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Food allergy and intestinal inflammatory conditions. Study in animal models.

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Bajo la dirección de los doctores:

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Introduction

CHAPTER 1: INTRODUCTION

1. INTESTINAL MUCOSA

The main interfaces between the host and its external environment are the skin, the gastrointestinal and the respiratory tracts (Biancheri, Di Sabatino, Corazza, & MacDonald, 2012) and although the skin is the most visible site of interface, the intestinal mucosa is one of the largest surface areas of the body that is exposed to and interacts with the external environment.

Intestinal tract has some of the most important functions for vital maintenance such as food processing, absorption of nutrient, and the defence against external aggression which is developed inside and outside of our intestine. For this exclusive mission, anatomical structure of intestinal tract has a particular complexity that let to carry out this difficult activity.

The mucosa layer is constituted by the epithelium, the lamina propria and the submucosa, being the epithelium the nearest part from the lumen and the external aggression. Under the epithelium, lamina propria is a thin layer of connective tissue where immunological cells are located and where the immunity response is placed. Then, several muscular layers included in the intestinal structure, closed to a very complex net of neuronal connections (sympathetics and parasympathetics), arterian, venous and lymphatic vessels allow the intestine to respond mechanically to external conditions like stress or the presence of solids or liquids. At the end, covering the epithelium, mucosal secretion protects the epithelial cells and help to fulfil the intestinal mission (see figure 1)

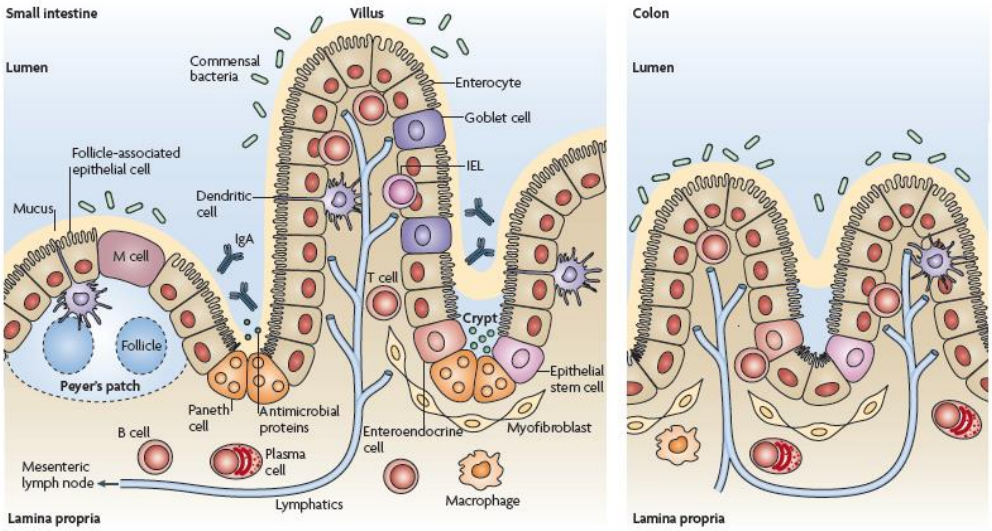

Figure 1.- Anatomy of the intestinal immune system in small and large intestine. IEL= Intraepithelial lymphocytes. IECs= Intestinal epithelial cells. There are several structural and functional differences between small and large intestine. The large

bowel differs in physical form from the small one, in its greater diameter and in its invaginations instead the evaginations of the small intestine (villi), among others. An IECs monolayer separates the gut lumen from the lamina propria. There are several types of IECs such as enterocytes, goblet cells, Paneth cells and enteroendocrine cells in the small intestine and enterocytes in the large intestine. The small intestine has areas of specialized epithelium and M cells that overlie the Peyer´s patches. Figure adapted from: (Abreu, 2010).

The ability to keep the delicate balance between absorbing essential substances or nutrients while inhibiting entry and replying harmful elements is the main characteristic of the intestinal mucosal barrier. Thus, two main functions are attributed to the gastrointestinal mucosa: first, it concerned with digestion and absorption of dietary nutrients, and a second function with the defence against many harmful dietary substances, microbes and toxins (Jankowski, Goodlad, & Wright, 1994). Both are intimately connected because a deficient digestion of nutrient can produce a dysfunction in the immunological response. Moreover, this mucosal defence consists of non-specific barrier mechanisms and specific immunological responses, and are placed in the epithelium and lamina propria layers. Both elements contribute equally in both missions (see section 1.3.).

Although the intestinal epithelial layer is the obvious physical frontier between the external and internal environment, there are other elements that form the intestinal mucosal boundary.

1.1. External mucosal barrier components

The first line of defence consists in limiting the contact between the luminal microbial community and the intestinal surface and therefore, prevents the penetration of most bacteria. This line of resistance embraces: i) the commensal microbiota; ii) the mucus layer; iii) secretory immunoglobulins (sIgA) and iv) the antimicrobial peptides.

i) Commensal Microbiota

Epithelial surfaces are under threat of potential harmful pathogens but they also give a refuge to many beneficial microorganisms and at the same time the intestinal mucosa has to deal with absorption and digestion of nutrients. Concerning that, it should be noted that the large intestine contains most of the microbiota while the small intestine is the main place for the others activities named beforehand (Reis & Mucida, 2012).

A large number of the microbes that altogether constitute the microbiota are highly advantageous for our health and well-being. The host and its symbionts seem to have co-evolved on the way to a mutually beneficial state of co-existence (Moran,

McCutcheon, & Nakabachi, 2008). This symbiosis exists to contribute to nutrient absorption, obstructs and inhibits pathogen invasion, as well as helps in the development and optimal functioning of the host immune system (Round & Mazmanian, 2009).

Colonization of the gut starts immediately upon birth and it is developed by the largest population of symbiotic bacteria, nevertheless we must remember that archeal, fungal and viral species are also present. This community of bacteria is formed by about $1x10^{14}$ specimens (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Dethlefsen, McFall-Ngai, & Relman, 2007) with an estimated human microbiome contents of more than 1000 bacterial species, with more than 160 different species commonly present in each person (Qin et al., 2010). Anyway, there are predominantly four bacterial phyla adapted to the intestinal niche: *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* (Gill et al., 2006; Qin et al., 2010).

Commensal bacteria develop its effects forming a resistance to pathogen colonization by the production of antimicrobial substances (such as bacteriocins), the alteration of luminal pH and directly competing against pathogens for nutrients. They also promote angiogenesis and the development of the intestinal epithelium (Artis, 2008).

These beneficial effects of the microflora have led to the selection of specific species with assumed health-promoting skills for the treatments of conditions where the gut bacteria compositions are disrupted. These microorganisms are known as probiotics and have been used in the prevention and treatment of gastrointestinal infections, inflammatory bowel disease and allergic diseases (Yan & Polk, 2012).

ii) Mucus layer

One mechanism to maintain the integrity of the intestinal mucosal barrier is the production of a thick mucus layer that overlies the entire intestinal epithelium.

In the epithelium layer, the goblet cells secrete large amounts of Mucin glycoprotein (3 litres/day) (Jankowski et al., 1994), via the expression of the Mucin 2 gene, that get together to form a protective gel-like layer that can have a thickness up to as much as 800 μm in the colon (Atuma, Strugala, Allen, & Holm, 2001).

The bacteria have the option to use the high number of glycans on the mucins as attachment sites and all the oligosaccharides present on the mucins as an enormous food source (M. Johansson et al., 2011). Reciprocally, symbiotic bacteria are responsible to induce the production of mucus layer, since outer mucus layer of germ-free mice is thicker than that of bacteria-colonized mice (M. E. Johansson et al., 2008).

While the great number of microorganism in the lumen can be found in the outer mucus layer, there is an inner, protected and unperturbed layer that is directly adjacent to the epithelial surface and is rather sterile. This sterility is a product of the

retention of a high concentration of antimicrobial peptides (such as defensins, cryptidines, etc) produced by various intestinal epithelial types of cells, including enterocytes and Paneth cells above all (M. E. Johansson et al., 2008).

iii) Secretory Immunoglobulin A (SIgA)

IgA is a relatively small component of serum antibodies, nevertheless the abundance of IgA-secreting cells in normal mucosa means that this isotype actually constitutes at least 70 % of all immunoglobulin produced in mammals. In fact, gut mucosa constitutes the body's largest effector organ of humoral immunity (Male, 2006). The IgA appears to be related to innate immune responses (IgA induction by commensal intestinal microbes), and also to highly adaptive responses (functional neutralization of pathogenic molecules and microbes).

The induction of mucosal IgA secretion takes place mainly in the organized gut-associated lymphoid tissue (GALT) and specifically in follicular B cells from Peyer 's Patches above all (see section 1.3.). But there are additional mesenteric lymph nodes and isolated lymphoid follicles (ILFs) that also contribute to the generation of IgA-producing plasma cells in the intestinal mucosa (Pabst et al., 2006).

It was demonstrated that the expression of SIgA on the apical luminal surfaces (Jankowski et al., 1994) carry out an essential activity maintaining luminal bacteria compartmentalization and limiting the penetration of bacteria into the host. SIgA sequester the bacteria within the mucus and keep them away from the epithelial cells, which represents an additional mechanism to protect the epithelium apart from the layer of mucus loaded with antimicrobial peptides. SIgA also opsonizes luminal bacteria that become internalized into the lamina propia as a means of scrutinize the external environment (Macpherson & Uhr, 2004). This specific process is mediated by the dimerization of SIgA which has an abnormally large diameter in relation to its molecular weight, leading to restrict the penetration of IgA-coated microbes through the surface epithelium (Phalipon & Corthesy, 2003).

