PhD Thesis

‘PROBIOTIC BACTERIA ADMINISTRATION AS AN IMMUNO-MODULATORY APPROACH FOR FOOD ALLERGY TREATMENT: PRECLINICAL STUDIES IN MOUSE MODELS’

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DISCUSSION
INTRODUCTION

1. Oral tolerance

Recent estimates indicate that up to 8% of children and 3–4% of adults in Westernized countries are affected by food allergy (Wang J and Sampson HA, Immunol Res 2012). Furthermore, in the last decades, an increase in the prevalence of the allergic diseases (not only food allergy) has been recorded. Several factors have been discussed as possible cause, including the improvement of diagnosis methods as well as the environmental and climate changes (Shea et al., J Allergy Clin Immunol 2008), but a very fitting explanation to this phenomenon seems to be the concept underlying the ‘hygiene hypothesis’ (Strachan DP, BMJ 1989). It is based on the potential influence of improved hygiene and better infection control on the development of allergies. It has been proposed that a reduced and/or altered exposure to, and colonization by, micro-organisms, particularly early in life (as a result of a Westernized lifestyle), leads to the polarization of allergen-specific T cell memory towards the Th2-type instead of the Th1 immune response (Sepp E et al, Acta Paediatr 1997; Ernst P et al, Am J Respir Crit Care Med 2000). Whether reduced microbial burden and/or impaired colonization by microbes are the only environmental stimuli influencing this immune effect is unclear, but this particular environmental exposure has received the most attention. The initial immune model of the Th1/Th2 imbalance associated with the ‘hygiene hypothesis’ (Romagnani S, Immunology 2004) has recently been questioned by further evidences that peripheral T-cell regulation plays a crucial role in the control of harmful T-cell responses. Indeed, the description of a new subset of CD4+ T cells named ‘regulatory T cells (T reg)’ by Sakaguchi et al. (Sakaguchi S et al., J Immunol 1995) revisited the ‘immune regulation’ concept. Then, it has been proposed that an impairment in the induction of regulatory responses (responsible for mucosal tolerance) is the underlying mechanism involved in the
differentiation of pathogenic T cells driving to inflammatory responses including aberrant Th2-shift that drives to allergic diseases (Wills-Karp M et al., Nat Rev Immunol 2001).

Subsets of T reg cells with distinct phenotypes and mechanisms of action include the naturally occurring CD4+CD25+Foxp3+ T reg population, generated in the thymus (Powrie F et al., J Exp Med 1994; Shevach EM, J Exp Med 2001) and anyone of many inducible T reg cell populations, that are derived in the periphery from CD4+Foxp3- precursors upon activation in presence of differentiating signals like TGF-β and IL-10 (e.g. Th3, Tr1 cells)(Haribhai et al., Immunity. 2011; Pot C et al., Semin Immunol 2011). In addition, subsets of CD8+ T cells, γδ T cells, dendritic cells (DC), IL-10-producing B cells, natural killer (NK) cells and resident tissue cells, which might promote the generation of T reg cells, could contribute to suppressive and regulatory events (Akdis M and Akdis CA, J Allergy Clin Immunol 2007; Bellinghausen I et al., Clin Exp Allergy 2006). T reg cells are also involved in a reciprocal relationship with Th17 lymphocytes and the manipulation of this pathway may result either in the induction of T reg that will suppress autoimmunity or in the induction of effector Th17 cells that initiate tissue inflammation (Bettelli E et al., Nature 2008).

T reg cells maintain immune homeostasis through various effector functions. In order to exert their functions, peripheral T reg cells act to abolish specific antigen-stimulated lymphocyte proliferation and suppress Th1 and Th2 cytokine production (Akdis CA et al., Allergy 2004). This can occur by direct mechanisms (through cell-cell interactions) or indirect effects (by mediators production).

The term ‘oral tolerance’ refers to the physiologic induction of tolerance that occurs in the gut-associated lymphoid tissue (GALT) and more broadly at other mucosal surfaces such as the respiratory tract (Faria AM and Weiner HL, Immunol Rev 2005; Iwata M et al., Immunity 2004; Mora JR et al., Science 2006). The mechanisms responsible for the oral
tolerance induction are related to the anatomical organization of immune system at gut level as well as to its interaction with resident microbiota. The GALT is the largest immune system in the body. Its physiologic role is the processing of dietary antigens in a manner that does not result in untoward immune reactions and protection of the organism from pathogens.

Bacteria colonizing the human intestinal mucosa (approximately $10^{12}$ microorganisms per gram of stool) are able to influence the local immune milieu (Qin J et al., Nature 2010; Arumugam M et al., Nature 2011). In turn, the microbiota is in a reciprocal balance with gut immune system because the latter plays a decisive role contributing to shape microbial environment and harmonizing interactions between the host and gut microbes. When the environment changes, the immune system must follow and adjust to the fluctuations. In this way, complex metabolic functions are maintained and damaging effects of microbial components are minimized (Cohen IR, Nat Rev Immunol 2007; Eberl G, Mucosal Immunol 2010).

There are several distinctive features of GALT and they play different roles in the gut immune system. The inductive sites for immune responses in the gut are Peyer’s patches (PP), which are macroscopic lymphoid aggregates in the submucosa along the length of the small intestine, and mesenteric lymph nodes (MLN), which are the largest lymph nodes in the body. MLN serve as a crossroads between the peripheral and mucosal recirculation pathways. In addition, there are lymphocytes scattered throughout the epithelium and lamina propria of the mucosa. A single layer of epithelial cells separates the gut microflora from the main elements of the gut immune system. In the GALT, also intraepithelial lymphocytes (IEL, mainly CD8+ T cells) are present.

Currently, different mechanisms of oral tolerance induction are known. In this process, the combination of commensals (Strober W, Immunity 2009), T cells (Izcue A et al., Immunol Rev 2006), and DC (Rescigno M, Adv Immunol 2010) collaborate in the set-
up of a tolerogenic environment in the gut. Major factors conditioning the gut to be a
tolerogenic environment are interleukin-10 (IL-10), retinoic acid (RA), and transforming
growth factor-β (TGF-β), which has many functions including to act as a switch factor for
IgA, the predominant immunoglobulin of the gut that offer a ‘protection barrier’ to the
mucosa (Li MO and Flavell RA, Cell 2008).

In particular, gut DC are unique in that they can drive T reg differentiation from
Foxp3- cells. These properties of DC relate to their being conditioned by commensal bacteria,
TGF-β and IL-10 from gut epithelial cells, and their expression of RA, which is provided in
the form of vitamin A in the diet and appears to be constitutively expressed by gut DC. The
DC plasticity and capacity to integrate microbial, environmental and self-derived signals make
them also able to select the type and the intensity of the response upon the encounter with a
pathogen. Therefore, besides their role in the development of tolerogenic T cells, they can also
promote effector T cell responses (Coombes JL and Powrie F, Nat Rev Immunol 2008)
which are in balance with the former to regulate immune homeostasis in the gut (Belkaid Y

CD11b+ monocytes may also play a role in the induction of T reg cells which occurs
in the MLN and involves both C-C motif receptor 7 (CCR7) and CCR9. Also macrophages
are stimulated to produce TGF-β after up-taking apoptotic epithelial cells or apoptotic T cells
following high-dose tolerance. Tolerance induction mode also depends on the dose of antigen.
In fact, lower doses of antigen favour the induction of T reg, whereas higher doses of antigen
favour anergy/deletion as a mechanism of tolerance induction. The liver may also play a role
in oral tolerance induction because for example, antigen (high dose) may be rapidly taken up
by this organ, where it is processed by plasmacytoid DC that induce anergy/deletion and T reg
cells. As a result, a number of different types of T reg cells may be induced or expanded in the
gut including CD4+CD25+Foxp3+ inducible T reg, natural T reg, Tr1 cells, Th3 positive for the latency-associated peptide (LAP), CD8+ T reg, and γδ T cells (Figure 1I).

2. What determines the development of a T regulatory response rather than a T pathogenic one?

GALT is exposed to oral and microbial antigens which can encounter gut APC (antigen presenting cells) in a number of ways. Besides the antigens, whole microorganisms may also get access to APC as a result of tissue damage, or via the acquisition of invasive potential (Sansonetti PJ, Nat Rev Immunol 2004).

Intestinal APC can be distinguished on the basis of the expression of surface markers such as CD11c, CD11b, CD8α, CCR6, CCR7, CD103 and CX3CR1. They can be found in lamina propria (LP) and GALT including MLN, PP and isolated lymphoid follicles (ILF). Their specific location within lymphoid tissues is related to their functions. Gut APC can be roughly divided into CX3CR1-CD103+ DC and CX3CR1+CD103- phagocytes (Rescigno M, J Leukoc Biol 2011).

CD103+ DC play many typical DC functions like antigen sampling and activation of T cells (Annacker O et al., J Exp Med 2005). They migrate from LP to MLN to present antigens to T cells in steady-state as well as in inflammatory conditions. In contrast to CD103+ DC, CX3CR1+ phagocytes are not able to migrate and have limited capacity to activate T cells in vitro and in vivo (Schulz O et al., J Exp Med 2009). Their primary function is to sample intestinal luminal antigens directly across the epithelial cell layer and influence initial stages of immune activation (Rescigno M et al., Nat Immunol 2001; Niess JH et al., Science 2005). They are also involved in re-stimulation of T cells after an antigen challenge (Hadis U et al., Immunity 2011; Niess JH and Adler G, J Immunol 2010). Taking into
Oral antigen crosses from the intestine into the GALT (gut associated lymphoid tissue) in a number of ways. It can enter via M cells, be sampled by DC processes that penetrate the lumen, or be taken up by intestinal epithelial cells. DC (dendritic cells) in the gut are unique in that they can drive Treg differentiation from Foxp3− cells. These properties of DC relate to their being conditioned by commensal bacteria, TGF-β and IL-10 from gut epithelial cells, and their expression of retinoic acid, which is provided in the form of vitamin A in the diet and appears to be constitutively expressed by gut DC. CD11b monocytes may also play a role in the induction of Tregs, and the induction of Treg occurs in the MLN (mesenteric lymph nodes) and involves both C-C motif receptor 7 (CCR7) and CCR9. Co-stimulation by PDL1-programmed cell death ligand (PDL) is also important for the induction of Treg. Macrophages are stimulated to produce TGF-β after uptaking apoptotic epithelial cells or apoptotic T cells following high-dose tolerance. Lower doses of antigen favor the induction of Treg, whereas higher doses of antigen favor anergy/deletion as a mechanism of tolerance induction. The liver may also play a role in oral tolerance induction and antigen (high dose) may be rapidly taken up by the liver, where it is processed by plasmacytoid DC that induce anergy/deletion and Tregs. A number of different types of Treg may be induced or expanded in the gut including CD4+CD25+Foxp3+ iTregs, nTregs, Tr1 cells, LAP+ Tregs (Th3 cells), CD8+ Tregs, and γδT cells.
account the functional differences between CX3CR1+ and CD103+ DC and the fact that CX3CR1+ phagocytes originate from monocyte precursors rather than conventional myeloid DC precursors (Bogunovic M et al., Immunity 2009), some authors suggested that CX3CR1+ cells should not be considered as a class of “genuine” DC but rather as a group of phagocytes whose identity lies somewhere in between DC and macrophages (Hume DA, Mucosal Immunol 2008; Pabst O and Bernhardt G, Eur J Immunol 2010).

Other DC subtypes that are not present in LP but in PP are: CCR6+ DC (CCR6 is the receptor of CCL20) that drive preferentially Th2 type responses (Salazar-Gonzalez RM et al., Immunity 2006) and CD8+ DC involved in the development of Th1 cells (Iwasaki A and Kelsall BL, J Immunol 2001). PP also contain non-conventional plasmacytoid DC with specialized features: they are impaired in type I IFN production (Contractor N et al., J Immunol 2007) and drive B cell immunoglobulin isotype switching to IgA in a T cell-independent manner (Tezuka H et al., Immunity 2011).

Luminal microbes shape the composition and concentration of cytokines, chemokines and other factors in LP by interacting directly with DC and/or by engaging the pattern recognition receptors (PRR) on intestinal epithelial cells (IEC). These receptors recognize characteristic structural motifs of the microorganisms called ‘microbe associated molecular patterns’ (MAMP). Once ligated, PRR can initiate signaling pathways in DC, influencing their phagocytic activity, expression of co-stimulatory molecules and cytokine production (Medzhitov R, Nature 2007). Simultaneous involvement of different types of PRR on DC helps to identify the nature of the encountered bacterium and is required to initiate a proper type of immune response. Together with the PRR action, different combinations and concentrations of the conditioning factors in the tissues influence DC functions. In particular they jointly determine whether a DC promotes differentiation of T reg, Th17, Th1 or Th2 cells. For example, TFG-β may be involved in development of Th17 cells or T reg depending
on other accompanying factors (IL-6 or RA) that are released by DC or IEC. TSLP induces non-inflammatory DC capable to drive Th2 but not Th1 responses and is involved in fostering tolerogenic DC in the human system together with TGF-β and RA. CX3CR1+ cells may respond to bacteria-produced ATP and induce or re-stimulate Th17, or release IL-10 and re-stimulate T reg during ‘steady-state’. Hence, both CD103+ and CX3CR1+ phagocytes can carry out opposite functions depending on the local cytokine milieu that is the consequence of interaction of the microorganisms, IEC and DC. Balance between different types of T-cells is decisive about the type of induced immune response and the maintenance of intestinal homeostasis (Figure 2I).

