of allergen-specific CD4$^+$ T cells, several studies use allergen-specific CD4$^+$ T cell lines or clones (Prickett et al. 2011, 2013), while others use primary CD4$^+$ T cells, without addition of cytokines or repeated stimulation (Frischmeyer-Guerrerio et al. 2011; Hofmann et al. 2012). Although T cell lines overcome the frequency limitation, previous in vitro expansion can alter cell phenotypes or bias the results through the selection of the rapidly proliferating clones (Pascal et al. 2013).

16.4   General Protocol

16.4.1   T Cell Isolation Protocols

Because of the ease of access to peripheral blood, PBMCs, which contain T and B lymphocytes and monocytes, are the major source of human T cells used in most studies. There is a variety of available techniques for isolation and enrichment of T cells, including those based on their unique ability to bind and form rosettes with sheep red blood cells, as well as those based on differential adherence properties T cells to nylon wool. Despite their widespread use, the resulting T cell population is not very pure compared to the level of purity achieved with other procedures, and several reports suggest alterations in T cell functionality when these techniques are used (Wohler and Barnum 2009).

Human T cells can also be purified on the basis of their cell-surface display if specific antigens can be recognized by monoclonal antibodies. This is currently carried out by two main methods: fluorescence-activated cell sorting (FACS) and immunomagnetic cell separation (Martino et al. 2012; Prickett et al. 2013). FACS requires sophisticated technology, highly trained personnel; it is time consuming, expensive and may result in a significant cell loss. Conversely, immunomagnetic

separation methods are faster, relatively inexpensive and do not require state of the art technology (Lancioni et al. 2009).

Immunomagnetic separation methods are based on the attachment of small magnetizable particles to cells via antibodies. The physical basis for such separation procedures involve the coupling of antibodies to magnetic beads, which subsequently allows the rapid capture of the cells specifically recognized by these antibodies among a mixed population of cells placed in a magnetic field. There are two types of magnetic cell isolation technologies, column and tube-based. Both work on the same principle, but the strength of the magnetic field required is different because they use beads of different sizes. In particular, the *column-based* technology uses beads consisting of iron oxide and polysaccharide of, approximately 50 nm in diameter, which require a very strong magnetic field. The main disadvantages of this system (exemplified by MACS produced by Milteny Biotech) lay in its high initial and running costs, although it provides cells with high purity and optimal viability and functionality (Li et al. 2012).

The immunomagnetic *tube-based* separation system utilizes micro-sized beads that can be selected using a magnet applied to a tube. The most commonly used beads, produced by Dynal, are 4.5 μm, uniform, spherical beads, which do not have any residual magnetism outside a magnetic field. They consist of an iron-containing core surrounded by a thin polymer shell to which biomolecules may be adsorbed. These beads can be attached to cells via a coating of primary or secondary antibodies. The cells, surrounded by a "rosette" of beads, may then be separated from the unlabeled population in a magnetic field using a relatively small, but powerful magnet (Neurauter et al. 2006).

There are two main strategies for cell isolation using micro-sized beads: *positive* and *negative selection*. Positive selection can be performed using either direct or indirect approaches (Neurauter et al. 2006). Following *direct positive isolation*, the appropriate antibody-coated beads are mixed with the sample and the target cells bound to the coated beads are subsequently collected with the aid of a magnet. For *indirect positive isolation*, a cell population is exposed to saturating amounts of a primary antibody which binds to the target cells and, once the unbound antibodies are washed away, secondary-antibody coated beads bind to the primary antibodies on the surface of the target cells. For further functional applications, and in order to avoid modifications of the phenotype, the cells bound to antibody-coated beads should be detached by exposure to a soluble antiserum against Fab fragments (e.g., DETEACHaBEAD). However, surface bound antibodies may elicit the transmission of signals across the cell membrane. This can be avoided by negative isolation, which does not require attachment of antibodies to the cells of interest at any time (Neurauter et al. 2007). By *negative isolation*, the cells are selected by removing all other cell types from the sample. Generally, a cocktail of monoclonal antibodies is incubated with the sample, followed by depletion of the undesirable cells using secondary antibody-coated beads (Biddison 1998; Mayer et al. 2013). Positive and negative separation strategies can be combined in the sequential sorting of T cell subsets, such as CD25^{+} FoxP3^{+} regulatory T cells, where depletion of unwanted cells is followed by positive selection of CD25^{+} T cell population (Mayer et al. 2013).

The procedure described in this chapter is based on the indirect positive isolation of a subset of human T cells relevant to food allergy, such as CD4^{+} T cells, by anti-CD4 and anti-IgG-coated beads (Biddison 1998; Neurauter et al. 2006). This methodology is very efficient in removing target cells due to its fast cell capture kinetics, whereas the use of secondary-antibody coated beads makes it a flexible protocol to isolate any cell of interest (Neurauter et al. 2007).