Thus, the protective role of sIgA in the gut has been demonstrated in different studies. For example, the natural SIgA antibodies can inhibit early invasion and horizontal fecal-oral spread of *Salmonella typhimurium (Wijburg et al., 2006) or the* influenza infection is abrogated by IgA-neutralizing antibodies to the virus (Renegar, Small, Boykins, & Wright, 2004). However, take in consideration that IgA is highly induced in the gut but only in animals containing intestinal microbes since in those mice kept in a germ-free state the number of intestinal IgA-producing plasma cells is 1-2 orders of magnitude lower (Mueller & Macpherson, 2006).

iv) Antimicrobial peptides

The antimicrobial peptide is one of the crucial effectors of the innate immunity, and is common in the multicellular organisms. In mammals, one family of antibacterial

peptides named defensins plays a central role in host defence, especially in the epithelial surface such as oral cavity, skin and intestine. Paneth cells, which are part of the epithelial cells, are the producers of antimicrobial peptides (defensins, cryptidines and lysozymes) in the small intestine and induce a negative feedback loop that limit bacterial access to the gut epithelial surfaces (Duerkop, Vaishnava, & Hooper, 2009). Paneth cell α-defensins have selective activities against commensal bacteria which could be associated with compositions of intestinal microbiota and homeostasis of the entire intestine (Masuda et al., 2011). In this sense, the expression of a human specific alpha defensin in the mouse dramatically changes the microbiota composition, including the loss of segmented filamentous bacteria (Salzman et al., 2010). Other antimicrobial peptide such as calprotectin in neutrophil granules, chelates zinc and manganese so it can be involved in host defence against bacterial infections (Corbin et al., 2008).

Although the microbicidal action of antimicrobial peptides is often considered mild; concentrations in the crypts can reach levels sufficient for strong bacterial lysis (Ayabe et al., 2000). Recently, the importance of the antimicrobial peptides has been widely recognized; however, the role that they play in host defence to pathogens remains unknown.

1.2. Intestinal epithelial cells

The second line of defence is formed by the epithelium, a continuous monolayer of columnar epithelial cells that are connected together by several dynamic junctional complexes. This monolayer is constituted by four lineages that arise from a pool of pluripotent stem cells that are located at the crypt region of the intestine (Barker, van de Wetering, & Clevers, 2008). These cells include enterocytes, goblet cells, enteroendocrine cells and Paneth cells (see figure 1). While enterocytes are responsible of bidirectional traffic of substances, goblet cells are mucus secreting cells, enteroendocrine cells are responsible of hormone secretion (serotonin), and finally, Paneth cells are the antimicrobial peptides producers.

This intestinal epithelium has two major functions; the first one is to limit the entrance of harmful intraluminal items, including foreign antigens, microorganisms and their toxins (Blikslager, Moeser, Gookin, Jones, & Odle, 2007). Second, to act as a selective filter, allowing the translocation of vital dietary nutrients, electrolytes and water from the intestinal lumen to the circulation (Blikslager et al., 2007).

In this sense, the enterocytes mainly work as absorptive entities which have an extensive system of microvillar extensions at the apical surface that form the brush border which increase the contact surface between the luminal content and epithelium. They also contain many of the digestive enzymes and transporter receptors that are necessary in metabolism and uptake of dietary antigens (Barker et al., 2008)

1.3. Intestinal immunological system

The mucosal immune system, developed in the higher mammals, is the first line of defense of the organism. The mucosal membranes are the main route of entry of microorganisms, allergens, and carcinogens so the host must protect itself against this threat. Nevertheless, absorption of nutrients and controlled uptake of antigens is crucial for life and for the maturation of the mucosal immune system. Consequently, in addition to physical barrier, well-regulated intestinal immune responses are necessary to meet this challenge (P. Brandtzaeg, 1998).

The maturation of the immune system does not occur until 2-3 years of life. Until that age, the child has more infections than adult due to the predominance of immature T and B cells leading to a slow and incomplete immune response. As child comes in contact with new antigens through infection and the acquisition of an optimal microbiota, the child expands his immunological repertoire and creates his memory cells. This fact along with the appearance of antibodies allows a greater anti-infectious defence with more rapid and effective immune responses (Martin et al., 2010).

In mucosal immune system there is a continuous immune activity, in contrast to what occurs inside the body (for example, in the spleen, liver or kidney), where the continuous presence of foreign agents on mucosal membranes is unendurable. In this sense, the mucosal immune system induces: a) a specialized response which generates tolerance against beneficial molecules; b) a no-sterilizing immune response to normal flora as a result of the adaptation to the environment and c) a sterilizing immunity against pathogens (Goto & Kiyono, 2012). This triple function determines the main differences between the internal immune system and the mucosal one.

Anatomical and functionally, in the gastrointestinal mucosa are special sites or compartments for the stimulation of the immune response, known as *inductive sites*, and others in which immune function is developed, called *effector sites*. In each of these compartments of the intestinal mucosa, several cell types are located in a characteristic manner. As an example, 85% of total lymphocytes and 67% of immunoglobulin's total production are linked to the intestinal mucosa immune system to regulate its high antigenic burden.

The Inductive sites as GALT, which includes tonsils, Peyer's patches, lymphoid follicles, appendix and caecal patch; whereas, the effectors' sites include lamina propria and epithelial layer (see figure 2) (Per Brandtzaeg & Pabst, 2004). Innate and adaptive immune cells are accumulated in these mucosal immune compartments and they are coordinated to keep a state of limited mucosal activation (oral tolerance) and to initiate active immune responses to harmful microbes (Slack et al., 2009).

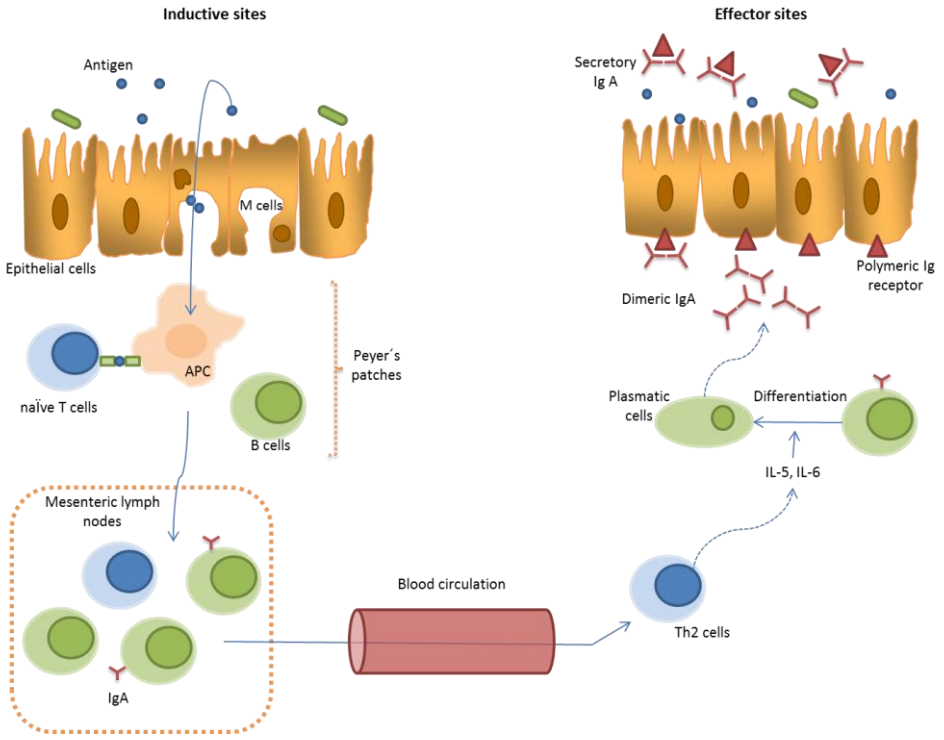

Figure 2.- Inductive and effector sites in the gastrointestinal mucosa.

The gut-associated lymphoid tissue (GALT) is the most important inductive place of the mucosal immune system. It is formed by organized and specialized lymphoid tissue with anatomical features that differentiates it from other secondary lymphoid tissues (not have a defined capsule or afferent lymphatic vessels). It contains well-defined structures such as Peyer's patches which are the most recognised inductive sites of the GALT (see figure 2).

Thus, Peyer´s patches are the major portal of entry of bacteria, and together with mesenteric lymph nodes, constitute the major IgA inductive site in the intestine. They are preferentially located in the lowest portion of the small intestine but they can also be found in the ileum, and also in the rectum (Craig & Cebra, 1971). Peyer's patches development occurs during fetal life independently of gut colonization by bacteria and they consist of a germinal center comprised of several B lymphocytes follicles surrounded by areas containing lymphocytes T (T cells), macrophages and dendritic cells (DCs) and also high endothelial venules are mainly present in the interfollicular areas (Fagarasan, Kawamoto, Kanagawa, & Suzuki, 2010; Hase et al., 2009). Moreover, the microfold cells (M cells) are coating the surface of Peyer´s patches and they are responsible for checking the intestinal tract contents. M cells are inefficient at up-taking soluble protein antigens but they are specialized in taking up particulate antigens or those antigens for which these cells express receptors (eg, poliovirus). M cells are specially designed to continuously sample luminal contents through endocytosis and deliver them to underlying macrophages (Corr, Gahan, & Hill, 2008).

Intestinal macrophages are the largest population of mononuclear phagocytes in the gut, especially in the large intestine. They are mainly located in the lamina propria, adjacent to the epithelium, but they can be found all over the gut tract both in the mucosa, submucosa and muscularis mucosae (A. M. Mowat & Bain, 2011). Some of them, that are positive for the marker CX3CR1 (the receptor for the chemokine CX3CL1), can also extend dendrites through the epithelium to actively sample bacteria and soluble antigens from the intestinal lumen. This fact together with the expression of class II major histocompatibility complex (MHC-II) and CD11c explain the misclassifying of intestinal macrophages as DCs that have been done until a few years ago (Bogunovic et al., 2009; Niess et al., 2005; Rescigno et al., 2001). However, the identification of new and more specific markers present in these CX3CR1+ cells, such as F4/80, CD68 and Ly6C (Bain et al., 2012; Rivollier, He, Kole, Valatas, & Kelsall, 2012) have let to accurate characterize these intestinal macrophages (CX3CR1+ CD103- CD11c+ MHC-II+ F4/80+ CD68+ Ly6c+). In addition, these cells have a different ontogeny in the intestine and appear to be derived from monocytes contrary to CD103+ CX3CR1- DCs which have the named pre-DC as the specific precursor (Bogunovic et al., 2009; Varol et al., 2009).