At gut level, it has been shown that Foxp3+ T reg are induced by tolerogenic DC expressing CD103. CD103 marker is the α chain of the αβ7 integrin and is expressed not only on DC but on a number of other epithelium-associated cell types, including CD4+CD25+ and CD4+CD25- regulatory T cells subsets (Lehmann J et al., Proc Natl Acad Sci U S A 2002), γδ T cells, IEL (Brenan M and Rees DJ, Eur J Immunol 2000) and tissue-resident memory αβ T cells (Gebhardt T et al., Nat Immunol 2009; Boyman O et al., Trends Immunol 2007). First studies have been proposed that the induction of Foxp3+ T reg cells by CD103+ DC occurs via a mechanism dependent on TGF-β, RA (Coombes JL et al., J Exp Med 2007; Sun CM et al., J Exp Med 2007) and on the expression of indoleamine 2, 3-dioxygenase (IDO) (Matteoli G et al., Gut 2010). Despite these first investigations, Worthington JJ et al. report that in mice RA is dispensable in the induction of Foxp3+ T reg by CD103+ DC and that these cells promote this type of regulatory response via an integrin αvβ8-mediated activation of latent TGF-β (LAP). In particular, it is shown that mouse DC lacking of the integrin expression have a reduced ability to activate LAP and induce Foxp3+ Treg in vitro and in vivo (Worthington JJ et al, Gastroenterology 2011). The TGF-β signaling is also pivotal in T cells for the expression of CD103 itself on Foxp3+ Treg cells (Reynolds LA and
Integrated action of conditioning factors in the lamina propria. Luminal microbes shape the composition and concentration of cytokines, chemokines and other factors in lamina propria by interacting directly with DC and/or by engaging PRR (pattern recognition receptors) on epithelial cells. Different combinations and concentrations of the conditioning factors in the tissues influence specific DC functions. In particular they jointly determine whether a DC promotes differentiation of Treg, Th17, Th1 or Th2 cells. For example TFG-β may be involved in development of Th17 cells or Treg depending on other accompanying factors (IL-6 or RA) that are released by DC or IEC. TSLP induces non-inflammatory DC capable to drive Th2 but not Th1 T cells and is involved in fostering tolerogenic DC in the human system together with TGF-β and RA. CX3CR1+ cells may respond to bacteria-produced ATP and induce or restimulate Th17, or release IL-10 and restimulate Treg during steady-state. Hence both CD103+ and CX3CR1+ phagocytes can carry out opposite functions depending on the local cytokine milieu that is the consequence of interaction of the microorganisms, IEC and DC. Balance between different types of T-cells is decisive about the type of induced immune response and the maintenance of intestinal homeostasis.
Maizels RM, J Immunol 2012), confirming the fact that CD103 expression is regulated by TGF-β (Robinson PW et al, Immunology 2001).

Notably, the induction of a population of CD4+LAP+ inducible Treg has been demonstrated to be involved in suppressing inflammation in a TGF-β-dependent manner in mouse experimental models of colitis and autoimmune diseases (Di Giacinto C et al, J Immunol 2005; Boirivant M et al., Gastroenterology 2008; Chen ML et al., J Immunol 2008). LAP expression has been also found on activated mouse and human Foxp3+ T reg cells (Andersson J et al., J Exp Med 2008; Tran DQ et al., Blood 2009).

3. Food allergy and its mechanisms of pathogenesis

The term “food allergy” is used to describe an adverse immunological response to food proteins (allergens) that is potentially life-threatening. It is important to distinguish between food allergy and other non immune-mediated adverse reactions to foods (e.g. lactose intolerance, reactions to histamine produced by scombroid fish contaminated by Salmonella organisms, to caffeine in coffee causing jitteriness, tyramine in aged cheeses triggering migraine). Other conditions which are associated with symptoms similar to food allergy include auriculo-temporal syndrome (a disorder characterized by facial flushing and salivation that may follow trauma to the parotid gland), and gustatory rhinitis (Sampson HA, J Allergy Clin Immunol 2004).

Food-induced allergic disorders are broadly categorized into those mediated by non-IgE (immunoglobulin E antibodies)-mediated mechanisms or by IgE-dependent processes. Non-IgE-mediated (cell-mediated) food allergy is less common and include: dietary-protein-induced enterocolitis and proctitis, celiac disease and its related skin disorder dermatitis herpetiformis.
IgE-mediated allergic responses are the most widely recognized form of food allergy and are characterized by the rapid onset of symptoms after ingestion. They may occur with a variety of clinical conditions, such as oral allergy syndrome, atopic dermatitis, gastrointestinal reactions (IgE-mediated immediate reaction), urticaria, anaphylaxis, exercise-induced anaphylaxis, respiratory reactions (rhinitis, asthma), urticaria / angioedema / anaphylaxis syndrome, "hypersensitivity pneumonitis". In some very sensitive individuals, the consumption of foods containing even small amounts of the sensitizing protein can be life-threatening. These individuals may show severe anaphylactic reactions, both local and systemic as bronchospasm, choking, nausea, vomiting, hypotension and shock, with possible fatal outcome.

An IgE-mediated food reaction occurs as soon as a food allergen generally enters the body through the mucosal surface of the gastrointestinal tract. Food proteins are directly internalized or transferred to DC, processed by them into peptide fragments and presented on the cell surface by class II MHC molecules. DC migrate to draining lymph nodes (MLN, mesenteric lymph nodes) for T cell activation (Hashimoto D et al., Immunity 2011). Peptide/MHC II complex is presented to naïve T cells resulting in T helper cell priming and activation. This event initiates humoral and cellular events associated with food allergy. In individuals with a genetic predisposition for allergic disease, the activation of T helper cells results in the secretion of cytokines that stimulate B cells to synthesize IgE allergen-specific antibodies in the sensitisation phase of the immune response. Th2 cells are associated with the secretion of various interleukins including IL-4, IL-5, IL-9, and IL-13. In the effector phase of the immune response, the allergen-specific IgE antibodies are bound primarily on mast cells and basophils by high-affinity surface IgE receptors (FcεRI). After ingestion, the food protein (not carbohydrate or fat) crosslinks with the specific IgE antibodies on mast cells and basophils releasing various inflammatory mediators including histamine, prostaglandins,
leukotrienes, and platelet-activating factor (PAF). Additionally, tumour necrosis factor (TNF-α), IL-5, and chemokines produced at local level result in the activation and recruitment of eosinophils responsible for the release of other inflammatory mediators (Gleich GJ, J Allergy Clin Immunol 2000).

4. Seafood and peanuts represent two common sources of potentially dangerous allergens

The most common food allergens causing reactions in children include milk, egg, wheat, soy, peanuts, tree nuts, fish and shellfish. While allergies to milk, egg, wheat and soy are commonly outgrown in childhood, allergies to peanut, tree nuts, fish and shellfish often persist in adulthood.

Despite limitations in epidemiologic studies and variations in methodologies, it is noteworthy that there are several geographic associations with food allergy. It appears that geography can affect both the prevalence of particular food allergies and the pattern of immunologic reactivity to individual allergenic components within the food, thus affecting the clinical expression of food allergy. For instance, American patients often had higher levels and higher frequencies of IgE antibodies to peanut allergens: Ara h 1, Ara h 2, and Ara h 3 (56.7% to 90%, respectively) and tended to present with more severe symptoms. Spanish patients recognized these three recombinant peanut allergens less frequently (16% to 42%, respectively) but had higher sensitization rates to the lipid transfer protein Ara h 9 (60%). Swedish patients had the highest sensitization rate to the Bet v 1 homolog Ara h 8 (65.7%) (Lack G, J Allergy Clin Immunol 2012).

In shellfish and seafood, tropomyosin, a heat-stable muscle protein, is the major allergen. Invertebrate tropomyosin belongs to a family of highly conserved proteins displaying
allergenic activity. These homologies are responsible for the cross-reactive allergies observed between various types of edible crustaceans and molluscs, including shrimps, lobsters, crabs, squids and mussels, as well as inhalant household allergens such as the ones derived from mites, cockroach and silverfish. Shrimp tropomyosin (ST) is the only major allergen of the majority of shrimp species, being recognized by >80% of shrimp allergic subjects (Daul CB et al., Int Arch Allergy Immunol 1994), and it accounts for most of the allergenic activity of whole shrimp extract. The IgE-binding regions of ST from Penaeus aztecs (named Pen a 1) have been analyzed allowing the identification of five major allergenic regions (Ayuso R et al., Int Arch Allergy Immunol 2002). Due to the wide cross-reactivity within shellfish and, more broadly, between different phyla of invertebrates, ST can be considered an invertebrate panallergen (Reese G et al., Int Arch Allergy Immunol 1999). The clinical consequences of this panallergenicity include the possibility of different cross-sensitization routes, including ingestion, inhalation and, interestingly, parenteral immunotherapy with mite extracts. Hence, the tropomyosin represents a clinically relevant allergen.

Peanut allergy accounts for the majority of cases of food-induced anaphylaxis. Exposure to even trace quantities of peanuts in a sensitized individual can lead to a fatal reaction. Peanut allergy is increasing in prevalence among children and adults worldwide, with only about 20% of affected children outgrowing it (Husain Z and Schwartz RA, J Am Acad Dermatol 2012). The cause of a rising prevalence of peanut allergy is unknown. Although genetic and environmental factors likely play a role, several theories have emerged to explain this recent phenomenon. Possible explanation are: the ‘hygiene hypothesis’, the maternal ingestion of peanuts during pregnancy and lactation as well as the timing of peanut introduction to infants or children, increased peanut consumption, use of peanut-containing non-food products, and early introduction of potentially cross-reacting proteins such as soy or carob. Commonly used processing techniques, including roasting, emulsifying, and mixing
additives with peanuts, possibly cause increased allergenicity and may be implicated in the rising incidence of allergy (Scurlock AM and Burks AW, *Ann Allergy Asthma Immunol* 2004; Sicherer SH and Sampson HA, *J Allergy Clin Immunol* 2007).

The allergenic components of peanuts are the proteins of the cotyledon. Eight peanut allergens have been identified, from Ara h 1 to Ara h 8, named after the scientific name for this legume, *Arachis hypogaea*. A new member of the LTP allergen family is Ara h 9 that seems to play an important role in peanut allergy for patients from the Mediterranean area (Krause S et al., *J Allergy Clin Immunol* 2009). Ara h 1 and Ara h 2 are the major peanut allergens implicated in most reactions and belong to the vicilin and conglutinin families of storage proteins, respectively. More than 90% of peanut-allergic patients have IgE antibodies to these two proteins. Similarly, 45% to 95% of peanut-allergic patients have IgE antibodies to Ara h 3, a related protein. Allergenic epitopes are generally resistant to acid, heat, and enzymatic degradation. Common foods cross-reacting with peanuts via an IgE-mediated mechanism are some legumes (e.g. pea, bean, etc.), tree nuts (e.g. almond, cashew, etc.) and seeds (sesame). Some research suggests a strong genetic component in peanut allergy (Hourihane JO et al., *BMJ* 1996; Sicherer SH et al., *J Allergy Clin Immunol* 2000) as well as an influence by sex. In different studies, the male/female ratio of children with peanut allergy is higher than that found for adults whose male/female ratio is less than 1 (Sicherer SH et al., *J Allergy Clin Immunol* 2003; Emmett SE et al., *Allergy* 1999). A similar pattern was seen for tree nut allergies. Many types of food allergies can be also associated with genetic polymorphisms in genes as those encoding the cytokines IL-10 or IL-13 (Campos Alberto EJ et al., *Pediatr Allergy Immunol* 2008; Liu X et al., *J Allergy Clin Immunol* 2004) depending on the examined population. Regarding peanut allergy, Dreskin et al. showed that variations in the 2 important SNP (single nucleotide polymorphisms) of CD14 (rs2569190 and rs2569193) are associated with the presence of peanut allergy and increased levels of total IgE and the
frequency of eczema in patients with peanut allergy (Dreskin SC et al., *Ann Allergy Asthma Immunol* 2011). Furthermore, more recent studies suggest important gene-environment interactions in the development of food sensitization, not only for peanut. An example is offered by the relationship between breast-feeding and the risk to develop food allergy depending on the polymorphisms in genes encoding IL-12 receptor β1, TSLP (thymic stromal lymphopoietin) and TLR-9 (Toll-like receptor 9) (Hong X et al., *J Allergy Clin Immunol* 2011). These data also suggest that innate arm of immune system has a considerable influence on the pathogenesis of a food allergic response similarly to what occurs in airway allergic inflammation.

Other factors as dietary fat, vitamin D, antioxidants and obesity can influence the development of food allergy.

5. Troubles in the management of food allergy and probiotic bacteria as ‘immunomodulators’

The rapid increase of allergy prevalence around the world over the last decades has highlighted the need to develop preventive and/or therapeutic strategies, but up to now no effective treatment is available for food allergy and its primary management still consists of strictly avoiding relevant allergens. Pharmacological therapy not always turns out to be effective: antihistamines decrease partially the symptoms induced by oral allergy syndrome and the IgE-mediated symptoms at skin level. The administration of anti-inflammatory drugs is indicated only in the case of gastroenteritis and allergic eosinophilic esophagitis (Sicherer SH and Sampson HA, *J Allergy Clin Immunol* 2010). The efficacy of specific immunotherapy (SIT) is still controversial because of native allergens injection causes severe adverse reactions, as demonstrated for peanut allergy (Rolland JM et al., *Pharmacol Ther* 2009). A new
promising attempt with few adverse reactions is being experimented: the oral immunotherapy (OIT) consisting in the oral administration of allergen (e.g. peanut) (Ismail IH and Tang ML, Isr Med Assoc J 2012) alone or in combination with monoclonal anti-IgE antibodies (Omalizumab) (Nadeau KC et al., J Allergy Clin Immunol 2011). An other possibility explored by preclinical models is offered by the use of ODN-CpG immunostimulatory sequences administered together allergen (Bashir ME et al., J Immunol 2004; Adel-Patient K et al., Int Arch Allergy Immunol 2007).

Among the immunomodulatory approaches there is also a growing interest to use probiotic bacteria and/or fermented ingredients (prebiotics) that promote the activity of the gastrointestinal microbiota. Both these measures have the potential to confer benefit(s) to host health. Probiotics are defined as ‘viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects’ (Food and Agriculture Organization. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group report on drafting guidelines for the evaluation of probiotics in food. London: Food and Agriculture Organization, 2002). They belong to Lactobacillus, Streptococcus and Bifidobacterium genera but also some non-pathogenic strains of Escherichia coli and other microorganisms such as the yeast Saccharomyces boulardii mirror the definition of probiotic bacteria. Since a decade, epidemiological studies have established that the development of allergy in genetically predisposed subjects is associated with a different proportion of the bacterial phyla belonging to the gut microbiota in comparison with normal subjects (Björkstén B et al., J Allergy Clin Immunol 2001; Kalliomäki M et al., J Allergy Clin Immunol 2001). From these observation, the rational for using ‘normal commensal bacteria’ in the attempt to restore in the gut conditions as close as possible to the healthy subjects is logically derived, and has been applied to the prevention and therapy not only of allergic diseases but also other inflammatory disorders (Veerappan GR et al., Curr

Recent studies in both human and experimental animal models highlight the importance of immune modulation exerted early in life by the commensal bacterial flora in the gastrointestinal tract, in directing the development of tolerance (Iweala OI and Nagler CR, Immunol Rev 2006). Probiotics are claimed to beneficially affect the immune system in several physiological and pathological conditions including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), obesity, type I diabetes and allergies.