16.4.2 Indirect Positive Isolation of Human CD4^{+} T

16.4.2.1 Preparation of Cells and Antibodies

PBMCs could be obtained from whole blood by density gradient of Ficoll-Hypaque (Martino et al. 2012). It is convenient to determine the approximate number of CD4^{+} T cells in the starting population of PBMCs by flow cytometry using anti-CD4 antibody. It is also helpful to establish the saturating concentration of anti-CD4 mouse-IgG monoclonal antibody to be used by flow cytometry. Generally, 1 µg/mL per 1×10^6 cells works well as a saturating concentration. However, pretitration may allow the use of down to a tenfold lower concentration.

16.4.2.2 Coating of PBMCs with CD4 Antibody

PBMCs ($\leq 200 \times 10^6$ cells) are suspended in PBS/BSA and anti-CD4 mouse IgG antibody is added (10×). After incubation during 45 min at 4 °C with gentle tilting and rotation, cells are centrifuged 10 min at 600×g at room temperature. The supernatant is discarded and the pellet resuspended in pre-cooled PBS/BSA. This step is repeated twice. Finally, washed cells are resuspended in 10 mL of pre-cooled PBS/BSA and kept on ice for 15 min.

16.4.2.3 Magnetic Beads Washing Procedure

On the basis of the number of CD4^{+} cells estimated by flow cytometry, the number of required goat anti-mouse IgG-coated magnetic beads is calculated. Five to ten magnetic beads are needed for each specific lymphocyte. Taking into account the concentration supplied by the manufacturer, the coated beads are resuspended in PBS and the desired volume of beads are transferred to a tube. PBS is added to the bead suspension and the tube is placed on a magnetic separation device for 2 min. The supernatant is then discarded by leaving the beads clinging to one side of the tube. The tube is removed from the magnet and the beads are washed following the previous step. Finally, the washed beads are resuspended in pre-cooled PBS/BSA and held on ice for 15 min.

16.4.2.4   Separation of T Cells

Anti-CD4-coated PBMCs are mixed with washed anti-IgG-coated beads and incubated for 45 min at 2–8 °C with gentle tilting and rotation. Afterwards, the tubes are placed on a vertical magnet for 2 min and the supernatant is carefully discarded. It is important not to disturb the beads that are clinging to one side of the tube. Once the tube is removed from the magnet, the beads are gently resuspended in 5 mL of Lymphocyte Culture Medium (LCM), which contains RPMI-1640 medium, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 5 % (v/v) fetal bovine serum (FBS) (heat-inactivated during 1 h at 56 °C) (Prickett et al. 2013). Then, the tube is placed on the magnet for another 2 min and the non-collected cells aspirated, as in the previous step. The same procedure is repeated adding 5 mL of LCM to the tube. Finally, 2.5 mL of supernatant are carefully removed and the tube gently tapped to resuspend the T cells-coated-bead suspension.

16.4.2.5   Detachment of T Cells from Beads

Polyclonal anti-mouse Fab antiserum (200 μL) is added. Approximately 10 μL of Fab antiserum are needed per 10^7 beads, although a smaller amount of Fab antiserum solution may be used when starting with smaller numbers of lymphocytes (see manufacturer's instruction). This is mixed gently by tilting and rotation during an incubation period of 45–60 min at room temperature (incubation at 4 °C reduces the number of detached cells, while 37 °C does not increase detachment efficiency). Due to the relatively small sample volume, care should be taken that the cells remain on the bottom of the tube during agitation. 5 mL of PBS/BSA are added to the tube to resuspend the beads. Tubes are placed on a vertical magnet for 2 min. The supernatant containing the detached cells is then aspirated and left aside, while the beads remain attached to the wall of the tube by the magnet. It is important not to disturb the beads clinging to the side of the tube. This step is repeated four times to improve the isolation yield, saving and combining detached cell-containing supernatants from each separation in the same tube. Then, the tube with the detached T cells is centrifuged 10 min at 600×*g* at room temperature. The supernatant is discarded and the cells resuspended in LCM. Finally, T cells are counted and the number of viable cells determined by trypan blue exclusion. It is convenient to determine the purity of the CD4+ T cell population by flow cytometry using anti-CD4 antibody.

16.5   Assess Viability

Once PBMC-derived T cells have been isolated and detached from the magnetic beads, they are usually resuspended in LCM (Prickett et al. 2013). Alternatively, Iscove's modified Dulbecco's medium (IMDM), containing 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL gentamicin and 5 % of heat-inactivated FBS,

could also be used (Frischmeyer-Guerrerio et al. 2011). In the above proposed T cell culture media, FBS could be replaced by 5 % of heat-inactivated human serum or 10 % heparinized human plasma. While most protocols use serum to provide optimal conditions for T cell viability, reactivity and expansion, a number of serum-free media have been developed (e.g., AIM-V, Invitrogen Corporation; or X-VIVO, Lonza) (Shreffler et al. 2009). These media are specifically formulated to support the culture of T cells and incorporate defined quantities of purified growth factors, lipoproteins, and other proteins, which are usually provided by the serum.