Unlike blood monocytes or macrophages present in other tissues, the most of resident intestinal macrophages express low levels of co-stimulatory molecules (CD40, CD80, and CD86), low levels of MHC-II and they do not produce inflammatory cytokines in response to TLR (Toll-like receptor) ligands, whole bacteria, or nonmicrobial stimuli such as interferon-γ (IFNγ) (Rivollier et al., 2012). This means that these cells are not able to contribute to effector T cell activity but they product IL-10. IL-10 support the survival of local $Foxp3^{+}$ regulatory T cells (Treg cells) (Hadis et al., 2011). All these facts together with other local factors keep resident macrophages in a state of partial activation that lets them to internalize and kill microbes, help to protective immunity, and maintain homeostasis (Platt & Mowat, 2008). After epithelial damage, or pathogenic invasion, the mucosa is infiltrated by a great number of inflammatory macrophages which express lower levels of CX3CR1, are TLR-responsive and they are derived from recently divided inflammatory $CX3CR1^{lo}$ $Ly6C^{hi}$ CCR2+ monocytes in the bloodstream. Inflammatory macrophages produce large amounts of pro-inflammatory mediators including tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6, and nitric oxide that drive local inflammation and promote the function of effector T cells. Consequently they are also important effectors cells in some pathologies such as inflammatory bowel disease (IBD) (Bain et al., 2012).

In contrast to intestinal macrophages, DCs express high levels of CD103 and low to intermediate levels of F4/80 and CX3CR1. They have the skill to migrate to the gut draining mesenteric lymph nodes where they can interact with and cause differentiation of naïve T cells, after the presentation of the antigen that it has been previously processed by DCs. DCs, therefore, initiate adaptive immune responses in local lymph nodes, whereas CX3CR1+ macrophages are able to modulate immune responses directly in the mucosa and serve as first line barrier against invading pathogens (Schulz et al., 2009).

Finally, in these inductive sites the naïve T and B cells are normally found in Peyer´s patches (see figure 2). After being activated, naïve T and B cells proliferate and become memory/effector cells that migrate from GALT to mesenteric lymph nodes through the lymph (P. Brandtzaeg, 2010). From mesenteryc lymph nodes, T and B cells travel through the blood and reach other mucosal areas. Activated cells reach the mucosa to experiment their last differentiation and become effector cells, either plasma cells producing antibodies in the case of B cells or cytokine-producing cells in the case of T cells (see figure 2). Mucosal sites that hold effector and memory cells are called effector sites which include for example lamina propria in the gut tract (Slack et al., 2009).

In the standard gastrointestinal tract, T cells represent one-third of the cells in the lamina propria; the phenotypic distribution of $CD4^+$ and $CD8^+$ T cells in this area is similar to what occurs with peripheral blood lymphocytes, with a supremacy of $CD4^+$ and αβ-T cell receptor (TCR)-positive cells. Finally, the plasma cells that produce IgA are in equal percentage to T cells (Macdonald & Monteleone, 2005).

The fact that the epithelial layer of the small bowel contains a ratio of about 1 T cell for every 10 epithelial cells, and in the colon the proportion is 1:20, lead us to think that these T-cell population which is called intraepithelial lymphocytes (IELs) are extremely important for gut mucosa. IELs are very heterogeneous, and the various subsets are spread differently in the epithelium of the small and large intestine which is probably influenced by the different digestive functions and the physiological conditions (Sheridan & Lefrancois, 2010). However, these IELs also share features that distinguish them from the conventional periphery T cells. Gut IELs are almost only T cells, and include a relevant percentage of $TCR\gamma\delta^+$ cells, which can constitute up to 60% of small intestinal IELs in mice although the proportion in humans is smaller (Abadie, Discepolo, & Jabri, 2012; Bonneville et al., 1988). They are mainly antigen-experienced T cells belonging to both $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ lineages, that characteristically express activation markers, such as CD44 and CD69 (Cheroutre, 2004). In addition, IELs express CD103 which interacts with E-cadherin (epithelial cadherin; transmembrane protein expressed on enterocytes that plays a key role in cell adhesion), allowing cell-cell interactions with epithelial cells. A good review of the different subsets of IELS in the gut mucosa have been recently described by Cheroutre and coworkers (Cheroutre, Lambolez, & Mucida, 2011).

IELs offer immediate and increased immune protection to avoid initial access and spreading of pathogens by their straight contact with the enterocytes and by their immediate closeness to antigens in the gut lumen. Consequently, the most of IELs contain abundant cytoplasmic granules for cytotoxic activity, and they can express effector cytokines, such as IFNγ, IL-2, IL-4 or IL-17 (Muller, Buhler-Jungo, & Mueller, 2000; Shires, Theodoridis, & Hayday, 2001) which can be released to fight against the foreign antigen.

However, IELs also need to display regulatory functions and avoid unnecessary inflammatory immune responses that could put in danger the integrity of the barrier;

because of that reason, they characteristically express both activating and inhibitory types of innate natural killer cell receptors, which typify them as stress-sensing although highly regulated immune cells (Bhagat et al., 2008; Denning et al., 2007). In this sense, TCRγδ$^+$ natural IELs have been implicated in various regulatory roles, including: antibody class switching and IgA production, IL-10-dependent oral tolerance, and clearing necrotic epithelium and mending damaged epithelium (Komano et al., 1995; Locke, Stankovic, Funda, & Harrison, 2006).

Despite all the beneficial actions that IELs develop, their elevated activation status and their close nearness to the intestinal epithelium suggest that these cells may contribute to initiate and/or exacerbate immunopathological responses and inflammatory diseases, such as IBD and coeliac disease, or promote cancer development (Abadie et al., 2012; Kanazawa, Ishiguro, Munakata, & Morita, 2001). In fact, the induced IELs population gradually increases with age (together with several pathologies) in response to exposure to foreign antigens and this fact allows the mucosal immune system to acquire an almost personalized mucosal immune catalogue that is directed against those environmental antigens that are most likely to be met again by a particular individual (Manzano, Abadia-Molina, Garcia-Olivares, Gil, & Rueda, 2002).

Apart from effector IELs, the epithelium contains another T-cell population, the regulatory T cells (Treg cells). Naturally arising Treg cells represent 1% to 2% of peripheral CD4 T cells and it is supposed that they preserve tolerance toward self-antigens. Inducible Treg cells, which like naturally Treg cells express Foxp3$^+$, develop from naïve CD4 T cells in the presence of TGF (transforming growth factor)-1 (Fantini et al., 2004; Rao, Petrone, & Ponath, 2005). Treg cells include T cells that express the transcription factor forkhead box protein 3 (Foxp3$^+$), Th3 cells and Tr1 cells (Fontenot, Gavin, & Rudensky, 2003; Kronenberg & Rudensky, 2005). Treg cells produce a high amount of IL-10 and therefore they play a key role in induction and keeping of immunological tolerance (Veenbergen & Samsom, 2012). IL-10 is a potent cytokine with antiinflammatory and immunoregulatory functions. It downregulates the production of Th1 cytokines (such as TNFα, IL-1, IL-12, and IFNγ) and it decreases the expression of MHC-II and costimulatory molecules on DCs, maintaining them in a tolerogenic state. Moreover this cytokine regulates the activation of mast cells, as well as directly suppress T-cell proliferation (R. A. Peterson, 2012). In recent years it has strongly arisen a theory in which mucosal inflammation comes from an inadequate activity of Treg cells. In this model, effector T cells, which react to the microbiota or other antigens, are kept in check by a population of Treg cells; therefore, imperfections in these cells lead to gut inflammation (Schmetterer, Neunkirchner, & Pickl, 2012). Thus, as a conclusion, it has been suggested that the differentiation of the adaptive immune response conducts to the destruction of pathogenic antigens or the activation of tolerogenic responses to innocuous antigens (P. Brandtzaeg, 2010) and a deregulation of destruction/tolerance mechanisms against antigens can lead to several pathologies.

At the end, it is important to mention that apart from the mucosal immune system, there are nonspecific defence systems which are of critical importance to maintain a healthy state and they include gastric acid, bicarbonate and mucus secretion, intact epithelial layer forming tight junctions, digestive enzymes, peristaltic movement, alternative complement pathways, phagocytes, and antimicrobial peptides (M. Johansson et al., 2011; Masuda et al., 2011). All of those mechanisms participate in the prevention of infection, control of invasion, and replication of pathogens and maybe allergen exposure within the intestinal tract.

2. PHYSIOLOGICAL RESPONSE TO FOREIGN SUBSTANCES

Physiological behavior against the presence of stranger molecules (antigen or allergen) will be different depending on the place (skin or mucosa) and the characteristics of the milieu where the contact has been produced. In non-pathological conditions, the inflammatory and immunological responses are developed resulting in the destruction of attacker agent (bacteria, viruses, parasites, etc.) and the reconstruction of damage tissue (see section 2.2.1.). In other situations, organism's response includes induction of immunoglobulin E (IgE) specific antibodies with a typical allergic reaction that will be reproduced every time the agent contacts with the body (see section 2.2.2.). Also, a chronic inflammation can occur when a deficient anti-inflammatory response or excessive immune response had been developed leading to several pathologies including allergy or inflammatory bowel disease (IBD) (see section 2.2.2. and 2.2.3.).

Thus, in normal conditions, the physiological response to a foreign agent is the absence of response, called "immunological tolerance", that is very important to live in harmony with our symbiotic microflora and to permits mutual benefits. The "oral tolerance" occurs in the gastrointestinal tract for the oral contact with food protein and antigen. Tolerance is also the physiological mechanism to discriminate self and non-self-protein to trigger the immunological defense only against non-self-components. When tolerance is broken, autoimmune and other inflammatory diseases are developed (Faria & Weiner, 2005) (see section 2.2.).

2.1 Oral tolerance

Gut homeostasis is accomplished not only by the regulation of barrier function but also by down-regulating the normal immune response to bacteria and food antigens through different immune cells such as the intestinal epithelial cells which play a key role in the maintenance of the intestinal homeostasis (L. W. Peterson & Artis, 2014). This last event was named "oral tolerance" because it is induced after oral challenge with particular antigens. One of the most accepted definitions of "oral tolerance" was given by Chase in 1946 and it refers to *a state of active inhibition of immune responses to an antigen by means of prior exposure to that antigen through the oral route* (Chase, 1946) This circumstance was first described in animals but it also exists in human, and it is important to highlight that it confers not only a local tolerance but also a systemic one against orally given antigens (Gutgemann, Fahrer, Altman, Davis, & Chien, 1998; Nagler-Anderson, 2000). Oral tolerance is a strong adaptive immune function because people absorb considerable amounts of intact food antigens after meals, corresponding to 10^{-5} of the intake reaching to a daily uptake of 130-190g (Scurlock, Burks, & Jones, 2009). The importance of food proteins to systemic immunity can also be realized by the fact that up to 0.5% of ingested proteins can be found intact, a few hours after ingestion, in blood circulation (Husby, Jensenius, & Svehag, 1985).