The probiotic concept remains controversial, because the precise mechanisms by which probiotic microorganisms exert their protective effects in vivo have not been well defined. Different mechanisms of action have been proposed for explaining how probiotic bacteria and their metabolic products (short-chain fatty acids, vitamins) exert their beneficial effects. They may involve modification of gut pH, antagonism against pathogens through production of antimicrobial compounds, competition for pathogen binding, receptor sites, nutrients and growth factors, stimulation of immunomodulatory cells, and production of lactase (Collado MC et al., Curr Drug Metab 2009). They also influence barrier functions by the inhibition of bacterial translocation and reinforcement of barrier, stimulation of mucosal defence both at the level of immune and epithelial function by increasing the production of secretory IgA, by stimulation of protective epithelial substances (such as mucins), by regulation of mucosal cytokine production, and by production of nutrients of special importance to the intestine such as short-chain fatty acids and vitamins (Gionchetti P et al., Gastroenterol Clin N Am 2005).

Clinical studies on different allergic populations yielded controversial results on the efficacy of prophylactic or therapeutic treatments with probiotics and the anti-allergic effects of these bacteria are still not completely defined (Gourbeyre P et al., J Leukoc Biol 2011;
Szajewska H, Isr Med Assoc J 2012). In particular, the combined effects of mixtures of different species of probiotic bacteria have been only in part explored in suitable animal models to better understand the in vivo processes that modulate allergy responses.

In murine models and clinical studies, VSL#3 probiotic preparation, a high concentration mixture of eight live freeze-dried bacterial species that are normal components of the human gastrointestinal microbiota, has been shown to prevent or ameliorate several gastrointestinal pathologies, such as IBD (Di Giacinto C et al., J Immunol 2005; Chapman TM et al., Drugs 2006), irritable bowel disease (Camilleri M, J Clin Gastroenterol 2008), pouchitis (Gionchetti P et al., Dis Colon Rectum 2007; Pronio A et al., Inflamm Bowel Dis 2008), chemotherapy-induced diarrhoea and autoimmune diabetes.

In vitro studies on human dendritic cells demonstrated the VSL#3 potential to induce IL-10 production and the down-regulation of costimulatory molecules (Hart AL et al., Gut 2004). In other in vitro studies, the probiotic mixture was able to decrease inflammatory cytokine production and to increase the release of regulatory mediators, but also to maintain the integrity of the “tight junctions” (Otte JM and Podolsky DK, Am J Physiol Gastrointest Liver Physiol 2004). In preclinical studies, the administration of VSL#3 resulted in decrease of neutrophils at inflammation sites (Shibolet O et al., Inflamm Bowel Dis 2002), the levels of pro-inflammatory IFN-γ e TNF-α (Madsen KL, Clin Invest Med 2001), the induction of T reg cells (Di Giacinto C et al., J Immunology 2005). Another study in an animal model of colitis demonstrated the involvement of DNA (CpG motifs)-TLR-9 axis in the reduction of disease severity (Rachmilewitz D et al., Gastroenterology 2004). A recent paper proposes another mechanism of action underlying the protective effects of VSL#3 probiotic bacteria in mouse models of gut inflammation and cancer: the colonization with VSL#3 probiotic bacteria would modulate gut microbial diversity and favour local production of conjugated
linoleic acid (CLA) in the colon that targets myeloid cell peroxisome proliferator-activated receptor γ (PPARγ) to suppress colitis \textit{(Bassaganya-Riera J et al., PLoS One 2012)}.

Furthermore, a prophylactic intranasal treatment with VSL#3 preparation is able to prevent the development of Th2-biased responses in a murine model of sensitization to a clinically relevant respiratory allergen \textit{(Mastrangeli G et al., Int Arch Allergy Immunol 2009)}.

On these bases, we sought to investigate the immunomodulatory activity of VSL#3 probiotic mixture in \textit{in vitro} and \textit{in vivo} models with the aim to clarify the immunological mechanisms of its therapeutic/preventive potential on aberrant Th2 responses developed against two clinically relevant food allergens as shrimp tropomyosin \textit{(Schiavi E et al., Allergy 2011)} and peanut \textit{(Barletta B et al., Mol Nutr Food Res 2013)}. 
AIM

The troubles in the management of allergic diseases, including food allergy, have given rise to the need for the research on new preventive/therapeutic approaches based on immunomodulatory/immunostimulatory properties. In the previous section, anti-IgE antibodies, ODN-CpG immunostimulatory sequences, OIT and probiotics have been presented as strategies of this type versus the standard pharmacological therapy and the avoidance of sensitizing allergen which are not ever feasible means for treating food allergy.

The rational for the use of probiotics to manage the allergic diseases comes from epidemiological data on recent increase in the prevalence of allergic diseases probably due to a diminished exposure and/or colonization by microbes (‘hygiene hypothesis’) as well as evidences of a differential composition of microbiota in allergic children compared to non-allergic ones. Also the successful use of microbial preparations (e.g. the VSL#3 mixture used in the studies presented here) in inflammatory disorders involving the gut, diabetes, chemiotherapy-induced diarrhoea and liver diseases have strengthened the hypothesis on the immunomodulatory activities of probiotic bacteria and their compounds.

Clinical trials evaluating the efficacy in prevention and treatment of allergic diseases by probiotics’ use have so far produced controversial data and suitable animal models lack to explain the mechanisms underlying the benefits of probiotic administration. Furthermore, the studies in this field are complicated by the different approaches used regarding the choice of a single strain of probiotic bacteria versus mixtures of bacteria strains.

Hence, the need for developing food allergy mouse models in order to evaluate the preventive and/or therapeutic potential of probiotics’ administration as well as the pathways
exploited by these ‘friendly microbes’ to exert the modulation of pathogenic immune responses.

The aim of this project has been to investigate by in vitro and in vivo studies the effects of VSL#3, a probiotic mixture made up by eight different bacteria strains (Lactobacillus, Bifidobacterium and Streptococcus genera) on ‘steady state’ conditions and established Th2 immune responses. The results obtained in in vitro studies prompted us to perform next in vivo studies to evaluate the potential of VSL#3 as therapeutic or preventive strategy. The choice of the mouse models of food allergy investigated in these phase was due to the clinical relevance of shrimp tropomyosin (ST) and peanut as food allergens. The promising results obtained upon the use of VSL#3 in the treatment of local and systemic anaphylaxis induced by the exposure to allergen and the suppression of Th2 inflammation in the gut were common features to both animal models. In the peanut allergy mouse model, an in vivo selective blocking study was performed to better understand the mechanism underlying VSL#3 therapeutic benefits and to demonstrate the functional role of the regulatory pathway involved.
MATERIALS AND METHODS

1. Preparation of allergenic material

**Preparation of purified shrimp tropomyosin (ST).** ST protein from *Metapenaeus ensis* was extracted and characterized in our laboratory according to the protocol and techniques described in Capobianco F et al. (Capobianco F et al., Int Immunol 2008).

**Preparation of peanut extracts.** For the preparation of peanut flour extract (PFE), seeds from *Arachis hypogaea* plant purchased from “La Montanara” (Napoli, Italy) were crushed in a ceramic mortar in presence of nitrogen until a fine flour was obtained. Three grams of the flour were added to 30ml of ‘gavage buffer’ (PBS/sodium bicarbonate 7.5% weight/volume, 8:2 ratio) and stirred with glass beads for 10min. The homogenate obtained was then transferred onto filters Falcon Cell Strainer Nylon 10µm (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The particulate was recovered and left to dry for 2h at 100° C.

Peanut aqueous extract (PAE) was prepared by modification of published protocols (Koppelman SJ et al., Allergy 2003; Porterfield HS et al., Clin Exp Allergy 2009). Seeds from *Arachis hypogaea* were stored at -80°C until use. They were ground with a grinder at full speed and then defatted with diethyl ether for 2h and dried at room temperature (r.t.) overnight (o.n.). Protein extract was obtained by mixing 25g of ground peanut with 250ml of 20mM Tris buffer (pH 7.2). After 2h stirring at r.t., the aqueous fraction was collected by centrifugation (3000g at r.t., for 30min). The aqueous phase was subsequently centrifuged (10000g at r.t. for 30min) to remove residual traces of fat and insoluble particles. The supernatant obtained by centrifugation was dialyzed against distilled water for three days. The extract was lyophilized and stored at -20° C. Protein concentration was determined on lyophilized samples (re-suspended in PBS) by Bradford analysis in which serial dilutions of
bovine serum albumin (BSA) were used to build a standard curve. The protein yield was typically about 45%. Reducing SDS-PAGE from the extract showed protein bands between 14kDa and approximately 100kDa.

2. VSL#3 probiotic preparation

The probiotic VSL#3 (VSL Pharmaceuticals, Fort Lauderdale, FLA) was kindly provided by Prof. C. De Simone (University of L’Aquila, Italy) as a lyophilized mixture consisting of 8 different Gram-positive organisms (\textit{Lactobacillus acidophilus}, \textit{Lactobacillus delbrueckii} subsp. \textit{bulgaricus}, \textit{Lactobacillus casei}, \textit{Lactobacillus plantarum}, \textit{Bifidobacterium longum}, \textit{Bifidobacterium infantis}, \textit{Bifidobacterium breve}, \textit{Streptococcus salivarius} subsp. \textit{thermophilus}) at the concentration of $3 \times 10^{11}$ live total bacteria/g, without any excipient. The concentration of each strain is unknown and covered by patent. The powder was resuspended in sterile PBS without additives for \textit{in vitro} studies and \textit{in vivo} oral administration.

3. Generation of Bone Marrow-Derived Dendritic Cells and VSL#3 co-culture

Bone Marrow-derived Dendritic Cells (BMDC) from naïve Balb/c (purchased from Charles River (Calco, Italy)) mice have been generated according to \textit{Lutz MB et al.} (\textit{Lutz MB et al., J Immunol Methods 1999}). Briefly, after killing, tibiae and femurs were opened at both ends and bone marrow cells were extruded with cold RPMI 1640 (Invitrogen Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT, USA), 1mM sodium pyruvate, 0.1mM non-essential amino acids, 2mM L-glutamine, 25mM HEPES, 100U/ml penicillin, 100mg/ml streptomycin (all by Invitrogen Life Technologies) and 0.05mM 2-ME (Merck, Darmstadt, Germany; complete medium). Thereafter, $2 \times 10^6$ cells were cultured in a bacteriological Petri dish (100 mm diameter; LP-
Italiana, Milan, Italy) in a volume of 10ml complete medium supplemented with 20ng/ml of GM-CSF (PeproTech, Frankfurt, Germany). On day 3, 10ml of DC media containing 20ng/ml of GM-CSF were added to the dishes. On days 6 and 9, 5ml of medium were removed and 5ml of fresh complete medium containing 20ng/ml of GM-CSF were added. On day 11, non-adherent and loosely adherent cells were harvested, washed twice with cold PBS and used for the experiments with VSL#3. After preliminary kinetic and titration experiments of BMDC and VSL#3 co-incubation, in vitro studies were performed incubating 10^6 cells with 10^7 bacteria in 1ml of volume culture for 18h. LPS (1µg/ml) and medium alone were used as positive and negative controls, respectively.

4. Animals

Eight-week-old female C3H/HeJ and C57BL/6 mice were purchased from Charles River. Animals were housed in the Animal Care Unit of the Istituto Superiore di Sanità and treated according to the local guidelines for animal care (D.L. 116/92, which has implemented in Italy the requirements of the European Directive 86/609/EEC on laboratory animal welfare). Groups of 5-10 mice were treated according to the detailed protocols described in the following sections.

5. In vivo experimental protocols

VSL#3 administration to naïve mice. Naive mice were daily administered with 7.5 × 10^8 live bacteria resuspended in a total volume of 50µl per mouse through the oral route by using a Gilson micropipette (Gilson Italia, Milano, Italy) for 40 days. Control groups received 50µl of sterile PBS. Then, mice were killed, mesenteric lymph nodes (MLN) were collected and single-cell suspensions were prepared for DC and T cells ex vivo assay (see Figure 1PR).
Figure 1PR. VSL#3 administration in naive mice

VSL#3 daily oral treatment
7.5 x 10^8/mouse/day

0 40 41 43

MLN collection
ex vivo DC assays

T cell assays
(48h aCD3/28)
**Mice sensitization and challenge.**

**ST sensitization.** Eight-week-old female C3H/HeJ were sensitized by intragastric (i.g.) route with 100µg of ST associated to 10µg of cholera toxin (CT) as mucosal adjuvant in 400µl of ‘gavage buffer’ (PBS/sodium bicarbonate 7.5% weight/volume, 8:2 ratio) on days 0, 7, 14, 21 (see Figure 2PR). Blood samples from retro-orbital plexus were collected on days 0 and 28. On day 28, spleen isolation was done from a group of immunized animals to evaluate the in vitro VSL#3 modulation properties on an already established Th2 response. ST-sensitized mice were challenged on day 35 with 600µg of ST per mouse. The appearance of symptoms of systemic anaphylaxis was observed during 1h after challenge. Symptoms were evaluated according to the following scoring system reported by Li XM et al. *(Li XM et al., J Allergy Clin Immunol 1999):* 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhoea, pilar erection, reduced activity and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; 5, death. Faecal samples were also individually collected and processed as previously described *(Capobianco F et al., Int Immunol 2008),* to evaluate local IgA antibody response and histamine content.