Commonly, T cells are seeded at a final concentration of $1–2.5 \times 10^6$ cells/mL in 24 or 48-well cell culture plates (Pascal et al. 2013). Although, in T cell proliferation ($[H^3]$-thymidine) and viability (MTT or XTT) assays, 96-well plates are used and only 100 µL of the cell solution are added, resulting in $1–2.5 \times 10^5$ cells per well (Martino et al. 2012). T cell culture assays are usually carried out in sample (three different wells) and biological triplicates (three different plates) (Prickett et al. 2011).

All incubations of T cell cultures must be performed at 37 °C in a humidified 5 % CO_2 incubator. The incubation period for T cell assays varies depending on the initial cell population, percentage of damaged cells and stimuli used. Proliferation following mitogen-induced activation generally peaks after 2–6 days of culture (Bohle et al. 2007). An incubation period of 72 h is also used when proliferative responses to food proteins (such as OVA or peanut extracts) are studied (Prickett et al. 2011; Martino et al. 2012). However, for gene expression and cytokine detection assays, the response may not peak until after 5–10 days. Thus, the effect of Ara h 2 peptides has been analyzed after 4 days of incubation (Pascal et al. 2013), milk allergenic proteins after 5 days (Shreffler et al. 2009) and Ara h 1 peptides after 7 days (Prickett et al. 2013). To ensure that treatments do not affect cell health, a viability test should be performed. An array of methods and kit-based assays are available for cell viability, such as the measurement of mitochondrial reductase with tetrazolium salts (e.g. MTT, XTT and Alamar blue).

16.6 Samples

Hydrophilic samples, such as polar food proteins, are usually added to the cells diluted in PBS or directly in the culture medium at different final concentrations, which range from 10 to 200 µg/mL of culture (Pascal et al. 2013; Wing et al. 2003). When samples contain polyphenols or alcohols, they need to be resuspended in PBS or culture media containing 10 % of the aprotic and highly polar solvent DMSO. Potent T cell activators, such as mitogens (lectins like PHA or Concanavalin A), antigens (tetanus toxoid) or antibodies (anti-CD3/CD28) should be included as positive controls in the assays to test both cell viability and functionality (Bohle et al. 2007; Pascal et al. 2013).

A critical parameter in the evaluation of the response to proteins of T cells is sample quality. This is crucial for recombinant proteins which are produced in bacterial systems. These can carry over contaminants from the host cells to the final

recombinant preparation, including irrelevant proteins and lipopolysaccharide (LPS). LPS, synthesized by gram-negative bacteria, has profound effects on T cell responses. LPS drives the development of Th1 subsets and it is also associated with toxicity (McAleer and Vella 2008). Because of these reasons, the purity of the proteins, and particularly the LPS concentration, should be determined by available commercial kits before application to T cell assays (Hofmann et al. 2012).

16.7   Experimental Readouts

In vitro T cell functional measures to stimuli may be monitored by assays that detect proliferation, cytokine secretion and the expression of genes of interest, or characterizing proliferating cells to identify phenotype and frequency. Measurement of the proliferative responses of T cells is fundamental for the assessment of their biological reaction to various stimuli, such as food allergens or hypoallergenic preparations. T cell proliferation is commonly determined by estimating incorporation of [H^3]-thymidine into the DNA, a process which is closely related to underlying changes in cell number. [H^3]-thymidine methodology has been widely utilized in allergen-specific T cell proliferation studies (Prickett et al. 2011; Shreffler et al. 2009). Alternatively, carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling prior to T cell culture enables direct measurement of cell division and it may be used in combination with cell surface markers to identify the target cells by flow cytometry (Foster et al. 2011; Prickett et al. 2013).

Cytokine secretion in response to a stimulus may be detected by measuring either cytokine production (by ELISA) or enumerating individual cytokine producing T cells (by ELISPOT). ELISA has been used to evaluate T cell behavior towards food allergens in several studies (Glaspole et al. 2011; Hofmann et al. 2012). In contrast to ELISA, ELISPOT allows the visualization of the secretory products of individual activated or responding T cells. Thus, it provides both qualitative (kind of cytokine) and quantitative (number of antigen-specific T cells) information (Prickett et al. 2011; Faresjö 2012). Flow cytometry has been also used for the measurement of T cell cytokine production by fluorescent bead arrays (e.g., Cytometric Bead Array™—CBA, BD Biosciences) (Kücüksezer et al. 2013; Pascal et al. 2013). Flow cytometry can also be used for phenotypic analysis of T cell subsets, frequency determination (Shreffler et al. 2009; Hofmann et al. 2012) and detection of intracellular cytokines (DeLong et al. 2011). Finally, Real-Time quantitative PCR (RT-qPCR) has been largely used for the study the expression of cytokine genes (Kücüksezer et al. 2013). Gene expression has also been assessed in purified CD4+ T cells by DNA microarrays to characterize T cell signaling pathways of allergic and non allergic children (Martino et al. 2012).

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