The induction of oral tolerance has been demonstrated that depends on several mechanisms including clonal anergy, clonal deletion and the generation of antigen-specific Treg cells (Chen, Kuchroo, Inobe, Hafler, & Weiner, 1994; Whitacre, Gienapp, Orosz, & Bitar, 1991). Briefly, T cell clonal anergy is promoted by a set of stimuli like actuation of IL-10 and/or the presence of intercellular adhesion molecule-1/leukocyte function antigen-1 (ICAM-1/LFA-1) among other (Schwartz, 2003). Clonal deletion is characterized by an increase of T cells apoptosis followed by phagocytosis and induction of TGFβ from macrophages. Finally, generation of Treg cells is induced by anti-inflammatory cytokines TGFβ and IL-10 secreted by intestinal resident macrophages which participate in maintenance of oral tolerance (Faria & Weiner, 2005).

At the beginning, it was generally accepted that feeding of a high dose of antigen results in tolerance obtainment mediated by T cell clonal anergy and apoptosis (deletion), whereas low doses would favour active suppression leading to the induction of Treg cells (Melamed & Friedman, 1993; Tsuji, Mizumachi, & Kurisaki, 2001). For ethical reasons, the existence of mucosal induced tolerance in humans is based mainly on circumstantial evidence, however, it is now admitted that both mechanisms may take place simultaneously and cannot be completely differentiated (von Boehmer, 2005). It is also important to remark that mucosal and systemic immune responses are mainly stimulated by particulate antigens, captured through M cells in the GALT, whereas soluble dietary antigens, transported by enterocytes, are the responsible probably of inducing tolerance (P. E. Brandtzaeg, 2002).

In fact, recent studies have further clarified induction and effector phases of oral tolerance. It is considered that antigen-sampling by lamina propria APCs (including CX3CR1 macrophages) followed by transporting and presentation by migratory CD103+CCR7+ cells in the mesenteric lymph nodes is crucial for the generation of Foxp3+ Treg cells (crucial cells to oral tolerance induction) (Coombes et al., 2007). It has also been exposed that after Treg cells induction, their migration to the lamina propria and expansion mediated by CX3CR1+ macrophages is essential for the effector phase (Hadis et al., 2011). The result of the immune response can differ from no detectable response to the induction of antigen-specific antibodies in large amounts depending on many factors such as the genetic background of the individual, the nature of the antigen or its resistance to protease.

2.2 Breakdown of oral tolerance

Among mechanisms of defence in our body it can be found external physical barrier as skin and mucosal, chemical barrier as stomach pH and biochemical defences as inflammatory response which involve cells, cytokines and soluble proteins that try to destroy the external invader (viruses, bacteria, and fungi) and also internal dysfunction (cancer). The main characteristic of inflammatory response is the high speed and efficacy of response. However, inflammatory response includes an opposite anti-inflammatory reaction to control the tissular damage that inevitably occurs during

the biological fight. To finish the inflammatory reaction, an intense reconstruction takes place to repair the inflamed zone.

2.2.1. Inflammatory response

Inflammation can be classified as either *acute* or *chronic*. *Acute inflammation* is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes), neutrophils (primarily), basophils (inflammatory response), eosinophils (response to helminth worms and parasites), and mononuclear cells (monocytes, macrophages) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue; this response is immediate and last for few days and can result in to resolution of the inflammatory process, to an abscess formation or to a chronic inflammatory process (see table I). Prolonged inflammation, known as *chronic inflammation*, leads to a progressive shift in the type of cells present like mononuclear cells (monocytes, macrophages, lymphocytes, and plasma cells), fibroblasts at the inflammatory loci and is characterized by simultaneous destruction and healing of the inflamed tissue; this response are delayed and can take months or years to end, the outcomes of this process can give rise to the tissue destruction, fibrosis and necrosis (Table I) (Singer AJ, et al. 1999), that can develop into different pathologies such as allergy, inflammation and autoimmune diseases.

Table I. Division in the immune system

	Acute inflammation	**Chronic inflammation**
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies or autoimmune reactions
Cells involved	Neutrophils (primarily), basophils (inflammatory response), eosinophils (response to helminthic worms and parasites), mononuclear cells (monocytes, macrophages)	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFNγ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis, necrosis

Acute inflammation is initiated by an injury which activates some resident cells in tissues such as mastocytes, macrophages and dendritic cells. These cells express different intracellular and membrane receptors known as pattern recognition

receptors (PRR) that are activated by several conserved structural motifs, known as pathogen associated molecular patterns (PAMPs), which are present on bacteria, virus etc. One of the principal PAMP is the Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria (Nurtanio & Yang, 2011; Shibolet & Podolsky, 2007).

Briefly, in this acute inflammation, the main components are changes in flow and vascular calibre, increased vascular permeability and inflammatory exudates and also the passage of leukocytes from the vascular space to the extravascular (Table I). The origin of these disorders is due to the release of chemical mediators such as TNFα, IL-8, IL-6, IL-1, prostaglandin E2 (PGE2) and nitric oxide (NO). These substances also participate in the activation of leukocytes, inducing phagocytosis and bacteria lysis. The activation of coagulation system, fibrinolysis and complement enhance the inflammatory process. Increased expression of adhesion molecules on endothelial cells, such as ICAM-1, VCAM-1 (vascular adhesion molecules class I) or E-selectin facilitates diapedesis (migration of immune cells to inflammatory loci). In the first 6 to 24 hours, neutrophils are found in local inflammation but they are replaced by monocytes and macrophages arriving at loci within 24 hours (Kolaczkowska & Kubes, 2013). These cell types are responsible to phagocytise microorganisms, altered cell and tissue debris (Table I). At this stage the acquired immune response is triggered by antigens, resulting from phagocytosis by macrophages, DCs and B cells when the innate immune response is not capable to eliminate the pathogen or eradicate the inflammation. These antigens are displayed by APCs through their MHC-I or MHC-II to CD8+ cytotoxic T cells or CD4+ helper T cells respectively (Sherwood & Toliver-Kinsky, 2004). Then, naïve T helper cells can be activated by MHC-II antigens in macrophages or DCs or by the presentation of the antigen by B cells. In each of these cases the T cells are known by different names: Th1, Th2 or Th17 cells respectively. Thus, the decision of which phenotype will acquire T cells is determined by the co-stimulatory signals (Kuchroo et al., 1995). The immune response is also regulated by the cytokines environment being this the deciding factor in the differentiation to one or another phenotype (Th1, Th2 or Th17). According to that, IL-12 together with IL-18 (both produced by APCs) induce Th1 differentiation; IL-4 (released by natural killer (NK) cells, mast cells, basophils and mature CD4+ cells) is responsible for Th2 development; and IL-23 (secrete by macrophages and DCs) conduce to Th17 cells (Ghilardi & Ouyang, 2007).

Moreover, it was established in the past that Th1 and Th2 cells inhibit each other by the cytokines they release. For example, activated Th1 cells produce IFNγ, a cytokine capable to activate macrophages which will phagocyte the pathogen once again. If the cell mediated response occurred, IFNγ in addition to activate macrophages, it will inhibit the antibody response by the plasma cells. Furthermore, Th2 cells can cooperate with and can activate B cells by the release of cytokines (IL-4, IL-10 and IL-13). These cytokines will induce B cells to proliferate and to give rise to memory cells and plasma cells that will produce antibodies against these specific pathogens. When this antibody response takes place, IL-10 produced by Th2 cells will prevent Th1 cells form producing IFNγ, suppressing the cell mediated response once

the tissue is clear of invaders, leading to inflammation resolution. However, some experiment has put in evidence that a clear separation does not exist among both ways of responses, significantly in complex intestinal human pathologies (Zenewicz, Antov, & Flavell, 2009). Moreover, it seems that the Th1/Th2 balance is also regulated by more recently characterized CD4+T-cell subtypes, Treg cells, which down-regulate both types of immune responses by secreting TGFβ (Th3 cells) and IL-10 (Tr1 cells) (Allez & Mayer, 2004).

Chronic inflammation is characterized by the cellular infiltrate, mainly macrophages, lymphocytes and plasma cells, overriding the formation of fibrous tissue. This process of chronic inflammation can occur for various reasons such as the progression of acute inflammation or recurrent episode of inflammation by acute and frequent intracellular infections. Most of the cases lead to chronic pathologies such as arthritis rheumatoid, asthma, IBD and cancer among others (Garn, Neves, Blumberg, & Renz, 2013; Rieder & Fiocchi, 2009).

Lymphocytes play an important role in this type of inflammation which is present in continues manner in the locus, using substances produced by macrophages to their migration. Consecutively, secrete IFNγ (activator of macrophages) establishing the basis for the persistence of the inflammatory reaction. Macrophages also produce biologically active substances such as TNFα and IL-1β, among others substances, that can cause damage when produced in an uncontrolled manner. The accumulation of macrophages persists in chronic inflammation for the continued recruitment of monocytes from blood flow to the inflammatory loci. They will differentiate in macrophages after migration and proliferate in the inflamed area (Table I). On the other hand, plasma cells produce antibodies against the antigen that persist in the inflamed area or against altered tissue components. All of this produces a vicious cycle that leads to chronic inflammation in which all immunological cells are involved (Matsuzaki et al., 2012).

Therefore, in summary, mucosal tissue homeostasis results from the perinatal establishment of mucosal induced immune tolerance. Perinatal defects in the induction of mucosal tolerance are associated with the later development of different pathological situations such as intestinal inflammatory diseases, for instance IBD, food allergy and also celiac disease; autoimmune diseases (such as rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus) and chronic inflammation of the respiratory mucosa (Fasano, 2011; Tulic et al., 2011). Some of these pathologies are described below.

2.2.2 Allergic response

The allergic diseases are specific inflammatory disorders in which susceptible individuals have an aberrant immune response to innocuous environmental antigens. The decision of the immune system to respond to allergens is highly dependent on factors including the type and load of allergen, behaviour and type of APCs, innate immune response stimulating substances in the same micro-milieu, the tissue of

exposure, interactions between T and B lymphocytes, co-stimulators, and genetic propensity known as atopy. In summary, among Th2-type cytokines, IL-4 and IL-13 are responsible for class switching in B cells, which results in production of allergen-specific IgE antibodies that bind to specific receptors on mast cells and basophils. After re-exposure to the sensitized allergen, this phase is followed by activation of IgE Fc receptors on mast cells and basophils resulting in biogenic mediator release which is responsible for the symptoms and signs of anaphylaxis (see figure 3) (Stone, Prussin, & Metcalfe, 2010).