**Peanut sensitization.** In the set-up phase of peanut allergy model, each mouse was immunized by i.g. administration of 5mg or 25mg of PFE total weight and 1mg or 10mg of PAE protein content plus 10 µg per mouse of cholera toxin (CT) (Gentaur, Brussels, Belgium) as adjuvant in 400µl of ‘gavage buffer’ on days 0, 7, 14, 21 (see Figure 3PR and 4PR). Blood samples from retro-orbital plexus were collected on days 0 and 25. PAE-sensitized mice were challenged one week after the last immunization (day 28) by receiving one i.g. dose of PAE (10mg/ per mouse). Symptoms were evaluated according to the scoring system reported by
Figure 2PR. Therapeutic treatment with VSL#3 in ST allergy mouse model
Figure 3PR. Therapeutic treatment with VSL#3 in peanut allergy mouse model
Figure 4PR. Preventive treatment with VSL#3 in peanut allergy mouse model

**I.G. immunizations**
1 mg of proteins/mouse of PAE + 10 μg per mouse of CT

**I.G. challenge**
(10 mg of proteins/mouse of PAE)

-9 0 7 14 21 23 28

VSL#3 daily oral treatment
7.5 x 10^8/mouse/day

Blood sampling (Ab assay)
Faecal collection (histamine and IgA assay)

Blood sampling (Ab assay)
Faecal collection (histamine and IgA assay)

Blood sampling (Ab assay)
Faecal collection (histamine and IgA assay)

Killing, tissue sampling (MLN, jejunum)
Sun et al and consisting in the following scheme: 0, no symptoms; 1, repetitive mouth/ear scratching and ear canal digging with hind legs; 2, puffiness around the eyes and mouth, diarrhoea, pilar erection, reduced activity and/or decreased activity with increased respiratory rate; 3, wheezing, laboured respiration and cyanosis around the mouth and the tail, periods of motionless for more than 1 min; 4, no response to whisker stimuli, reduced or no response to prodding; 5, endpoint: tremor, convulsion, death (Sun J et al., J Immunol 2007). Serum samples were individually collected within 1h after challenge to assess systemic histamine release.

The choice of the different symptom score systems to evaluate the anaphylactic reactions after antigen challenge is related to the different genetic background of mice used in the two food allergy mouse models.

**VSL#3 administration.**

**Therapeutic treatment of ST-allergic mice.** The daily oral VSL#3 treatment was performed from day 40 to day 60 in ST-sensitized and challenged mice. The probiotic mixture was administered as described for naive mice experiments. On day 65, the animals were re-challenged as described above. Faecal samples were collected within 1h to evaluate histamine release and post-treatment IgA antibody response. On day 67, mice were killed to collect jejunum.

**Therapeutic and preventive treatment of peanut-allergic mice.** In the therapeutic VSL#3 protocol (Figure 3PR) daily oral VSL#3 treatment was administered from day 29 to day 49 to PAE-sensitized and challenged mice. The probiotic mixture was received in the same conditions described above for ST-sensitized and challenged animals. Control groups received 50µl of sterile PBS. On day 50, mice were re-challenged as previously mentioned.
Serum and faecal samples were collected within 1h to evaluate histamine release and post-treatment IgA antibody response, respectively. On day 52, mice were killed to collect MLN and jejunum.

In the preventive setting (Figure 4PR), daily oral VSL#3 treatment was administered from day 9 to day 23 during the immunization period with PAE. The probiotic mixture was received in the same conditions already described. Control groups received 50µl of sterile PBS. On day 28, mice were challenged as previously described. Serum and faecal samples were collected within 1h to evaluate histamine release and post-treatment IgA antibody response, respectively. On day 29, mice were killed to collect MLN and jejunum.

_In vivo blockade of TGF-β in peanut allergy mouse model._ In a separate set of experiments, one day before the second challenge with PAE (day 51), VSL#3 therapeutically-treated mice were administered with the neutralizing anti-TGF-β1, 2, 3 antibody derived from the hybridoma cell line 2G75A9 (kindly donated by Dr. J. Letterio, Dept. Pediatrics, Case Western Reserve University, Cleveland, OH). Distinct subgroups of animals were injected through intraperitoneal route with 100µg of antibody per mouse in a total volume of 200µl of sterile PBS, or with 200µl of PBS vehicle as control.

6. **Measurement of histamine levels**

In ST allergy mouse model, histamine levels were determined on faecal samples using an enzyme immunoassay kit (Immunotech, Marseille, France), validated for the use on faecal material (Mastrangeli G et al., Int Arch Allergy Immunol 2009; Adel-Patient K et al., Allergy 2005). Faecal extracts were analyzed at different dilutions: 1:50 for pre-immune (day 0) and post-immunization (day 28) samples, 1:200 for post-challenge samples (day 35 and day 65).
In peanut allergy mouse model, histamine content was assayed on post-challenge sera from PAE-allergic and treated mice (as well as from PBS control group animals) by using the same enzyme immunoassay kit according to the manufacturers’ instructions. Sera samples were analyzed at 1:10 dilution.

7. Evaluation of local IgA antibody response in faecal extracts

Allergen-specific IgA response by individual mice was monitored by ELISA on faecal extracts according to Marinaro M et al. (Marinaro M et al., J Immunol 1995) with some modifications. Plates (Greiner Bio-One, Frickenhausen, Germany) were coated with purified ST (5µg/ml) or with PAE (20µg/ml) in PBS overnight at 4°C. After blocking with PBS/0.05% Tween 20/10% foetal bovine serum (FBS, Hyclone) (PBS/Tween/FBS), faecal extract samples were diluted 1:10, added in duplicates and incubated overnight at 4°C. Individual faecal extracts from naïve mice were used in parallel as controls. Bound IgA antibodies were detected by adding biotin-labelled rat monoclonal anti-mouse IgA (BD Biosciences PharMingen, San Diego, CA, USA) for 5h at room temperature, at the 1:3000 dilution, followed by avidin–peroxidase (Sigma-Aldrich, Milan, Italy) at 2.5µg/ml. PBS/Tween/FBS was used in all the steps as the ‘diluent buffer’. After enzyme reaction and colour development, results were expressed as AU/ml which were calculated from a standard curve obtained with a pool of ST or PAE-immune mouse faecal extracts.

Total IgA antibodies were evaluated in faecal extracts of individual mice by sandwich ELISA kit (BD Biosciences Pharmingen) according to the manufacturer’s instructions, and expressed as ng/ml.
8. Evaluation of allergen-specific antibody responses in serum samples

Systemic antibody responses against ST or PAE by individual mice were monitored by ELISA test. Briefly, plates (Greiner Bio-One) were coated with purified ST (5µg/ml) or PAE (20µg/ml) in carbonate/bicarbonate buffer, pH 9.6, for 3h at 37°C plus overnight at 4°C. Serum samples were added in duplicates (diluted 1:20 for IgE measurement, 1:200 for IgG1 and 1:50 for IgG2a) and incubated overnight at 4°C in the IgE ELISA or 2h at 37°C in the IgG ELISA. Pre-immune sera from each individual mouse were used in parallel as controls. Bound antibodies were detected by adding peroxidase-labelled rat monoclonal anti-mouse IgE (Southern Biotechnologies Associates, Birmingham, AL, USA) for 5h at room temperature or peroxidase-labelled rat monoclonal anti-mouse IgG1, IgG2a (BD Biosciences PharMingen) for 1h at 37°C. All the secondary antibodies were diluted 1:1000. The peroxidase substrate ortho-phenylenediamine chloride (Sigma–Aldrich) was then added, and absorbance was determined with an ELISA reader (Bio-Rad) at 490nm. Results were expressed as optical densities at 490nm (OD 490) or AU/ml which were calculated from a standard curve obtained with a pool of antigen-immune mouse serum samples.

9. In vitro stimulation of cells: cytokine production, fluorescent-activated cell sorting (FACS) analysis and intracellular cytokine staining.

Individual spleen cell suspensions from ST-sensitized mice were prepared and cultured as described in Capobianco F et al. (Capobianco F et al., Int Immunol 2008), with the optimal dose of ST (10µg/ml) alone or in combination with live VSL#3 (10⁷ bacteria/ml, as determined in previous set-up experiments) for 72h. Co-culture with live VSL#3 alone was also set as a control. Cell viability was checked by dye exclusion assay (cell count after Trypan blue staining) at 48h and 72h with different concentrations of VSL#3 (10⁶, 10⁷, 10⁸
bacteria/ml or medium alone). The supernatants were then collected and stored at -20°C for cytokine analysis. Levels of cytokines IL-5, IFN-γ, IL-10 and IL-13 (BD Biosciences Pharmingen) were determined by sandwich ELISA kits, according to the manufacturers’ instructions. Sensitivity for each assay was: 4pg/ml (IL-5 and IL-13), 30pg/ml (IL-10), 15pg/ml (IFN-γ). To identify T cell subpopulations, FACS analysis was performed using antibodies for T-cell surface markers, including anti-CD3 and anti-CD4 (eBiosciences, San Diego, CA, USA). To determine intracellular cytokine production, spleen cells from *in vitro* culture above described were incubated for further 4h with PMA (10ng/ml) and ionomycin (250ng/ml) (Sigma-Aldrich) in the presence of Golgi Stop (BD Biosciences). At the end of the stimulation period, cells were harvested, washed and stained for surface markers. Cells were fixed and permeabilized with BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer’s protocol, and then stained with anti-IFN-γ, anti-IL-4, anti-IL-10 and anti-IL-17 antibodies (BD Biosciences). The cells were analyzed using a FACScanto system (BD Biosciences), and the results were processed using BD FACSDiva software (BD Biosciences).

Individual MLN cell suspensions were prepared from naïve as well as PAE-allergic mice (after the challenge with PAE at day 50 and day 28 for therapeutic and preventive settings, respectively) untreated and treated with VSL#3 mixture, after aseptic removal and mincing of the lymphoid organs. Cells were re-suspended in RPMI 1640 (Invitrogen Life Technologies) supplemented with 1mM sodium pyruvate, 0.1mM non-essential amino acids, 2mM L-glutamine, 25mM HEPES, 100U/ml penicillin, 100mg/ml streptomycin (all from Invitrogen Life Technologies), 0.05mM 2-ME plus 10% FBS (complete medium) and counted. Cells were maintained in culture for 18h without stimuli and then stained for DC and T cell markers for FACS analysis as well as cytokine production in culture supernatants. FACS staining was performed using antibodies for cell surface markers, including anti-MHC II class,
anti-CD11c, anti-CD86 and anti-CD103 (eBiosciences). To determine intracellular cytokine production, MLN cells were incubated for 4h with PMA (10ng/ml) and ionomycin (250ng/ml) (Sigma-Aldrich) in the presence of Golgi Stop (BD Biosciences). Cells were harvested, washed and stained for surface markers. Cells were fixed and permeabilized with BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer’s protocol, and then stained with anti-IL-10 antibody (BD Biosciences). T cell analysis was performed using antibodies for cell surface markers, including anti-CD3, anti-CD4, anti-CD25 (eBiosciences). Cells were treated, fixed, permeabilized and stained with anti-IL-10 antibody (BD Biosciences) as described above for DC. The cells were analyzed using FACSCan system (BD Biosciences) and BD FACSDiva software (BD Biosciences).

The supernatants from MLN cell cultures were collected and stored at -20°C for cytokine analysis. Levels of cytokines IL-10 and TNF-α (BD Biosciences Pharmingen) were determined by sandwich ELISA kits, according to the manufacturers’ instructions. Sensitivity of ELISA assays was 30pg/ml for IL-10 and 8pg/ml for TNF-α.

The MLN cells isolated from PAE-sensitized and VSL#3-treated animals were also cultured for 4 days under antigen stimulation (PAE: 50µg/ml) or medium alone (as control) and the supernatants were tested for IL-13 production (BD Biosciences Pharmingen).

10. Cytokine expression in the gut

On the basis of previous studies (Capobianco F et al., Int Immunol 2008), at the end of the experiments (day 67 for ST allergy mouse model and day 52 for the peanut allergy model), a two-centimetre section of the jejunum immediately distal to the duodenum was collected from individual mice, to evaluate the effects of probiotic therapeutic treatment on the expression of cytokines at the local level. Two one-centimetre distinct fragments were cut
to evaluate gene expression and protein production. Naïve mice were used to determine basal level of cytokine mRNA and protein expression.

The fragment devoted to mRNA expression study was immediately placed in RNaLater RNA stabilization solution (Ambion, Austin, TX, USA) and treated according to the manufacturer’s instructions. Total RNA was extracted by using RNeasy Mini Kit (Qiagen Italy, Milan, Italy). Quantity and quality of RNA was assessed by measuring the A260/A280 absorbance ratio and by analysis on ethidium bromide-stained agarose gel. Total cDNA was obtained by retro-transcription of 500ng of RNA per 25µl final reaction volume by using the TaqMan Reverse Transcription Reagents kit (Applied Biosystem, Perkin-Elmer Italia, Milan, Italy) following the manufacturer’s protocol. In order to evaluate quantitative differences in cytokine gene expression in the jejunum of treated and control mice after probiotic treatment and in vivo anti-TGFβ blockade, Real-time PCR was performed on cDNA (Capobianco F et al., Int Immunol 2008) using the SYBR Green PCR Master Mix (Applied Biosystem) according to the manufacturer’s instructions. The following specific primers were used:

- **HPRT** (used as “normalizer gene”) forward CTGGTGAAAAGGACCTCTCG
- **HPRT** reverse TGAAGTACTCATTATAGTCAAGGGCA
- **FOXP3** forwardAGAGTTTCTTCCACAACATGGACTACTT
- **FOXP3** reverse GATGGCCCATCGGATAAAGG
- **TGF-β** forward ACCGCAACAACGCCATCTAT
- **TGF-β** reverse GCACTGCTTCCC
- **IL-13** forward AGACCAGACTCCCCTGTGCA
- **IL-13** reverse TGGGTCCTGTAGATGGCATTG
- **IL-17** forward AAGTGAGCTCCAGAAGGCC
IL-17 reverse GGTCTTCATTGCGGTGG

For Real-Time PCR testing IL-27 mRNA expression, commercially available primers were used. All the primers have been used at the final concentration of 0.4µM. Results for each gene have been analysed by comparison to the HPRT values. Fold increases have been calculated according to the $2^{-\Delta\Delta CT}$ method.

A second fragment was used to prepare total protein extracts according to Boirivant M et al. (Boirivant M et al., Gastroenterology 2006). Briefly, tissue fragments were lysed in a suitable volume of lysis buffer (Hepes 0.01M pH 7.9, EDTA 0.001M, KCl 0.06M, Nonidet P40 0.2%, dithiothreitol 0.001M, phenylmethylsulfonyl fluoride 0.001M, aprotinin 10µg/ml, leupeptin 10µg/ml, Na$_3$VO$_4$ 0.001M, NaF 0.001M) (Sigma-Aldrich), incubated 1h on ice, under gentle shaking every 5min. After a centrifugation of 30min (16 000g, 4°C), supernatants were collected and stored at -80°C. Total protein content was evaluated by the Bio-Rad protein assay (Bio-Rad). Jejunum extracts were then analyzed for the content of IL-10, TGF-β, IL-4, IL-5, IL-13 and IFN-γ by sandwich ELISA kits (BD Biosciences Pharmingen), according to the manufacturers’ instructions. Sensitivity for TGF-β assay was 4.61pg/ml. Results were expressed as pg/mg of total protein.

A third fragment from PAE-allergic, treated and administered with anti-TGF-β blocking antibody was fixed o.n. in 10% formalin, then transferred in sterile PBS and stored at +4°C for histological and immunohistochemical analysis.

11. Histology and immunohistochemistry in the jejunum

A third one-centimetre distinct fragment from jejunum was cut in the mice of peanut allergy model to perform histological analysis. Naïve mice were used to determine basal level of histological markers expression.
For histology and immunohistochemistry analysis, intestinal samples were removed and frozen in liquid nitrogen or fixed in 10% buffered formalin for 48h, processed, and embedded in paraffin.

Sections, 3μm thick, were stained with hematoxylin-eosin, Toluidine blue for mastocytes evaluation, or processed for immunohistochemistry. For the immunohistochemical study, gut sections were allowed to adhere to pretreated slides (Bioptica, Milan, Italy) and then deparaffinized and rehydrated. Endogenous peroxidase activity was removed by incubation with 0.5% hydrogen peroxide in distilled water for 1h at room temperature. The following primary monoclonal (MAb) and polyclonal (PAb) antibodies were employed: a rat anti-human CD3 MAb (IgG1, clone CD3–12, MCA1477, AbD Serotec, Oxford, UK), diluted 1:10 in PBS; a rat anti-mouse FOXP3 MAb (IgG2a kappa, clone FJK16S, 14–5773, e-Bioscience), diluted 1:50; a goat anti-human latency-associated peptide (LAP)/TGF-β1 PAb (AF-246-NA, R&D Systems), diluted 1:100; a rabbit anti-mouse TLR9/CD289 PAb (Creative Biomart, Shirley, NY, USA), diluted 1:100. The following matched isotype controls have been respectively used: rat IgG1 MAb (clone YNB46.1.8, MA1–90035, Thermo Scientific Pierce Antibodies, Rockford, IL, USA); rat IgG2a MAb (PA5–33212, Thermo Scientific Pierce Antibodies); normal goat polyclonal IgG control (AB-108-C, R&D Systems); normal rabbit polyclonal IgG (AB-105-C, R&D Systems). Biotinylated rabbit anti-rat IgG (BA-4000, Vector Laboratories, Burlingame, CA), rabbit anti-goat IgG (BA-5000, Vector Laboratories), and goat anti-rabbit IgG (E0432, DAKO, Glostrup, Denmark), were used as secondary antibodies at 1:200-dilution.

Tissue sections were incubated overnight in a moist chamber at 4°C with the different primary antibodies diluted as above indicated, in Tris-buffered saline (TBS) containing 0.1% crystalline bovine serum albumin. Secondary antibodies were then applied for 45min at r.t.. After two 5-min rinses with TBS, tissue sections which had been incubated with primary
antibodies, were incubated with the avidin-biotin-peroxidase complex (Vector Laboratories) diluted 1:50 for 45 min at room temperature. The immunoreactions were then revealed by using the 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as chromogen. Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted. Specific primary antibodies replaced with TBS or nonimmune sera were used as negative controls in immunohistochemical techniques.

Histological examination included assessment of different parameters as: oedema, villi Ø, villi height, mononuclear cells infiltration, mastocytes infiltration, and GALT activity, evaluated by the mean Ø of lymphoid follicles associated to the intestinal mucosa. As these parameters varied from a score 0 to score 3, on the basis of their severity, according to parameters explicated by the pathologist in previous works (Cersini A et al., Infect Immun 2003; Martino MC et al., J Infect Dis 2005).

Histological criteria for normal jejunal tract characteristics included detection of no or only a few mononuclear cells per HPF and no or only a few scattered neutrophils or mastocytes interspersed throughout the mucosal lamina propria, without tissue changes (no interstitial thickening or oedema, and GALT activation).

For the immunohistochemical evaluation, each tissue sample was subdivided in five areas. Three fields per area were randomly selected at 40HPFs, and all the cells stained by the primary antibody were counted and recorded.

12. Statistical analysis

Groups of 5-10 mice/treatment were studied throughout the study. Data were presented as means of individual samples with SEM. The Mann-Whitney U test (rank sum) or the Wilcoxon matched pairs test when appropriate were used for the nonparametric analysis
of differences between different experimental groups of mice or different treatments, respectively. $P$ values of less than 0.05 were considered statistically significant.
RESULTS

1. In vitro studies

**VSL#3 in vitro shapes functions of immature DC and spleen cells from naïve mice**

In order to investigate the effects of VSL#3 bacteria on DC which are one of first cell type encountered by foreign agents at mucosal sites, we examined the ability of different amounts of the probiotic preparation to induce *in vitro* maturation and cytokine production in mouse BMDC differentiated starting from bone marrow precursors. Immature BMDC from naïve mice were left untreated or incubated with VSL#3. LPS, a well known agent enabling the maturation of DC, was used as positive control. The effects of VSL#3 on DC maturation were time- and dose-dependent and were maximal after 18 h of co-culture at the dose of $10^7$ bacteria, as established by preliminary kinetic and titration experiments. As shown in **Figure 1R (panel A)**, VSL#3 co-culture induced a significant up-regulation of surface expression of the activation/maturation- associated molecules CD83 and CD86 as well as MHC class II on DC. At the same dose, the bacteria induced a significant production of IL-10 and IL-12p70 cytokines by BMDC, significantly higher than those obtained upon LPS stimulation (**Figure 1R, panel B**). Thus, the probiotic mixture was as effective as the LPS control in inducing DC maturation and the release of polarizing cytokines by these cells.

The potential of VSL#3 to shape *in vitro* T cells profile was studied by evaluating cytokine production by spleen cells from naïve mice incubated with $10^7$ VSL#3 bacteria for 72 h. The probiotic mixture had the capacity to elicit a significant Th1/Treg-type cytokine profile characterized by IL-10 and IFN-γ production (**Figure 2R**), whereas no IL-5 production was induced (data not shown).
Figure 1R: VSL#3 shaping of BMDC functions. A) expression of maturation associated markers on the cells analyzed by FACS. B) production of cytokines by BMDC tested by ELISA assay. Experiments were independently repeated at least four times and each bar represents the mean ± SEM of data from all experimental replicates.

Figure 2R: Immunomodulatory activity of VSL#3 on spleen cells from naive mice. IL-10 and IFN-γ production by spleen cells from naive mice, stimulated in vitro with live VSL#3 bacteria or LPS, was evaluated by ELISA test in culture supernatants. Experiments were independently repeated at least four times and each bar represents the mean ± SEM of data from all experimental replicates.
These data indicate that VSL#3 is able to modulate the phenotype of both innate and adaptive immunity cells in the ‘steady state’ condition of naïve mice towards a regulatory/Th1 profile.

2. *In vivo* studies on naïve mice

**VSL#3 administration induces a regulatory environment in mesenteric lymph nodes (MLN) of naïve mice**

The next step was to evaluate the potential of an *in vivo* VSL#3 oral administration in affecting the cell environment in mesenteric lymph nodes (MLN) which are the gut-draining lymph nodes. Naïve C57BL/6 mice were daily fed by oral route with VSL#3 for 40 days, according to the protocol reported in Figure 1PR and MLN were isolated on day 41.

We wanted to test the VSL#3 potential to *in vivo* modulate the functions of local DC. We identified DC as CD11c and MHC class II double-positive cells in the mononuclear cell population isolated from MLN of VSL#3-fed mice. We found a significant increase in the frequency of CD11c+MHCII+ DC in probiotic-administered animals compared to PBS control group (Figure 3R, top). DC population was analyzed for its potential to be prone for the co-stimulation and to produce cytokines. *In vivo* probiotic treatment induced DC maturation as evidenced by increased frequency of CD86-expressing DC, as well as the induction of IL-10 in such cells (Figure 3R, middle).

Also a significantly higher frequency of CD4+CD25+ IL-10-secreting T cells was recovered from *ex vivo*-isolated MLN of VSL#3-fed naïve mice compared to PBS group MLN (Figure 3R, bottom).
**Figure 3R:** VSL#3-treated naïve mice showed a regulatory environment in the MLN. The probiotic feeding induced a significant increase of MHCII+CD11c+ DC (upper) with a greater portion of DC expressing CD86 and IL-10 positive in VSL#3 fed mice compared to PBS control group (middle). Also a significant increase of CD4+CD25+IL-10+ T cells was induced by probiotic mixture administration in naïve mice (bottom). Data are reported as mean ± SEM for n=5 mice/group. Data are representative of three separate experiments.
These results indicate that the oral feeding with VSL#3 preparation is able to shape the DC functions also in in vivo ‘steady-state conditions’ of gut-draining MLN from naïve mice. In particular, it induces the maturation of DC and a regulatory environment characterized by the production of IL-10 by both DC and T cells.

3. In vitro studies on cells from immunized mice

VSL#3 modulates in vitro Th2-polarized spleen cells

After the results obtained on immature DC and spleen cells in in vitro as well as in in vivo experiments on the ‘steady-state’ conditions of naïve mice, we decided to evaluate the potential of VSL#3 to modulate also Th2-polarized responses both in vitro and in vivo.

In vitro Th2 polarization induction by an allergen such as ST was the first step in order to achieve this goal. Purified ST was available in our laboratory since its purification from Metapenaeus ensis was performed for the development of ST allergy mouse model in C3H/HeJ strain, in vitro systems and ELISA tests (Capobianco et al., Int Immunol 2008). In order to study the response type induced by VSL#3 on immune cells in vitro, C3H/HeJ mice were sensitized with ST according to the protocol described in Capobianco F et al. (Capobianco et al., Int Immunol 2008). Spleens were aseptically removed from mice after four immunizations at day 28 (see Figure 2PR). Spleen cells were re-stimulated in vitro with the same allergen in the presence of 10⁷ live VSL#3 bacteria. Cell viability was checked at 48h and 72h by dye exclusion assay with different concentrations of VSL#3, and 74.96% ± 8.65 (mean ± SEM) of viable cells were recovered, without significant differences among the various culture conditions (data not shown). High IL-5 and IL-13 production in culture supernatants was induced in response to allergen-specific re-stimulation (Figure 4R, panel A, top), as expected in a typical Th2 response. The co-incubation with VSL#3 preparation significantly
(P < 0.05) reduced allergen-stimulated IL-5 and IL-13 production. Reduction of Th2 cytokines was associated with a significant increase of IFN-γ production by spleen cells stimulated with the antigen in the presence of live VSL#3 bacteria, which were also able to significantly increase IL-10 production both in the presence or in the absence of the antigen (Figure 4R, panel A, bottom).

Intracellular cytokine staining for IL-4, IFN-γ, IL-17 and IL-10 showed that the frequency of IFN-γ+ and IL-10+ CD4+ T cells was significantly (P < 0.05) increased by VSL#3 co-incubation during allergen re-stimulation, whereas the frequency of IL-4+ CD4+ T cell did not change in the presence of VSL#3. IL-17 expression by CD4+ T cells was not induced at spleen level by allergen re-stimulation nor affected by VSL#3 co-culture (Figure 4R, panel B and C).

Altogether, these results indicate that VSL#3 co-culture is able to induce in spleen cells from sensitized mice a Th1/T regulatory-type response associated with a reduction of the antigen-specific Th2 cytokine production.

4. In vivo modulation of a mouse model of food allergy to shrimp tropomyosin

Oral therapeutic administration of VSL#3 after protect mice against anaphylactic reactions after food allergen sensitization

The interesting results of previously described in vitro studies on immunomodulatory properties of VSL#3 encouraged us to investigate whether the probiotic mixture was able to affect an in vivo established allergen-specific Th2 response, when administered by the oral route in an experimental therapeutic setting to allergic mice. A VSL#3 oral treatment was performed in a mouse model of food allergy to ST in C3H/HeJ mouse strain, previously developed in our laboratory (Capobianco F et al. 2008) (see Figure 2PR).
Figure 4R: VSL#3 modulated *in vitro* allergen-induced cytokine production by immune spleen cells. *In vitro* co-culture with VSL#3 modulates allergen-induced T cell responses in spleen cells from ST-sensitized mice, in vitro re-stimulated with the allergen in the presence or absence of live VSL#3. A) IL-5, IL-13, IFN-γ and IL-10 production by individual spleen cells from ST-sensitized mice, was evaluated by ELISA in culture supernatants. Experiments were independently repeated at least four times and each bar represents mean ± SEM of data from all experimental replicates.
Figure 4R: VSL#3 modulated *in vitro* allergen-induced cytokine production by immune spleen cells. *In vitro* co-culture with VSL#3 modulates allergen-induced T cell responses in spleen cells from ST-sensitized mice, *in vitro* re-stimulated with the allergen in the presence or absence of live VSL#3. B) The frequency of IL-4+, IFN-γ+, IL-10+ and IL-17+ CD3+ CD4+ T cells was evaluated by FACS. C) A representative FACS diagram for IL-4+ and IFN-γ+ frequency in CD3+CD4+ gated cells. Experiments were independently repeated at least four times and each bar represents mean ± SEM of data from all experimental replicates.
Briefly, after four immunizations with 100µg of ST antigen associated to cholera toxin (CT) as adjuvant, ST-allergic mice were re-challenged by intragastric route with 600µg of ST and were observed for 1h after the challenge to evaluate allergen-induced local and systemic anaphylaxis. Symptoms were assessed applying a system of symptomatic score, derived from the literature (see “Materials and Methods” section) which gives a score between 0 (no symptoms) and 5 (death) depending on the observed anaphylactic reactions. Upon this first allergen challenge, ST-sensitized mice showed anaphylactic symptoms with severity ranging from 2 to 4. Then, mice were fed for three weeks with daily oral administrations of VSL#3 or PBS as control vehicle. Three days after the last oral treatment, the animals were re-challenged with allergen and the anaphylactic reactions were monitored and scored as in the first challenge. Symptom score was evaluated between the experimental groups (VSL#3- and PBS-fed mice) and compared with that one recorded before probiotic treatment. The therapeutic treatment with VSL#3 reduced the symptom score as compared with that one recorded in the control mice treated with PBS and with the score recorded in the animals before VSL#3 administration (Figure 5R). Histamine levels were evaluated in the faecal extracts samples obtained from faeces collected from mice during the challenge-induced anaphylactic reactions. Accordingly with the evaluation of the symptoms, gut histamine levels were decreased in the VSL#3-treated groups (Figure 6R).