Although mast cells are the most important effectors in early allergic response through histamine production, Th1 and Th2 cells are also present in delayed reactions of atopic persons. Histamine, TNFα, IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also released after stimulation of mast cells and are responsible of early and delayed-phase allergic symptoms, as well as of Th2 cells activation. Th2 lymphocytes induce eosinophils activation playing an essential role in the physiopathology of some diseases as eosinophilic gastroenteropathies or allergic asthma. Moreover, they are present in non-IgE allergic response. The presence of Th1 cells is complex and their appearance has been described in chronic lesions of atopic dermatitis patients (Montero Vega, 2006).

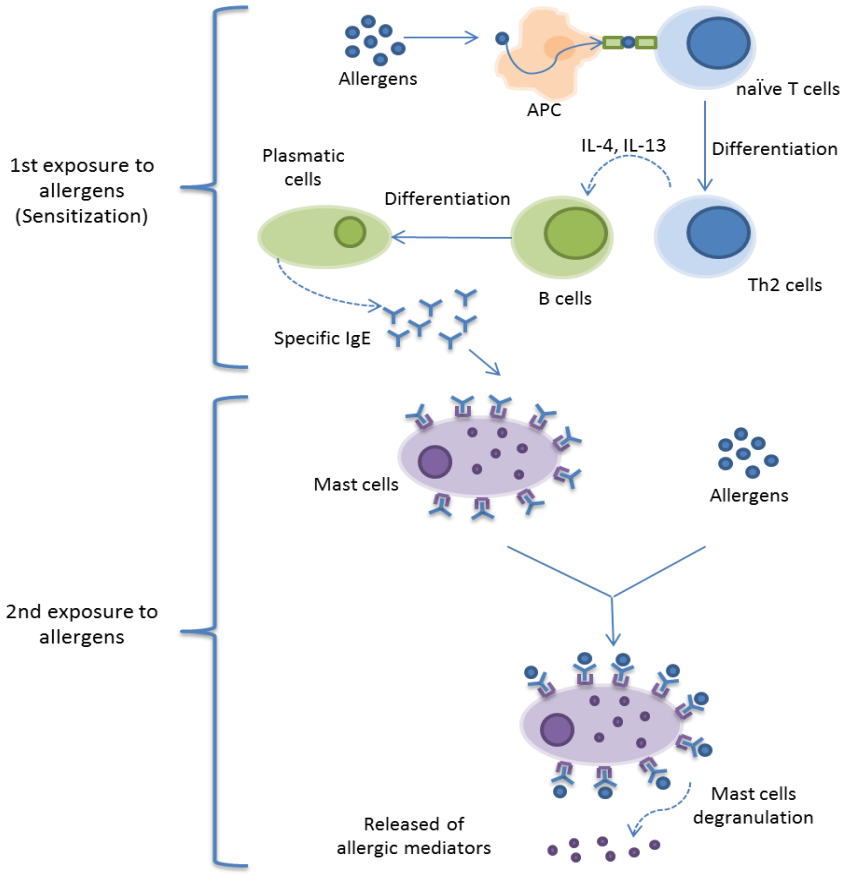

Figure 3.- General mechanism of allergy development. The allergy development occurs in various stages. Firstly the allergen is internalized, an antigen presenting cells present it to T cells that by cooperation T-B makes specific IgE secretion. These immunoglobulins will fix to the mast cells surface and when a second exposure to the

allergen will take place, it will bind to specific IgE and finally there will occur the mast cell degranulation will be given.

i) Food allergy: Cow's milk protein allergy

In 2010, the National Institute of Allergy and Infectious Diseases (NIAID, USA) published some guidelines were "food allergy" was defined as *an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food*. And food is described as anything designed to human consumption (Boyce et al., 2010).

Food allergy is a rising healthcare topic. Approximately 20% of the population of industrialized countries such as the United States, the United Kingdom and Germany, has experienced adverse reactions to food but only 1/3 of the reactions in children and 1/10 in adults are real food allergy in which there is an unusual immunological reaction to food. So, true food allergies are supposed to affect up to 6-8% of children younger than 10 years of age and 1-4% of adults with nuts, fruits and milk as the most common causes. Cow's milk protein allergy (CMPA) is the leading cause of food allergy in infants and young children under three years of age. However, true CMPA seems to have the highest incidence in the first year of life, with a prevalence of around 2% to 3% in the infant population. It then falls to <1% in children 6 years of age and older (Rona et al., 2007; Sicherer, 2011).

The reasons for the increased prevalence of allergic diseases during the past few decades are not completely clear yet, but it was suggested that the greater level of hygiene in urbanized populations in industrialized countries could play a fundamental role which was called "the hygiene hypothesis". According to this theory, modern hygiene, and dietary and medical practices affect the composition of the gut microbiota and limit exposure of infants to pathogens which are helpful to the correct development of the immune system. This change in the microbiota, in combination with genetic and epigenetic factors, influences not only the epithelial mucosal barrier but also perinatal maturation of the immune system, leading to disease susceptibility (Guarner et al., 2006). This hypothesis is sustained by the findings that probiotics such as lactobacilli and bifidobacteria strains can increase IgA responses in a T-cell dependent way and that non-pathogenic *Escherichia coli* or *Lactobacillus rhamnosus GG* reduce infection and protect against the progress of food-induced atopic dermatitis (Kalliomaki, Salminen, Poussa, Arvilommi, & Isolauri, 2003).

However, this hypothesis does not explain why a bigger Th2 reaction associated with inflammatory reaction or autoimmune diseases exists. It is proposed that any type of stimulations by pathogens, with Th1 or Th2 responses, induce Treg cells productions that control aberrant immune responses. Also, microbiota can induce this regulatory cells that control immune responsiveness. So, a drop in Treg cells production takes place in the reduction of contact with microorganisms and a failure in tolerance is originated (Montero Vega, 2006).

In food allergy, an increase in gut permeability has been observed, breaking the first physical barrier against foreign substances. This fact can be the origin or the consequence of allergic food reaction. Intestinal permeability is increased in two phases: fisrt, by a rise of transcellular permeability (transport of molecules of molecular weight (MW) > 600 Da (such as food antigens, peptides) through the epithelial cells) (Laiping So et al., 2000) that permit entry of allergen-IgE complex via the low-affinity IgE receptor (CD23, FcεRII) expressed on enterocytes. This form protects the allergen of metabolism into enterocytes. And a second phase where paracellular permeability (transport in the space between epithelial cells) has been enhanced by the presence of activated mast cells whose secretion of chymases affects tight junctions and open the otherwise sealed paracellular route. Histamine, TNFα, IL-13 and IL-8 secreted also from mast cells, influences in the intestinal permeability too. This increase of paracellular permeability triggering a massive passage of allergen molecules results in more severe local and systemic reactions (Perrier & Corthesy, 2011).

IgE-dependent food allergy can affect different organ systems such as the oral cavity and digestive tract, the skin, the respiratory tract and the cardiovascular system. The symptoms of allergy vary from slight inconveniences to life-threatening reactions if an anaphylaxis event occurs. Thus, food allergies manifest mainly with gastrointestinal (GI) symptoms in up to 50% of patients (Crespo & Rodriguez, 2003). The best characterized abnormal immunologic reaction to food is an immediate IgE-mediated hypersensitive to food allergens, also named as hypersensibility type I reaction. IgE production from B-cells is dependent of Th2-secreted cytokines (IL-4 and IL-13). But It also exists a non-IgE mediated food allergy form which is characterized by a delayed response with intestinal symptoms (diarrhoea) associated to TNFα production (Jyonouchi, 2012).

Related to the pathogenesis of the food allergy there is a continuous question with controversial answers and it is if alterations in intestinal permeability are the cause or the consequence of food allergy. This abnormality has been described in most inflammatory digestive diseases and although allergic inflammation leads to epithelial disorder with altered antigen handling most studies have resolved that increased gut barrier permeability is more a consequence rather than a cause of food allergy. Nevertheless it takes part in a self-perpetuating cycle which maintains allergic state (Heyman, 2005).

Nowadays relevant food allergens can be identified as the cause of gastrointestinal disorder in patients with food allergies. Taking this premise as a starting point, the standard of care of this alteration is the avoidance of the hurting allergen. However this option is limited by several reasons such as multiple food allergies, allergies to ordinary foods (cow´s milk, wheat, soy, eggs) or the inability to identify specific foods. The treatment of food allergy is still a matter of controversy and the current options are poorly evaluated. The general antiallergic drugs include oral preparations of disodium cromoglycate (mast cell-stabilizing drug which acts locally in

the GI tract) which is available for food allergy mild cases. Nevertheless there are not controlled studies confirming its efficacy and the studies accrediting the use of this drug are limited (Paganelli, Scala, Di Gioacchino, Bellioni, & Stefanini, 1996; Stefanini et al., 1995). In more severe cases, corticosteroids would be necessary and if anaphylaxis happens, therapy also with epinephrine and antihistaminic drugs might be inevitable.

There are several new immunomodulatory therapeutic strategies that offer the potential to be applied to food allergy. These involve allergen-specific and nonspecific therapies that have been recently reviewed by Virkud and Vickery (Virkud & Vickery, 2012).

Allergen-specific treatments include exposure to native allergen in different ways: oral, sublingual, and epicutaneous immunotherapy (desensitization). It also exists the possibility of giving modified food allergens such as peptide or plasmid DNA immunotherapy or mutated recombinant proteins co-administered with heat-killed bacteria, such as copiously heated (baked) milk and egg represent an alternative approaching to food oral immunotherapy and are changing the paradigm of strict dietary abstention for food allergy patients (Virkud & Vickery, 2012).

On the other hand, allergen-nonspecific approaches recently studied a Chinese herbal preparation which prevented anaphylaxis induced by peanut in a murine model and is currently being investigated in clinical trials (Wang et al., 2010). These approaches also include anti-IgE therapy, probiotics and even parasitic helminthic infections used as therapy, IL-4 or IL-5 receptor antagonists, Th1-type cytokines such as IL-12 or IFNɣ, and other experimental therapies as Glucagon-like peptide 2 that are directed to modify the intestinal barrier to make it less permeable to antigens (Land & Burks, 2012; Nowak-Wegrzyn & Sampson, 2011). Recently, it has been identified a new tactic to treat IgE-mediated food allergy which is the monoclonal antibody: omalizumab (Rafi et al., 2010). Nevertheless further investigations are necessary for clarify the pathophysiology of food allergy because this would lead to the development of reliable diagnostics methods and effective drugs for treatment of this disease.