The protection against anaphylaxis was associated at gut level with an increase of total IgA levels in the faecal extracts of ST-allergic mice treated with VSL#3 when compared with those found before treatment, whereas no significant changes were induced in control mice (Figure 7R). The ST-specific IgA levels resulted also increased upon the VSL#3 treatment but not at a significant level.
**Figure 5R.** VSL#3 modulation of anaphylactic symptoms in the mouse model of food allergy to ST. VSL#3 therapeutic treatment reduced the symptom score as compared with the score recorded in the control mice treated with PBS. Data are reported as individual values from a representative experiment out of three.

**Figure 6R.** VSL#3 modulation of histamine levels. VSL#3 therapeutic treatment reduced the histamine levels in faeces of ST-allergic animals compared to PBS control group and the mice before VSL#3 administration. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
Figure 7R. VSL#3 induced a protective IgA response. In the shrimp tropomyosin allergy model, probiotics treatment increased total IgA production at the gut level. Also the ST-specific IgA levels were increased by VSL#3 treatment but not in a significant manner. Total and ST-specific IgA were evaluated in faecal samples collected before (day 28) and after (day 65) therapeutic treatment, from groups of mice treated with VSL#3 (right) or PBS (left) as control. Data are reported as individual values from a representative experiment out of five.
Thus, oral therapeutic administration of VSL#3 after ST sensitization protect mice against systemic and local anaphylactic reactions and induces a protective IgA antibody response at gut level.

**Oral treatment with VSL#3 in ST-sensitized mice down-regulates Th2 allergic inflammation in the jejunum by inducing a mixed Th1/T regulatory response.**

At the day of sacrifice, jejunum sections were isolated from therapeutically-treated ST-allergic mice and PBS control group to evaluate the changes induced *in situ*. The choice of jejunum is due to previous investigations in which it was identified as the site preferentially affected by the oral sensitization with ST food antigen, where it induced the skewing of the response towards a prevalent Th2-like phenotype (*Capobianco F et al., Int Immunol 2008*).

In ST-allergic mice, VSL#3 therapeutic treatment attenuated Th2 inflammation in the jejunum. Therapeutic treatment with VSL#3 down-regulated the tropomyosin-induced Th2 response at the gut level, as indicated by the significant decrease in IL-4, IL-5 and IL-13 tissue content when compared with the amount measured in mice treated with PBS after sensitization (*Figure 8R, top*). According to the results of the *in vitro* studies, probiotic *in vivo* modulation induced a shift towards a prevalent T reg/Th1 response in the jejunum, characterized by increased FOXP3 mRNA expression, increased IL-10 and TGF-β tissue content (*Figure 8R, middle*), increased IL-27 mRNA expression and IFN-γ tissue content (*Figure 8R, bottom*). IL-17 mRNA expression in the jejunum was significantly increased in ST-sensitized and challenged mice, in comparison with naïve mice (data not shown), but it was not modulated by the probiotic treatment (*Figure 8R, bottom*).

Then, the suppression by VSL#3 therapeutic treatment in ST-allergic mice is associated with an induction of a mixed Th1/T regulatory response in the jejunum, mainly
Figure 8R. Therapeutic treatment with VSL#3 down-regulated the Th2 response in the jejunum tissue, where it induced a prevalent Th1/T regulatory profile. Th2 (upper), T regulatory (middle) and Th1/Th17 (bottom) polarization was evaluated in whole tissue homogenate from jejunum samples of individual mice. Cytokine (IL-4, IL-5, IL-13, IL-10, TGF-β, IFN-γ) content was evaluated by sandwich ELISA kit. Results are reported as mean ± SEM for n=5 mice/group. FOXP3, IL-17 and IL-27 mRNA expression in the same tissue samples from individual mice was assessed by real-time PCR. All the results were normalized against HPRT mRNA expression and are reported as mean ± SEM for n=5 mice/group. Data are representative of three separate experiments.
mirrored by an increase of IFN-γ, TGF-β release and up-regulation of FOXP3 mRNA expression, respectively.

**VSL#3 modulates the ST-specific systemic antibody response**

In the ST allergy mouse model, the down-regulation of local ST-induced Th2 response by VSL#3 was paralleled by changes of ST-specific antibody response in serum. After therapeutic treatment with the probiotic mixture, mice that received VSL#3 showed significant decreased levels of ST-specific IgE and increased levels of allergen-specific IgG2a (Figure 9R top and middle), whereas control mice showed no significant changes in the levels of serum ST-specific antibody. No changes were observed in ST-specific IgG1 levels upon VSL#3 treatment (Figure 9R, bottom).

These data support the broad effect of the probiotic mixture in modulating the ST-induced Th2 response not only at local but also systemic level.

5. *In vivo* studies on VSL#3 mechanism of action in a mouse model of peanut allergy

**Development of a peanut allergy mouse model: selection of the allergenic extract and experimental schedule for optimal sensitization**

Before their use in *in vitro* systems and *in vivo* development of a mouse model of peanut allergy in C57BL/6 mice, two types of seed extracts from *Arachis hypogaea* obtained according to the procedures described in “Materials and Methods” section were analyzed qualitatively by electrophoresis on polyacrylamide gel in the presence of SDS (SDS-PAGE) under reducing conditions. Figure 10R shows the electrophoretic pattern of peanut flour extract (PFE)
Figure 9R. Probiotic treatment decreased ST-specific IgE levels and increased ST-specific IgG2a in the serum. ST-specific antibodies were evaluated in serum samples collected before (day 28) and after (day 65) therapeutic treatment, from groups of mice treated with VSL#3 (right) or PBS (left) as control. Data are reported as individual values from a representative experiment out of five.
Figure 10R: Electrophoresis pattern of two different peanut extracts. The peanut flour extract (PF) compared to peanut aqueous extract (PAE) after Coomassie G-250 staining. Left arrows indicate the bands corresponding to standard molecular weights. Right arrows indicate the bands corresponding to the major peanut allergens.

Figure 11R: IgE responses induced by peanut extracts. Four doses of 1 mg protein per mouse of peanut aqueous extract (PAE) weekly administered was the best schedule to obtain an optimal intragastric sensitization. Data are reported as mean ± SEM for n=5 mice/group. Data are representative of three separate experiments.
compared to the peanut aqueous extract (PAE), after Coomassie G-250 staining. Both types of peanut extracts had similar molecular components between 97 and 14 kDa.

In the development phase of a peanut allergy mouse model, C57BL/6 mice were divided into four groups administered with different doses of antigen, prepared according to the procedure described in "Materials and Methods" section. In order to identify the best type of extract and the optimal dose to obtain an oral sensitization to peanut, the groups A and B were immunized with 1 mg and 10 mg of protein per mouse (contained in the PAE), respectively. C and D groups were administered with 5 mg and 25 mg of PFE, respectively. The allergen was administered by intragastric route (gavage), in the presence of 10μg of cholera toxin (CT) per mouse as mucosal adjuvant on days 0, 7, 14, 21 (see Figure 3PR and 4PR). Individual sera from mice collected after the four immunizations were analyzed by ELISA test to determine the highest antigen-specific IgE response. As shown in Figure 11R, the A group immunized with 1mg of PAE showed a marked increase in allergen-specific IgE levels compared to control animals. As expected, control mice administered only with 10μg of CT per mouse did not show allergen-specific IgE. This result suggested that four doses of 1 mg of PAE protein content per mouse weekly administered was the best schedule to obtain an optimal intragastric sensitization. The optimal dose for allergen challenge by intragastric route to obtain systemic and local anaphylaxis was also established as 10mg of PAE protein content.

The VSL#3 therapeutic treatment is effective to modulate the systemic anaphylaxis induced by the challenge with peanut extract.

Once the mouse model of peanut allergy was established, studies on the therapeutic and preventive potential as well as on the mechanisms underlying the benefits of VSL#3 mixture were performed.
According to the experimental therapeutic setting, after four immunizations with 1mg of PAE associated to cholera toxin (CT) as adjuvant, peanut allergic mice were re-challenged by intragastric route with 10mg of PAE (see Figure 3PR) and were observed for 1h after the challenge to evaluate allergen-induced anaphylactic reactions. A symptom score derived from the literature (see “Materials and Methods” section), ranging from 0 (no symptoms) to 5 (death) depending on the observed anaphylactic reactions, was applied. Upon this first allergen challenge, PAE-sensitized animals showed anaphylactic symptoms with severity ranging from 1 to 3. Then, mice were treated for three weeks with daily oral administrations of VSL#3 or PBS as control vehicle. Three days after the last oral treatment, the animals were re-challenged with 10mg of PAE protein content and the anaphylactic reactions were monitored and scored as for the first challenge. For each individual mice in any experimental group (VSL#3- and PBS-fed mice), symptom score was compared with that one recorded before probiotic treatment. Therapeutic treatment with VSL#3 reduced the symptom score in the probiotic-administered allergic animals whereas no changes were recorded in the score of control group after the PBS administrations (Figure 12R). Histamine levels were evaluated in the serum samples collected during the challenge-induced anaphylactic reactions. Accordingly with the evaluation of the symptoms, serum histamine levels were decreased in the VSL#3-treated group (Figure 13R).

These results demonstrated that the treatment with VSL#3 was able to modulate the systemic anaphylaxis induced by the challenge with allergen.
Figure 12R. **VSL#3 modulation of anaphylactic symptoms.** In peanut allergy model, VSL#3 therapeutic administration reduced the symptom score recorded after the challenge performed after the treatment period. No changes in the symptom score were revealed before and after the PBS administration in the control group. Data are reported as individual values from a representative experiment out of three.

Figure 13R. **VSL#3 modulation of histamine levels.** In the peanut allergy model, VSL#3 therapeutic treatment reduced the histamine levels in the sera of allergic mice when compared with the levels measured in the PBS control group. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
**Oral therapeutic treatment with VSL#3 induces a regulatory response in MLN of peanut-allergic mice**

In the same therapeutic setting of experiments, the VSL#3 potential to affect cell environment in the gut-draining MLN of PAE-sensitized mice was investigated. At day 52 (see Figure 3PR), MLN were isolated and processed after the killing of treated and re-challenged mice.

As in *ex vivo* studies on MLN of naïve VSL#3-treated mice, a significant higher frequency of CD11c^+^MHCII^+^ DC was found in the gut draining lymph nodes of allergic treated mice compared to PBS control group. Furthermore, taking into account the association between the expression of CD103 (integrin αIEL chain) and the induction of gut tolerance (*Worthington JJ et al., Gastroenterology 2011*), a study on CD11c^+^MHCII^+^ DC population for the expression of this marker was performed. It showed that VSL#3 treatment was able to significantly increase the frequency of CD103^+^ DC (Figure 14R, panel A) in the MLN of VSL#3-treated PAE-allergic mice compared to PBS control animals.

Supernatants from 18h MLN cell cultures contained significantly increased levels of IL-10 and decreased levels of inflammatory TNF-α in the VSL#3-treated group as compared with PBS-treated mice (Figure 14R, panel B).

MLN cells were also re-stimulated *in vitro* with PAE for 96h to assess antigen-specific T cell response. Antigen-induced IL-13 production was significantly reduced in probiotic-treated mice as compared with PBS-fed group (Figure 14R, panel C).

These results support the potential of VSL#3 therapeutic treatment to promote regulatory responses in the gut-draining LN of allergic mice.
Figure 14R. VSL#3 induced a regulatory environment and decrease inflammation in MLN of allergic mice. A) Mononuclear cell population was isolated from VSL#3-treated peanut-allergic mice. FACS analysis showed a significant increase of CD103+DC in the ex-vivo isolated MLN of probiotic-treated animals compared to PBS controls. In the FACS representative diagram, data are reported as mean ± SEM for n=5 mice per group. Data are representative of three separate experiments.
Figure 14R. VSL#3 induced a regulatory environment and decreased inflammation in MLN of allergic mice. B) Supernatants of MLN cells stimulated for 18h without any stimulus contained significantly increased levels of IL-10 and decreased levels of inflammatory TNF-α in the VSL#3-treated group as compared with PBS-treated mice. C) MLN cells were also in vitro re-stimulated with PAE for 4 days to assess antigen-specific T cell response. Antigen-induced IL-13 production was significantly reduced in probiotic-treated mice. Data are reported as mean ± SEM for n=5 mice per group. Data are representative of three separate experiments.
Oral treatment with VSL#3 in sensitized mice down-regulates Th2 allergic inflammation in the jejunum by inducing a T regulatory response

In peanut sensitized and challenged animals, the reduced severity of anaphylaxis after probiotic therapeutic treatment was associated with a significant decrease of both gene expression and protein level of IL-13 in the jejunum (Figure 15R, panel A), thus suggesting that oral therapeutic administration of VSL#3 was able to attenuate allergen-induced anaphylactic reactions and to suppress Th2-mediated allergic inflammation in the intestinal mucosa. No changes in IFN-γ levels in the jejunum of peanut-allergic mice therapeutically treated with VSL#3 were revealed when compared to PBS control group (Figure 15R, panel B). This suggested that unlike the ST- allergy mouse model, in this case a Th1 response induction was not associated with the VSL#3 therapeutic benefits. To further address the characteristics of the regulatory response promoted by probiotic treatment in PAE-allergic mice, TGF-β production in the jejunum of sensitized and treated mice was investigated. Both TGF-β gene expression and protein production was significantly up-regulated in the jejunum mucosa of VSL#3-fed mice, as resulted by quantitative RealTime PCR analysis and protein assay in tissue homogenates, respectively (Figure 15R, panel C). The up-regulation of TGF-β was associated with a significant increase of PAE-specific IgA antibodies in the gut (faecal extracts) induced by VSL#3 treatment in allergic mice compared with the levels of PBS control group (Figure 16R).