Cow's milk protein allergy (CMPA)

CMPA is clearly related to artificial breastfeeding and is due mainly to a deficient maturation of mucosal immune system. In the last decades it has been demonstrated a less incidence of allergy in children who receive mother´s milk than in children who are feeding with infant formula. However, CMPA symptoms can develop in exclusively breastfed infants with an incidence rate of 0.5%. It, therefore, raises questions about sensitization to cow's milk proteins through breast milk. Transfer of native bovine proteins, such as β-lactoglobulin, into the breast milk is controversial: some authors have found bovine proteins in human milk but others allude to cross-reactivity between human and cow's milk proteins (Mavroudi & Xinias, 2011).

The main concern of infant food allergy is the possibility that this early reaction is a determining factor to adult pathologies. So, CMPA is an important disease but is not an

isolated event. It is usually the beginning of the "atopic career" and therefore it has been described that CMPA could play an essential role in the pathogenesis of others atopic pathologies that children would suffer later such as others food allergies, rhinitis, asthma and mainly atopic dermatitis. As an example, between 30 and 80% of children who are affected with CMPA and/or atopic dermatitis will develop asthma or another respiratory allergy (Gustafsson, Sjoberg, & Foucard, 2000). In regard to atopic dermatitis, this skin disease is frequently seen in patients with Ig-E mediated food allergy (20-40%) and is mostly related to mutations associated with loss of function of the fillagrin gene, which is involved in the epidermal barrier function (Rance, Boguniewicz, & Lau, 2008). Similar mutations seem to predispose for the combination of atopic dermatitis and asthma (Weidinger et al., 2013).

These findings which link artificial breastfeeding, food allergy incidence and the posterior developing of different diseases apparently reflect that a damaged surface epithelium in any part of the body and immaturity of the immune system during neonatal time may be a predisposing condition for allergen penetration.

2.2.3 Inflammatory bowel disease

The term inflammatory bowel disease (IBD) referred to a chronic, relapsing-remitting inflammatory pathology of unknown aetiology. The most representative clinical pictures are Crohn´s disease (CD) and Ulcerative colitis (UC) which share several symptoms (abdominal pain, diarrhoea, general malaise and body weight loss) but otherwise they present well-established differences (Table II) in injuries distribution and others clinical manifestations (Stange et al., 2008; Van Assche et al., 2010).

Table II. Differential features between CD and UC

Crohn´s disease (CD)	**Ulcerative colitis (UC)**
- From mouth to anus	- Rectum and/or Colon
- Discontinuous affectation	- Continuous affectation
- Transmural	- Involve mucosa layer
- Pasty diarrhoea	- Liquid diarrhoea with blood, mucus and pus
- Frequent fistulas and intestinal stenosis	- Low frequency of fistulas and intestinal stenosis
- Pathologic anatomy: granulomas and lymphoid aggregates	- Pathologic anatomy: crypt abscess, mucin depletion and glandular distortion
- Fibrosis	

Substantial differences have been observed in the incidence of IBD across different geographic areas and over time. IBD is observed mainly in the developed countries of the world, with the highest incidence and prevalence rates in North

America and Europe. In a recent systematic review (Molodecky et al., 2012), it was published that the IBD incidence is higher in Canada (19.2 per 100,000 persons (0.019%) for UC and 0.02% for CD), Northern Europe (UC was 0.024% in Iceland and 0.011% for CD in the United Kingdom), and Australia (0.017% for UC and 0.029% for CD). In the same way, prevalence is higher in Europe and Canada. A North-South gradient has long been described for IBD, but this geographic difference has the tendency to disappear because the incidence appears to have risen in Southern and developing countries in recent years (Molodecky et al., 2012). There are not solid evidences about sex differences in IBD epidemiology. The IBD incidence peak seems to occur among the second to the fourth decade of life. On the other hand, there are few studies which have evaluated the influence of race/ethnicity in this pathology but they show the greatest incidence of IBD among white and Jewish people. However, the incidence of IBD in Hispanic and Asian people is gradually increasing (Hou, El-Serag, & Thirumurthi, 2009).

Although many advances in the IBD pathogenesis characterization have occurred, the specific etiology is still unknown. Nevertheless, IBD is now generally thought to be caused by the breakdown of oral tolerance through a mixture of genetic and environmental factors (Pabst & Mowat, 2012). An incipient model of the IBD pathogenesis proposes that there are three crucial factors: a breakdown in intestinal barrier function; exposure of luminal substances to immune cells in the lamina propria; and an overstated immune response (Clayburgh, Shen, & Turner, 2004). Patients with CD with clinically active disease have augmented intestinal permeability, and in inactive-disease patients, increased intestinal permeability is a clinical relapse prognostic. In addition to patients with IBD, increased intestinal permeability occurs in 10-25% of their healthy first-degree relatives, indicating that increased intestinal permeability likely preceded the beginning of clinical disease (Hedin, Stagg, Whelan, & Lindsay, 2012; Wyatt, Vogelsang, Hubl, Waldhoer, & Lochs, 1993)

But it is important to highlight that although intestinal permeability is a key participant in the development of IBD, data suggest that amplified intestinal permeability alone is not enough to predispose to IBD. Moreover, the breakdown in the protective barrier in IBD patients leads to an inflammatory infiltrate and higher production of cytokines and other mediators that can further contribute to alter the function of the barrier (Hering, Fromm, & Schulzke, 2012; Madara & Stafford, 1989; Mullin, Laughlin, Marano, Russo, & Soler, 1992). Until a few years ago, the dominant paradigm was that human CD promoted mainly a Th1-biased response associated with excessive IFNγ/TNFα/IL-12 secretion, whereas UC was mediated by an atypical Th1/Th2 inflammation pattern in which an increase in Th2 cytokines (without affecting IL-4 levels) was associated with the production of several Th1 pro-inflammatory markers as TNFα and IL-1β (Bailon et al., 2011). But in the last few years, the Th1/Th2 paradigm has in fact been replaced by the Th1-Th17 paradigm (Di Sabatino, Biancheri, Rovedatti, MacDonald, & Corazza, 2012). Genome-wide association studies (GWAS) have let to discover that genetic polymorphisms of the IL-23 receptor locus are associated with both CD and UC suggesting that the IL-17/IL-23 axis could have a role in the pathogenesis of both conditions (Duerr et al., 2006). In fact, it has been recently

demonstrated that levels of IL-17 and Th17 were increased in patients with UC and CD compared with controls, and these Th17 cells were found copiously in the mucosa and submucosa of UC and CD, respectively (Round & Mazmanian, 2009). IL-23 (which is also increased in IBD) promotes Th17 and Th1 responses, leading to inflammation of the GI tract (Kamada et al., 2008)

In addition, it has also been proposed the environment as a participant in the IBD unleashing. Industrialization and urbanization of societies are associated with changes at many levels (microbial exposures, hygiene, diet, lifestyle behaviours, medications, etc.), which have all been implicated as potential environmental risk factors for IBD. However, the exact connection between genetic susceptibility and the role of environment in the pathogenesis of IBD remains unknown (Molodecky et al., 2012).

Due to the chronical inflammation the symptoms depend on the affected segment of the intestinal tract and therefore there are some differences between UC and CD at this level. Some of the symptoms related to inflammatory damage in the digestive tract are diarrhoea, constipation, pain or rectal bleeding with bowel movement, tenesmus, abdominal cramps and pain. But there are also general features associated with IBD in some cases, such as fever, loss of appetite, weight loss, fatigue, night sweats, primary amenorrhea, etc. Apart from intestinal complications, it can also occur extraintestinal complications which affect up to 25% of patients, being arthralgia the most frequent problem (C. Mowat et al., 2011).

Medical treatment to cure the disease is not yet available, but actual therapies can control symptomatology in a very effective way which optimizes quality of life. IBD management often requires long-term treatment based on a combination of drugs to control the disease. Apart from changes in diet and lifestyle, drugs which are usually employed in IBD are summarized in Table III (C. Mowat et al., 2011; Sandborn, 2012).

Table III. Therapeutic agents in IBD

Aminosalicylates—anti-inflammatory agents	- 5-aminosalicylic acid (5-ASA) - Sulfasalazine, mesalamine, olsalazine, balsalazide
Corticosteroids	- IV (methylprednisolone, hydrocortisone) - Oral (prednisone, prednisolone, budesonide, dexamethasone) - Rectal
Immune modifiers	- Thiopurines: 6-mercaptopurine (6-MP) and azathioprine (AZA) - Calcineurin inhibitors: cyclosporin A, tacrolimus - Methotrexate (MTX)

Anti-tumor necrosis factor (anti-TNF) agents	- Infliximab - Adalimumab - Certolizumab - Golimumab
Antibiotics	- Metronidazole - Ciprofloxacin
Probiotics	- *Escherichia coli Nissle 1917* - *Lactobacillus rhamnosus GG*

There is also a large range of new treatment strategies which some of them are now being used in an off-label way and in a few years will probably supply several novel therapeutic agents, such as the selective anti- α4 integrin therapy with vedolizumab and natalizumab; the anti-interleukin 12/23p40 therapy with ustekinumab and Janus kinase 1, 2 and 3 inhibition with tofacitinib (Cohen, Nanau, Delzor, & Neuman, 2014; Sandborn, 2012).

3. ANIMAL MODELS

Animal models are useful tools to study the pathogenesis and molecular mechanisms of the human diseases. Some limitations could be the different anatomical, physiological and immunological features between man and animals and for that reason, caution is necessary in interpretation of the results from animal models to clinical approach. Nonetheless, although no animal model fully mimics the full range of clinical manifestations of the human diseases, many of them reproduce a collection of the features that characterize its most common forms.

Experimental models mimic basically biochemical changes in the response of immune system where are involved several cells, antibodies and cytokines that are responsible for the symptoms of the diseases.

3.1. Animal models of allergy

Mice are the most common species used in animal models of allergic diseases due to the availability of various immunological and molecular reagents, including transgenic animals. Nevertheless most of these allergic disease models are acute and transient after initial allergen sensitization and challenge, and there is not a correspondence with the usually chronic allergic human diseases. Furthermore, mice do not develop allergic disease spontaneously and chronic models are difficult to establish. However, mouse model offer the opportunity to explore detailed mechanisms of allergic reactions that would be impossible for ethic consideration in human, as well as studies in animal models provide major advances in the understanding of the disease concept (Takeda & Gelfand, 2009).

An ideal animal model should imitate human disease as closely as possible with respect to route of exposure, mechanisms underlying the disease and associated symptoms and therefore possess the following features: 1) having similar allergenic response as humans; 2) having a similar reactivity to proteins on an “allergenicity” scale compared to humans, thus discriminating allergens from non-allergens; 3) elicit response using typical routes of exposure for administration; 4) no adjuvant requirement, and 5) being reproducible, specific and sensitive (Ahuja, Quatchadze, Stelter, Albrecht, & Stahlmann, 2010).

A common problem related to allergic animal model is the high capacity of rodents to develop oral tolerance for antigenic protein, in contrast to what occur in human. This problem is getting around with the use of antigen plus adjuvants as described below.

Frequently, mouse strain Balb/c is used for its tendency to develop Th2-biased immune responses. Authors have employed a wide variety of antigens, application

routes, doses and sequences. Some of that antigens are frequently associated with asthma in humans as house dust mite or Aspergillus, and other such ovalbumin (OVA) from chicken egg is only for experimental use (Schroder & Maurer, 2007). OVA preferentially induces a Th2 response in T cells and offers the opportunity to perform experiments for study the presence of OVA-specific immunoglobulins in mice. Balb/c mice also exhibit clinical symptoms of food allergy such as diarrhoea, anaphylaxis, and eosinophils and mast cells accumulation apart from antibody responses (IgE, IgG1).

Usually, protocols for allergy induction in mice include a sensitization period, followed by a challenge period. Approximately 24 h after challenge, mice develop an increase of antigen-specific IgE and IgG1 antibodies but not IgG2a as an evidence of Th2-biased response. Many investigators have explored the differences between oral or intraperitoneal (i.p.) administration of protein in various models of food allergy and have observed that i.p. administration promote a strong IgE response while the oral exposure was found to be less sensitive. However, some authors have suggested that oral sensitization of mice requires low doses and intermittent protein intake (Ahuja et al., 2010; Bowman & Selgrade, 2009). So studies in food allergy animal model utilize systemic sensitization followed by oral challenge because the oral sensitization does not induce allergy but oral tolerance (driven by the Treg cells and others mechanisms). Two approaches are currently available to avoid oral tolerance in adult animals: to use an alternative route or to include an adjuvant (aluminium hydroxide, cholera toxin, complete Freund's adjuvant) in the oral exposure (Aldemir, Bars, & Herouet-Guicheney, 2009; Bowman & Selgrade, 2009).

In food allergy the degree of sensitization should take into account some considerations: i) the concentration of allergen; ii) the allergen should be taken in context with the food source; iii) the route and duration of allergen exposure; iv) age of animals; v) a genetic predisposition; vi) the use of adjuvants; vii) isotype specificity response, and viii) Th1/Th2 polarization (Helm, 2002). Studies of allergy are based on the notion that this disease is a consequence of Th2 response against the presence of a foreign protein, but in clinical studies it has been demonstrated that human T cells were not as obviously polarized in this way, and the concept that atopic disease reflects a Th1/Th2 imbalance is probably too simplistic (Takeda & Gelfand, 2009). Recently, Th1/Th2 inflammatory responses were reported to be influenced by the levels of LPS exposure in animal models. The exposure to a high level of LPS resulted in increased antigen-specific Th1 responses, while a low dose of LPS resulted in Th2 sensitization (Yamashita & Nakayama, 2008).

In this type of allergy the experimental endpoint includes systemic anaphylaxis symptoms such as fall in body temperature, loss of body weight or diarrhoea (Takeda & Gelfand, 2009). Allergen-specific IgE-mast cell interactions associated with Th2-type immune responses have been linked to the allergic reactions to foods such as rice or cow's milk. CD4+ T cells from Peyer´s patches were shown to be the prominent effectors cells as a source of Th2 cytokines (IL-4), whereas CD8+ T cells or Treg cells played potent suppressive roles controlling the oral tolerance induction. Furthermore,

protective roles for allergen-specific IgA, IFNγ or IL-10 have been shown in food allergy (R. A. Peterson, 2012).

Besides mice, others animals are useful to study different allergic reaction as asthma or food allergy. Guinea pigs, rats, dogs, horses, swine or monkeys have been frequently used but they are hard to handle and too expensive to use on a regular basis (Helm, Ermel, & Frick, 2003; Shin, Takeda, & Gelfand, 2009). Also, worms and flies are useful biological models to the intestinal inflammatory disease study (Lin & Hackam, 2011). Despite the benefits of animal models of food allergy, there are some limitations. Until now, there is not any animal model which can detect known food allergens, predict the allergic potential of novel food proteins or imitate the food-allergic sensitization and allergic responses in human (Takeda & Gelfand, 2009).

i) Allergy induced by Ovalbumin

Among different types of allergens, ovalbumin (OVA) derived from chicken eggs is frequently used for induce a robust allergic inflammation in mice and moreover is cheap, can be highly purified and the immunodominant epitopes have been well characterized. The way of administration is very important to develop allergic reaction without induction of tolerance. For OVA, i.p. injection with an adjuvant is the prominent route for sensitization. The responses observed in mice following i.p. administration have been compared with those observed following gavage administration and a stronger IgE response was observed following systemic exposure of mice to OVA (Ahuja et al., 2010).

Usually, acute sensitization requires multiple systemic administrations (14-21 days) of allergen in presence of an adjuvant. Aluminium hydroxide ($AlOH_3$) is one of the best choices for adjuvant since it promotes the development of Th2 immune response when it is exposed to antigen. Nevertheless, aluminium hydroxide is a non-physiological substance that can induce mast cell-independent allergic reaction. For that, some authors have demonstrated that this adjuvant is not required for the generation of acute allergic inflammatory response using OVA as antigen (Conrad et al., 2009). OVA has been used to trigger different allergic reaction as asthma, rhinitis and food allergy depending on administration's route (Golias et al., 2012; Moon, Kim, Rhee, Lee & Min, 2012; Schroder & Maurer, 2007). In all cases, OVA induce a systemic and characteristic strong IgE/IgG_1 specific response whose elevation can be measured by ELISA in plasma and in specific altered organ (lung, nose, intestine).

OVA-induced allergy is clearly a model with a high Th2-biased response demonstrated by increased levels of IL-4, IL-5, IL-13 and IL-10. These cytokines play an important role in the production of antigen-specific IgE and mucus secretion, muscle contractility and eosinophilia. However, there is also an elevation of IL-17 and IFNγ demonstrating a contribution of Th17 and Th1 subset. This fact is present also in allergic patients (Liu & Yang, 2011; Perrier, Thierry, Mercenier, & Corthesy, 2010).

ii) Cow's milk protein allergy

Cow's milk protein allergy is a major cause of transient food allergy hypersensitivity in children. It involves the skin, respiratory and gastrointestinal tract and can lead to systemic anaphylactic shock (Helm, 2002). Animal models of this allergy use oral immunization to mimic human food allergy by provoking food hypersensitivity after oral ingestion. To bypass the tendency of mice to develop oral tolerance is necessary to include an adjuvant as choleric toxin which stimulates a Th2 response, increasing the intestinal permeability and the production of IgG1 antibodies.

Just-weaned three weeks old mice are necessary for this model to induce a good allergic response. The strain used can be Balb/c or C3H/HeJ with the same results (Helm, 2002; Lara-Villoslada, Olivares, Jimenez, Boza, & Xaus, 2004; Li, Schofield, Huang, Kleiner, & Sampson, 1999). Cow´s milk-specific IgE antibody levels were shown to be significantly increased at 3 weeks and peaked at 6 weeks after the initial feeding. Intragastric challenge with cow´s milk at week 6 elicited systemic anaphylaxis accompanied by vascular leakage, significantly increased plasma histamine and increased intestinal permeability to casein. Histological examination of intestinal tissue revealed marked vascular congestion, oedema, and sloughing of enterocytes. Development of IgE-mediated cow's milk hypersensitivity in this model is likely to be Th2-mediated because in vitro stimulation of mice spleen cells from mice to cow´s milk induced significant increases in the levels of IL-4 and IL-5, but not IFNγ.

However, this model has some limitations that significantly reduce its utility such as it requires long periods of sensitization to achieve a sustained and measurable systemic immune response. Moreover, because of the need to use adjuvants during such a long period of time, the allergic response could be related to them.

iii) Dinitrofluorobenzene atopic dermatitis

Similar to previous diseases, atopic dermatitis is a Th2-related pathology associated with high IgE levels. Nevertheless, both the Th2 cytokines IL-4 and IL-5 and the Th1 cytokine IFNγ play important roles in the inflammation and hypertrophy of the skin in patients. The skin barrier dysfunction and filaggrin mutations in some patients are important factors that contribute to bacterial super-infection in the skin allowing the persistent and refractory disease (Takeda & Gelfand, 2009).

Mouse models of atopic dermatitis are difficult to establish probably due to the fact that very different characteristics exist between mice and human skin. Epicutaneous sensitization can be made with diverse allergen as OVA, Der p8 (house dust mite protein) or haptens as Oxazolone, Trinitrochlorobenzene and Dinitrofluorobenzene (DNFB) (Kaplan, 2010). Protocols include a first sensitization with the allergen in a clear skin, and then repeated cutaneous challenge in the same or different skin site. The number of challenge must be high in the case of haptens, since a low number of challenges elicit a Th1-biased response while a high number (9-10)

lead to a chronic Th2 dominated inflammatory response that is similar to human atopic dermatitis. Both, Balb/c and C57BL/6 strains of mice are capable of respond similarly.

Some authors have found a great similarity between man and mouse in the regulation and mobilisation of Langerhans cells in the skin. Like in human, mouse epidermal Langerhans cells need two signals for activation: TNFα provided from keratinocytes and IL-1β in autocrine fashion. This is an important fact because all results found in mice studies respecting cutaneous allergic reaction can be extrapolated to human skin allergy (Griffiths, Dearman, Cumberbatch, & Kimber, 2005).

3.2. Animal models of intestinal inflammation

As it has previously been described, inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract that comprises two major conditions: Crohn's disease (CD) and ulcerative colitis (UC).