To further analyze the effect of in vivo probiotic therapy at the gut level, we performed histological and immunohistochemical analysis of jejunum sections of PAE-allergic treated mice to evaluate mast cell infiltration and activation. The VSL#3 administration induced a significant reduction on histological score of inflammation parameters as oedema, villi height and mast cells infiltrate at local level (Figure 17R, panel A) as compared with PBS vehicle administration. The reduction of mast cells infiltrate upon probiotic treatment was confirmed
Figure 15R. VSL#3 modulation of Th2 inflammation in the jejunum. A) VSL#3 treatment induced a down-regulation of IL-13 cytokine level and its mRNA expression in the jejunum. B) Although not significantly, the mixture induced IFN-γ release and C) a significant increase of TGF-β protein content and mRNA expression at local level. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
Figure 16R. VSL#3 induced protective IgA response. In the peanut allergy model, VSL#3 increased the peanut-specific IgA levels in the faeces of allergic mice when compared with the same antibody levels measured in the faecal extracts of PBS control group. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
**Figure 17R. VSL#3 therapeutic treatment reduced allergic inflammation in the jejunum.** Panel A) Oedema, villi height and mast cells infiltrate were significantly reduced upon VSL#3 treatment in PAE-allergic mice and reported to naive score level. Data are reported as mean ± SEM. Data are the combined results of three separate experiments. *p* is referred to VSL#3 group vs PBS controls.
also by histological/immunohistochemical analysis with Toluidine Blue and anti-CD117 staining, respectively. In fact, a highly significant reduction of mast cell recruitment \textit{in situ} and down-regulation of their activation (as demonstrated by CD117 down-regulation) was observed in the jejunum of VSL\#3-treated mice, restoring the naïve condition (Figure 17R, panel B and C).

The same intestinal tract was studied for the expression of key cell markers of regulatory responses. In particular, immunohistochemical analysis of sections confirmed the induction of a regulatory pathway triggered by VSL\#3 treatment. CD3$^+$/FOXP3$^+$ T cells were significantly enriched in the jejunum of VSL\#3-treated mice (Figure 18R, panels A and B). A significantly higher number of CD3$^+$ T cells expressing latency-associated peptide (LAP) was also induced by VSL\#3 treatment as compared with PBS controls.

Notably, these cells were arranged in sub-epithelial lymphoid aggregates, as found by histomorphological analysis in the jejunal tract of VSL\#3-treated mice, not observed in PBS-treated or naïve groups (Figure 18R).

Then, these data suggest that the regulatory environment established by VSL\#3 in the MLN is the key to promote a suppression of Th2 allergic inflammation induced by peanut at jejunum level, and point to a central role played by probiotic-induced TGF-$\beta$ in the induction of a regulatory cell population and in the control of Th2-driven inflammation in the jejunum of peanut allergic-mice.

\textit{In vivo} TGF-$\beta$ blockade abrogates VSL\#3 therapeutic benefits

Because of the involvement of TGF-$\beta$ as regulatory cytokine locally induced by probiotic therapeutic treatment, we decided to investigate its role in the protective effect of VSL\#3 in peanut allergy mouse model. We tested this hypothesis by using a neutralizing anti-
Figure 17R. VSL#3 therapeutic treatment reduces mast cell infiltration and activation in the jejunum. Panel B) diagram and C) sections: a highly significant reduction of mast cell infiltration, evaluated by Toluidin Blue staining, and activation, as demonstrated by CD117 down-regulation, in the jejunum of VSL#3-treated mice, restoring the naïve condition. Aggregated data on mean counts are reported in B panel: values, representative of three separate experiments, are presented as mean ± SEM from six individual mice/group.
Figure 18R. The induction of a regulatory pathway by VSL#3 oral treatment was confirmed by immuno-histochemical analysis of the jejunum. A) diagram and B) sections: CD25+/FOXP3+ regulatory T cells were significantly enriched in the jejunum of VSL#3-treated mice. This correlated with the CD103+ DC found in MLN which have been associated with Foxp3+ T cells induction at gut level. These cells expressed surface TGF-β in the form of latency-associated peptide (LAP). Aggregated data on mean counts are reported in A panel: values, representative of three separate experiments, are presented as mean ± SEM from six individual mice/group.
TGF-β1, 2, 3 monoclonal antibody that blocked the activity of this cytokine. *In vivo* administration of this antibody (see Figure 3PR) to peanut allergic mice treated with VSL#3 one day before PAE re-challenge (day 51) abrogated the capacity of probiotics to control anaphylaxis and Th2 inflammation, as indicated by histamine serum levels and IL-13 gene expression in the jejunum, respectively (Figure 19R). Accordingly, the administration of anti-TGF-β significantly abrogated the inhibitory effect of VSL#3 on histological score of parameters as oedema and villi height in the jejunum of PAE allergic probiotic-treated animals (Figure 20R, panel A). Histological and immunohistochemical analysis also showed a significant increase in mast cell infiltration and activation after TGF-β blockade (Figure 20R, panel B and C).

Conversely, the administration of TGF-β blocking antibody counteracted the regulatory response induced by probiotic treatment in the jejunum mucosa, significantly reducing the presence of CD3⁺FOXP3⁺LAP⁺ T cells *in situ* (Figure 21R).

Upon VSL#3 treatment of PAE-allergic mice, we also observed the up-regulation of other markers in the gut. It is well known that VSL#3 is able to act through TLR-9 stimulation in mouse colitis because of immunostimulatory microbial DNA interaction with this PRR expressed on the intestinal epithelium or immune cells (DC, lymphocytes, NK cells) *(Rachmilewitz D et al., Gastroenterology 2004)*. In PAE-allergic mice, TLR-9 expression was up-regulated by probiotic therapy as demonstrated by immunohistochemical analysis (Figure 22R, panel A, left) but this effect appeared to be TGF-β-independent as suggested by the absence of significant difference in TLR9 expression between VSL#3-treated allergic mice receiving or not TGF-β blocking antibodies (Figure 22R, panel B, left). We performed also a study on CD127 (IL-7Rα) expression in the jejunum section and it revealed that this marker was up-regulated by VSL#3 treatment (Figure 22R, panel A, middle). This feature paralleled the probiotic-mediated up-regulation of CD103 integrin on the same intestinal tract.
Figure 19R. In vivo blocking of TGF-β before challenge abrogated the suppression of Th2 allergic response induced by VSL#3 treatment. *In vivo* administration of anti-TGF-β to peanut allergic mice treated with VSL#3 before PE re-challenge abrogated the capacity of probiotics to control anaphylaxis and Th2 inflammation, as indicated by histamine serum levels and IL-13 gene expression in the jejunum, respectively. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.

Figure 20R. In vivo blocking of TGF-β before challenge abrogated the anti-inflammatory effects induced by VSL#3 treatment. Panel A) *In vivo* administration of anti-TGF-β to peanut allergic mice treated with VSL#3 before PAE re-challenge abrogated the attenuation of oedema and mast cells infiltrate observed upon VSL#3 treatment. Data are reported as mean ± SEM. Data are the combined results of three separate experiments. *p* is referred to VSL#3 group *vs* VSL#3 *in vivo* blockade group.
**Figure 20R.** *In vivo* blockade of TGF-β before challenge abrogated the anti-inflammatory effects induced by VSL#3 treatment. Panel B) diagram and C) sections: histological and immunohistochemistry analysis showed a significant increase in mast cell infiltrate and activation after TGF-β blockade in PAE-allergic mice. Aggregated data on mean counts are reported in B panel: values, representative of three separate experiments, are presented as mean ± SEM from six individual mice/group.
**Figure 21R. In vivo blocking of TGF-β before challenge counteracted the regulatory response induced by VSL#3 treatment.** Panel A) diagram and B) sections: The administration of TGF-β blocking antibody counteracted the regulatory T cell population expressing FOXP3 and LAP induced by probiotic treatment in the jejunum mucosa. Aggregated data on mean counts are reported in B panel: values, representative of three separate experiments, are presented as mean ± SEM from six individual mice/group.
The effect of VSL#3 on the CD103 marker was significantly abrogated by TGF-β blockade in VSL#3-treated mice (Figure 22R, panel B, right), whereas the increase in CD127 expression was independent by TGF-β action (Figure 22R, panel B, middle).

Taken together, these results provide evidences about the functional role of TGF-β in promoting regulatory responses and in the protective effect of probiotic treatment against PAE-induced anaphylaxis and local Th2 inflammation.

**Preventive VSL#3 treatment approach is less efficient than therapeutic one to modulate peanut allergy**

A parallel setting of experiments were performed to evaluate the potential of VSL#3 to prevent the establishment of a Th2 allergic response to peanut allergen. At day 28, after a simultaneous treatment/sensitization period, C57BL/6 mice were challenged by intragastric route with 10mg of protein PAE (see Figure 4PR). After the challenge, mice were observed for 1h to evaluate allergen-induced local and systemic anaphylaxis. Symptoms were assessed applying the same symptomatic score used for therapeutic protocols.

As shown in Figure 23R, panel A, the preventive treatment with VSL#3 did not determine detectable differences between the score recorded in mice administered with probiotic bacteria and PBS control group. In fact, the assigned score reached the maximum value 1 in both groups and consisted mainly in itching and swelling around the nose and head. Prophylaxis with VSL#3 did not determine a change in the gut antigen-specific or total IgA levels (Figure 23R, panel B) nor in TGF-β content of jejunum homogenate in treated mice compared to PBS control animals (Figure 23R, panel C). As in the therapeutic approach, preventive VSL#3 treatment did not induce significant changes in IFN-γ levels (Figure 24R,
Figure 22R. The up-regulation of CD103 expression in the gut of PAE-allergic and VSL#3 treated mice was dependent by TGF-β action. Panel A) VSL#3 treatment was able to increase the expression of TLR9, CD127 and CD103 markers. Panel B) Only the VSL#3-mediated up-regulation of CD103 was dependent on the action of TGF-β as shown by the significant reduction of this markers expression upon TGF-β in vivo blockade. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
Figure 23R. Preventive treatment with VSL#3 did not affect the anaphylactic reactions nor induced a protective mucosal response at local level. As shown in the Panel A) the preventive treatment with VSL # 3 did not determine a detectable difference between the score recorded in mice administered with probiotic bacteria and PBS control group. Data are reported as individual values from a representative experiment out of three. Panel B) prophylaxis with VSL#3 did not determine a change in the gut antigen-specific or total IgA levels panel C) nor in TGF-β content of jejunum homogenate in treated mice compared to PBS control animals. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
left) in the jejunum of treated mice compared to PBS-administered animals. Conversely, no effects on jejunum IL-13 content were induced by the probiotic prophylaxis (Figure 24R, right).

In the MLN isolated from treated mice at day 29, preventive treatment with VSL#3 did not affect the frequency of DC expressing CD103 when compared to PBS control group (data not shown). The supernatants of 18h MLN cells cultures showed a decrease, although not statistically significant, of TNF-α levels compared to those measured in the PBS group (Figure 25R, left). Regarding IL-10 levels, the preventive treatment resulted in a decrease of this cytokine levels in the MLN cell culture from treated mice compared to PBS controls, although it was not statistically significant (Figure 25R, right).

Taken together, the lack of statistically significant differences as well as the presence of controversial features in these results (e.g. the trend of IL-10 and IFN-γ levels) suggest that VSL#3 preventive protocols may be less effective than therapeutic one in the modulation of a Th2 allergic inflammation in a peanut allergy mouse model.
Figure 24R. Preventive treatment did not affect Th1 and Th2 cytokines levels at local level. The IFN-γ (left) and IL-13 (right) levels were not significantly influenced by VSL#3 treatment in the jejunum of treated mice. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.

Figure 25R. Prophylaxis with VSL#3 did not induce a significant regulatory shift in the MLN. The supernatants of 18h MLN cells cultures showed a decrease, although not statistically significant of TNF-α levels compared to those measured in the PBS animals cell cultures. Regard as IL-10 levels, the preventive treatment decreased of this cytokine levels in the MLN cell culture from treated mice compared to PBS controls, although not in a statistically significant manner. Data are reported as mean ± SEM. Data are the combined results of three separate experiments.
DISCUSSION

Amongst allergic diseases, IgE-mediated allergies are a relevant public health problem in Westernized countries. Despite their low prevalence, they are potentially life-threatening because of anaphylactic reactions severity and lacking of effective strategies for their prevention and therapy.

In the last decade, the use of probiotic bacteria as an immunomodulatory approach in the management of allergic diseases (and other inflammatory chronic conditions) has been under investigation by preclinical and clinical studies. Some probiotic preparations are already commercially available and their use resulted successful in the treatment of human inflammatory diseases such as ulcerative colitis, Crohn's disease, pouchitis, irritable bowel syndrome, antibiotic-associated and travellers' diarrhea, diverticular disease and some liver diseases. As in colitis and diabetes animal models, preclinical studies on the modulation of allergies by probiotics have also given rise to encouraging data. Despite this feature, human clinical trials examining the effectiveness of therapeutic microbes for the prevention or treatment of allergic diseases are far less convincing. Furthermore, the global evaluation of the results of these studies is complicated by discrepancies in the use of single strains of probiotic bacteria or mixtures of them. Hence, the need for a better understanding of mechanisms exploited by probiotic microbes to exert their therapeutic and/or prophylactic effects in order to find the optimal single strain or combinations of more strains with synergistic properties. In fact, it is thought that some probiotic strains may be more effective than others due to their ability to induce oral tolerance and that ad hoc preparation of mixed strains might be more effective than single one. A number of factors have been proposed to explain the mechanisms behind the probiotic benefits, including the improvement of microbial balance within the gut
(some probiotics can exclude or inhibit pathogens), the enhancement of intestinal epithelial barrier function (modulating mucus and defensin production) and the generation of regulatory responses (resulting in both local and systemic effects). In addition, the molecular pathways involved in the signal transduction primed by the interaction between probiotic MAMP (microorganism-associated molecular pattern) and host PRR (pattern recognition receptors) exposed by innate immune cells at mucosal sites require further studies.

Depending on the type of disorder to be treated, probiotic applications require different properties. If the aim is to enhance the host immune responses, such as those used for the prevention of traveller’s diarrhoea or gastroenteritis, probiotics with a mild immunostimulatory activity seem to be required. In contrast, IBD treatment requires probiotic bacteria that can counterbalance the pro-inflammatory pathology, taking the altered epithelial barrier into account. In the field of allergic diseases, the main target for probiotic applications seems to be the dendritic cells which acquire tolerogenic properties that enable them to polarize T cell responses towards a regulatory phenotype. This is a common feature of immunoregulatory microbes (some *Bifidobacterium* and *Lactobacillus* species), which is being increasingly reported in animal models of colitis ([Di Giacinto C et al., J Immunol 2005](#)), food allergy ([Schiavi E et al., Allergy 2011](#)) and allergic airway responses ([Karimi K et al., Am J Respir Crit Care Med 2009](#)).

In order to study the potential benefits of VSL#3 mixture in the context of food allergies, *in vitro* and *in vivo* experiments on naïve and polarized cells as well as in naïve and allergic mice were performed and the results presented in the previous section were obtained.

The effects of VSL#3 on the cells of innate and adaptive branch of immunity were studied using BMDC and spleen cells from naïve mice, respectively. The mixture was able to induce the activation/maturation of BMDC and to stimulate the production of IL-12 and IL-10 cytokines by these cells. Moreover, on first encounter with DC, probiotic bacteria seemed
to favour the establishment of a tolerogenic/Th1 prone environment. These data were consistent with the production of IFN-γ and IL-10 by spleen cells from naïve mice.

The induction of a tolerogenic environment was also promoted by *in vivo* oral administration of VSL#3 to naïve mice. In fact, a significant increase of mature DC expressing CD86 and producing IL-10 was determined by probiotic mixture in the local mesenteric lymph nodes (MLN). This innate tolerogenic response was associated with the development of a T regulatory response characterized by an increase of CD4+CD25+IL-10+ T cells frequency in MLN induced by oral feeding with VSL#3.

Encouraged by the promising results obtained on naïve cells and in *in vivo* ‘steady state’ conditions of naïve animals, we studied the *in vitro* probiotic effects on polarized cells. A Th1/T regulatory-type response was also promoted by VSL#3 on mouse spleen cells isolated from allergen-sensitized mice, suggesting that probiotic preparation had the capacity to suppress (as suggested by the reduction of IL-5 and IL-13 levels) and redirect a Th2-type polarized response.

*In vivo* oral therapeutic administration of VSL#3 to ST- and peanut-allergic mice resulted in a significant reduction of systemic (as shown by a diminished symptom score and serum histamine release) and/or local anaphylaxis (lower faecal histamine levels) following allergen challenge. Furthermore, in both animal models of food allergy, at gut level the suppression of the anaphylaxis was accompanied by an increase of protective IgA response associated to an up-regulation of TGF-β.

However, there are some differences between the results obtained from the VSL#3 therapeutic treatments performed in ST- and peanut-allergic mice.

In the jejunum of ST-allergic animals treated with probiotic, IL-4, IL-5 and IL-13 tissue content was significantly reduced, whereas FOXP3 and IL-27 mRNA expression, IL-10,
TGF-β and IFN-γ tissue content were up-regulated. In the same intestinal tract, the inflammation was characterized by the contribution of Th17 subpopulation (IL-17 up-regulation) besides a Th2 involvement; however, the Th17 response was not affected by the in vivo probiotic modulation. Then, VSL#3 therapy in ST allergy mouse model induced Th1 type and regulatory cytokines and, notably, the up-regulation of IL-27 expression in the jejunum. IL-27 has been demonstrated to exert broad inhibitory effects on Th1, Th2 and Th17 T cells, probably through the expansion of inducible regulatory T cells (Stumhofer SJ and Hunter CA, Immunol Lett 2008; Yoshida H et al., J Leukoc Biol 2009). Interestingly, it has been pointed out that microbial products, through their interaction with Toll-Like Receptors (TLR), are able to finely tune the balance between the expression of IL-12 family members and thereby control the outcome of T-cell-mediated inflammation (Goriely S et al., Nat Rev Immunol 2008). In particular, EBI3 expression, one of the two IL-27 subunits, has been induced in human DC stimulated by TLR2, TLR4 and TLR9 ligands depending on MyD88 and NF-kB signalling (Wirtz S et al., J Immunol 2005). Moreover, the probiotic strain Bifidobacterium infantis has been reported to enhance IL-27 in vitro production in TGF-β plus IL-6-stimulated mouse spleen cells (Tanabe S et al., Int J Mol Med 2008). Other reports demonstrate that it is possible to modify the way T cells respond to cytokines such as IL-12 or IL-27 by selectively enhancing IL-10 secretion in IFN-γ producing Th1 cells, and thereby conveying anti-inflammatory capacity to otherwise pro-inflammatory Th1 cells (Rutz S et al., Proc Natl Acad Sci USA 2008). Such Th1 regulatory cells, induced in the presence of DC-derived IL-27, could be responsible for the therapeutic effect induced in the mouse model of ST allergy by VSL#3 treatment, in synergy and mutual relationship with other regulatory populations directly induced by probiotic components and characterized by the prevalent production of TGF-β and IL-10 (Figure 1D).
Figure 1D. Proposed mechanism for VSL#3 modulation in tropomyosin allergy model. VSL#3 probiotic mixture induces the production of cytokines as IL-27, IL-10, TGF-β by dendritic cells and/or gut epithelium through Toll-like receptors involvement. These cytokines promote Th1/T regulatory responses. In particular, the suppression of Th2 allergic response is mediated by IFN-γ and IL-10 produced by Th1reg and/or Tr1 and by iTreg Foxp3+ cells. Also the production of protective IgA by B cells induced by TGF-β at gut level might explain the modulation of Th2 inflammation.
The studies on VSL#3 therapeutic potentials in the jejunum of peanut-allergic mice revealed its capacity to suppress the Th2 inflammation, mainly driven by IL-13. Also in this case, the suppression was associated with a regulatory response but no Th1 shift of the allergic response was found. After the probiotic therapeutic administration, the finding of FOXP3+ T regulatory cells expressing Latency Associated Peptide (LAP), that we could identify as ‘Th3 TGF-β dependent (LAP+) Tregs’ in the jejunum of peanut-allergic mice, correlated with the recruitment of CD103+DC in local MLN. This feature is in agreement with published data by others on the tolerogenic properties of this DC subpopulation in inducing FOXP3+ T regulatory responses (Coombes JL et al., J Exp Med 2007; Worthington JJ et al., Gastroenterology 2011).

Furthermore, the treatment induced an increase in CD127 and CD103 expression in the jejunum confirming the activation of T regulatory cells (Simonetta F et al., Eur J Immunol 2010). Probably, TLR-9 stimulation by immunostimulatory bacterial DNA is upstream of TGF-β release by epithelial cells. This cytokine promotes the induction of FOXP3+ T reg cells in the gut establishing a regulatory response which counteracts the allergic inflammation at local level, as indicated by the reduction of TNF-α levels recorded in MLN and of IL-13 in the jejunum. In addition, the Th3 regulatory cells themselves produce TGF-β, favouring the B cell switch towards the production of protective IgA mucosal responses (Figure 2D). VSL#3-promoted counter-regulation of Th2 allergic inflammation at local level determined also a significant reduction of mast cells infiltrate. Taken together, these data strongly suggest that the establishment of the tolerogenic environment and the reduction of inflammation was mediated by TGF-β up-regulation promoted by probiotic bacteria. The functional role of this cytokine in the probiotic action was confirmed by in vivo anti-TGF-β blocking experiments performed in peanut-allergic mice. In fact, the blockade of this
Figure 2D. Proposed mechanism for VSL#3 modulation in peanut allergy model. VSL#3 probiotic mixture induces the production of TGF-β by gut epithelium and/or dendritic cells through Toll-like receptors (TLR-9). TGF-β is responsible for induction of iTreg LAP+ and/or Foxp3+ LAP+ Treg cells at gut mucosa level. Also the recruitment of CD103+ dendritic cells in the mesenteric lymph nodes correlates with the induction of a Foxp3+ response at local level. TGF-β production could be also the inducer of IgA protective antibodies produced by B cells.
regulatory cytokine reverted the effect of VSL#3 in recruiting CD3+FOXP3+LAP+ T cells and in reducing mast cells accumulation/activation in the jejunum.

It is known that Th3 cells are TGF-β-dependent and TGF-β-producing cells and this cytokine is able to induce FOXP3 expression. LAP is a pro-peptide that is non-covalently associated to the amino-terminal domain of TGF-β, forming a latent TGF-β complex. Membrane-bound TGF-β (LAP) is an important component of some T regulatory populations and may or may not be expressed on all FOXP3+ T reg cells. TGF-β itself may induce surface LAP expression on murine CD4+ T cells dependent or independent of FOXP3 induction (Weiner HL et al., Immunol Rev 2011). In addition, VSL#3 administration has already associated with an increased number of regulatory CD4+LAP+ T cells which ameliorated the severity of recurrent colitis in mice (Di Giacinto C et al., J Immunol 2005). VSL#3-treated patients affected by pouchitis showed a significant increase in the percentage of mucosal CD4+LAP+ T cells accompanied by a significant increase in FOXP3 mRNA expression in the gut tissue (Pronio A et al., Inflamm Bowel Dis 2008).

In contrast to conventional T cells, CD127 expression is increased on activated CD4+Foxp3+ T reg cells expressing also CD103 marker (Simonetta F et al., Eur J immunol 2010). In the jejunum sections of VSL#3 treated-mice, the up-regulation of this marker paralleled the probiotic-mediated up-regulation of CD103 integrin on the same intestinal tract but if the increase of CD127 expression was independent by TGF-β action, the effect of VSL#3 on the CD103 marker was significantly abrogated by TGF-β blockade in mice VSL#3-treated confirming the regulation of CD103 expression by the regulatory cytokine (Robinson PW et al, Immunology 2001).

The capacity of TGF-β to act as a modulator of mast cells functions has been already demonstrated. Gebhardt T et al. showed some TGF-β-mediated effects that can be found also
in our peanut allergy model treated with VSL#3: the down-regulation the expression of c-kit (also named CD117) on mast cells and the reduction of the histamine release by them. Other effects mediated by this cytokine on mast cells are the down-regulation of receptors for IgE expression, the reduction of cysteinyl-leukotrienes and TNF-α release and the up-regulation of the prostaglandin D2 generation and cyclo-oxygenase 1 and 2 expression (Gebhardt T et al., Gut 2005). Obviously, these data indicate that the change in mast cells mediator release profile induced by TGF-β might be of relevance for the control not only of mast cells associated disorders of the intestine such as food allergy but also Crohn’s disease, irritable bowel syndrome and parasitic infection.

In the peanut allergic mice, the probiotic mixture also induced the formation of isolated lymphoid follicles (ILF) that are absent in PBS control group and naïve mice. These complexes are composed of a large B cell area, including a germinal center, and epithelia overlying the clusters contain M cells. A large fraction of the lymphoid residents display an immature phenotype. The composition of IgA+ B cells is smaller but substantial and ILF are structurally and functionally similar to the follicular units that compose PP (Peyer’s patches) and are believed to be an equivalent or complementary system to PP for the induction of intestinal IgA antibody responses to a variety of luminal antigens and the maintenance of intestinal immune surveillance.

Upon the VSL#3 therapeutic treatment, effects on allergen-specific serum antibodies was revealed in ST-allergic VSL#3-treated mice. In particular, a prevalent shift of the ST-specific humoral response towards the Th1-driven isotype IgG2a, with a significant reduction in serum specific IgE levels, was observed in mice therapeutically treated with probiotics. The up-regulation of ST-specific IgG2a can be associated with the increase of IFN-γ production in the culture supernatants from spleen cells from probiotic-treated mice in vitro re-stimulated with ST, when compared with spleen cells from control mice. No changes were found in
serum antibody response of peanut-allergic mice upon VSL#3 therapeutic administration when compared to control group. These data suggested us that different mechanisms at local and/or systemic level could be involved in the anaphylaxis modulation by the probiotic preparation used, depending on the food allergen, on the mouse strain and the Th2 response course.

Then, from the results described above for the two mouse models of food allergy, we can conclude that a therapeutic use of VSL#3 mixture was effective to reduce the systemic and local anaphylaxis upon a challenge with high dose of allergen administered in both ST- and peanut-sensitized animals. The mechanism underlying the suppression of the Th2 responses at gut level show some differences in the two models, probably due to different allergens or mouse strain used. In particular, in the ST allergy model, VSL#3 determines the shift of Th2 allergic response to a Th1/T regulatory mixed profile with an effect also on allergen-specific systemic antibody response. In peanut allergy model, the suppression of the Th2 allergic inflammation occurred mainly through an induction of a T regulatory response performed by CD3+FOXP3+LAP+ T cells (Th3) probably induced by a TGF-β increased release from local cells (e.g. epithelial cells) and the Th3 cells themselves. A Th3 contribution in the T regulatory response observed in the ST allergy model is also suggested by the significant increase of TGF-β in the jejunum of ST-allergic VSL#3-treated mice.

In a preventive treatment setting of experiments performed in peanut-allergic mice, VSL#3 did not result as effective as in the therapeutic schedule. In fact, neither significant modulating effects on anaphylaxis were revealed nor a suppression/shift of the Th2 response upon probiotics administration. This result can be due to the use of adult instead of neonatal mice. Growing evidences exist about the importance of a “competence window” in which probiotic bacteria can still induce a protection against the development of inflammatory
disorders in animal models as well as in clinical trials on human (Feleszko W et al., Clin Exp Allergy 2007; Szajewska H, Nestle Nutr Inst Workshop Ser 2013).

These data suggest that a preparation consisting in Lactobacillus, Bifibacterium and Streptococcus genera of probiotic bacteria mixed in ad hoc concentrations is able to suppress an established Th2 response against two clinically relevant food allergens such as shrimp tropomyosin and peanut. The possibility that this type of therapeutic approach can be transferred to humans is strengthened by the use of VSL#3 in other inflammatory gut disorders and the similarity of our mouse models of food allergy with human counterpart: the route of allergen administration, the pattern of IgE binding to the allergens, the anaphylactic reactions triggered by the challenge with allergens, the pattern of Th2 allergic inflammation at gut level.
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