The majority of animal models exhibit Th1-dominant immune responses, and they have been widely used for studying CD. Although CD affects both small (ileitis) and large (colitis) intestines, only some CD models develop inflammation in the small intestine. In contrast to Th1-mediated models, mouse models developing Th2-mediated colitis for the study of UC are limited (Mizoguchi & Mizoguchi, 2010). Many animal models, including genetically engineered mice, congenic mouse strains, chemically induced models, and cell-transfer models have been established for studying IBD (Jurjus, Khoury, & Reimund, 2004). Nowadays, GWAS have revealed more than 100 genetic loci that show significant association with IBD, majority of these related to intestinal homeostasis. Notably, the results of human genetic studies are remarkably coherent with the data derived from experimental models (Saleh & Elson, 2011).

As it has been reviewed by Mizoguchi et al., there is a great amount of animal models to study IBD which are categorized mainly into chemically induced, cell-transfer, congenial mutant, and genetically engineered models (Mizoguchi, 2012). Three of these chemically induced animal models are detailed below.

i) Colitis induced by Dextran Sulfate Sodium

This type of colitis is induced by the oral administration of Dextran Sulfate Sodium (DSS) dissolved in water to mice or rats and for its simplicity is widely used. Among symptoms it is found hematochezia, body weight loss, shortening of the intestine, mucosal ulcers and neutrophils infiltration. Cytokines as IL-1 and TNFα and adhesion molecules as ICAM1 and VCAM1 are involved in the pathogenesis of this model like it occurs in human IBD.

Several factors may affect susceptibility to DSS-induced lesion, such as concentration, duration and frequency of DSS administration, molecular weight of DSS, intestinal flora present in the bowel, and genetic mouse factors. So, the most severe form of colitis in Balb/c mice is observed when mice are treated with DSS 40 kilo Dalton (kDa) molecular weight, presenting a severe and diffuse colitis in the middle and distal third of the large bowel, while mice treated with DSS 5 kDa develop milder form of colitis with lesions located at cecum and upper colon (Perse & Cerar, 2012). Acute colitis is usually induced by continuous administration of 2-5% DSS for short period (4-9 days) and chronic colitis may be induced by continuous treatment of low concentrations or cyclical administration of DSS (Perse & Cerar, 2012).

Major differences exist among mice strains in the response to DSS-induced colitis. For example, it has been observed that Balb/c mice are less susceptible than C3H mice, and C57BL/6 mice. Melgar and coworker have studied the differences between both strains of mice to develop chronic colitis when mice are exposed for five days to DSS (45 kDa, 3- 5%). Whereas C57BL/6 mice develop a chronic colitis for 4 weeks after DSS removal, in Balb/c mice any symptom or biochemical feature correlated with chronic colitis were observed in the same conditions (Melgar, Karlsson, & Michaelsson, 2005).

Cytokine profile of DSS acute colitis is consistent with acute inflammatory response characterized by a macrophage-derived cytokine profile, strong chemotactic pattern and a polarized Th1/Th17 panel (TNFα, IL-1β, IL-17) similar in both C57BL/6 and Balb/c mice. However, a Th2-biased profile is observed in DSS chronic colitis characterized by high levels of IL-4, IL-6 and IFNγ only in C57BL/6 strain (Alex et al., 2009).

ii) Colitis induced by Dinitrofluorobenzene/dinitrosulfonic acid

In 1992, Brkic et al. introduced a model of experimental colitis in mice induced by Dinitrofluorobenzene (DNFB) in sensitized animal, mimicking IBD by the intestinal histopathological and extra-intestinal manifestations (Brkic et al., 1992). Later, Rijnierse et al. presented this model as a non-IgE mediated hypersensitivity model with associated mast cells activation (Rijnierse, Koster, Nijkamp, & Kraneveld, 2006). A hypersensitivity reaction in the colon was observed with DNFB upon skin sensitization followed by a local intrarectal challenge with the corresponding Dinitrosulfonic acid (DNS) (Rijnierse et al., 2006). In Balb/c mice an acute response occurs within 6 h after challenge and is prolonged as far as 72h. This effect has been associated with direct mast cell activation and degranulation as indicate the presence of mast cell protease-1 (MCP-1) protein in plasma. Moreover, in colon tissue is possible to observe a hypertrophy of colonic patches and an increase in colonic TNFα levels. The activation of mast cells in this model trigger the activation of T cells which have a bidirectional interactions with mast cells resulting in a reciprocal activation (Rijnierse et al., 2006)

On the other hand, Wen-Bin Xiao and Yu-Lan Liu observed some changes in CD8+ T-cell in DNFB/DNS induced colitis in rats, such as an increase in the population of

CD8+CD28- Treg cells in control group. However, this change is not correlated with the biochemical features found in human IBD (Xiao & Liu, 2003).

In spite of this model reflects the important role of TNFα in human CD as well as UC, it only let's to examine partially the complex scene of human IBD.

iii) Colitis induced by Trinitrobenzene sulfonic acid

Trinitrobenzene sulfonic acid (TNBS)-induced colitis is a predominant Th1 response to hapten that resembles human Crohn´s disease. Granulomas with infiltration of inflammatory cells (T cells and macrophages) in all layers were seen in the intestine of animals after intracolonic administration of TNBS in 50% ethanol. The isolated macrophages produced large amounts of IL-12, and the lymphocytes produced large amounts of IFNγ and IL-2. Also, water absorption in the inflamed mucosa was markedly diminished (Liu & Yang, 2011). The similarities between the human disease and TNBS-induced colitis have allowed to show the immune system involved in the pathogenesis of IBD and to test therapeutic molecules that have the potential to be used in the clinic for the management of the human disease.

Even a strong Th-1 related response is evident in the cytokines profile of TNBS-induced colitis, participation of Th-2 derived cytokines is observed in IL-12-/- mice where a Th-1 biased response is impossible (Dohi & Fujihashi, 2006). In this case, the response is characterized by a crypt distortion and mononuclear cell infiltration limited to the mucosal layer.

Similarly to what occurs in others animal models, a chronic colitis model can be developed with chronic administration of low doses of TNBS and this permit to generate intestinal fibrosis (Zhu, Lu, Ou, Zhang, & Chen, 2012). Characteristic intestinal fibrosis mediated by TGF-β_1, platelet derived growth factor (PDGF), connective tissue growth factor (CTGF) and TNFα is observed in day 15 after TNBS administration. All together let the deposition of collagen I and III in a wide area of intestine (Zhu et al., 2012). Moreover, IL-17 has been found as an important key in the inflammation caused by TNBS administration. Jin et al. working with IL-17 knockout (KO) mice, IFNγ KO mice and anti-IL-17 monoclonal antibody have found that the presence of IL-17 at sites of local inflammation plays an important role in the pathogenesis of TNBS-induced acute colitis, while IFNγ in peripheral blood was related to systemic inflammation (Jin, Lin, Lin, & Zheng, 2012).

Resemblance between TNBS colitis mouse model and human CD is also evident in the contribution of pattern recognition receptors as TLR or nucleotide-binding oligomerization domain containing 2 (NOD2) in colitis seriousness. NOD2 was the first identified gene strongly related with susceptibility to CD and the loss of function by a mutation of NOD2 leads to reduced antimicrobial resistance and impaired innate immunity in CD (Comalada & Peppelenbosch, 2006). In NOD2-/- mice it has been described a reduction in IELs that include >70% of CD8+ T cells and comprise greater numbers of TCRYδ+ T cells. The fall of this T cells is responsible of loss of homeostasis

in intestinal mucosa. Moreover, the reduction in IELs contributes to the high susceptibility of NOD2-/- mice to TNBS induced colitis (Jiang et al., 2013).

REFERENCES

Abadie, V., Discepolo, V., & Jabri, B. Intraepithelial lymphocytes in celiac disease immunopathology. Semin Immunopathol. 2012;34(4):551-566.

Abreu, M. T. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 2010;10(2):131-144.

Ahuja, V., Quatchadze, M., Stelter, D., Albrecht, A., & Stahlmann, R. Evaluation of biotechnology-derived novel proteins for the risk of food-allergic potential: advances in the development of animal models and future challenges. Arch Toxicol. 2010;84(12):909-917.

Aldemir, H., Bars, R., & Herouet-Guicheney, C. Murine models for evaluating the allergenicity of novel proteins and foods. Regul Toxicol Pharmacol. 2009;54(3 Suppl):S52-57.

Alex, P., Zachos, N. C., Nguyen, T., Gonzales, L., Chen, T. E., Conklin, L. S., . . . Li, X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis. 2009;15(3):341-352.

Allez, M., & Mayer, L. Regulatory T cells: peace keepers in the gut. Inflamm Bowel Dis. 2004;10(5):666-676.

Artis, D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol. 2008;8(6):411-420.

Atuma, C., Strugala, V., Allen, A., & Holm, L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol. 2001;280(5):G922-929.

Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., & Ouellette, A. J. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol. 2000;1(2):113-118.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-1920.

Bailon, E., Cueto-Sola, M., Utrilla, P., Nieto, A., Garrido-Mesa, N., Celada, A., . . . Comalada, M. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis. 2011;17(10):2087-2101.

Bain, C. C., Scott, C. L., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., . . . Mowat, A. M. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C(hi) monocyte precursors. Mucosal Immunol. 2012.

Barker, N., van de Wetering, M., & Clevers, H. The intestinal stem cell. Genes Dev. 2008;22(14):1856-1864.

Bhagat, G., Naiyer, A. J., Shah, J. G., Harper, J., Jabri, B., Wang, T. C., . . . Manavalan, J. S. Small intestinal CD8+TCRgammadelta+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. J Clin Invest. 2008;118(1):281-293.

Biancheri, P., Di Sabatino, A., Corazza, G., & MacDonald, T. Proteases and the gut barrier. Cell and Tissue Research. 2012:1-12.

Blikslager, A. T., Moeser, A. J., Gookin, J. L., Jones, S. L., & Odle, J. Restoration of barrier function in injured intestinal mucosa. Physiol Rev. 2007;87(2):545-564.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., . . . Merad, M. Origin of the lamina propria dendritic cell network. Immunity. 2009;31(3):513-525.

Bonneville, M., Janeway, C. A., Jr., Ito, K., Haser, W., Ishida, I., Nakanishi, N., & Tonegawa, S. Intestinal intraepithelial lymphocytes are a distinct set of gamma delta T cells. Nature. 1988;336(6198):479-481.

Bowman, C. C., & Selgrade, M. K. Utility of rodent models for evaluating protein allergenicity. Regul Toxicol Pharmacol. 2009;54(3 Suppl):S58-61.

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., . . . Panel, N. I.-S. E. Guidelines for the Diagnosis and Management of Food Allergy in the United States: Summary of the NIAID-Sponsored Expert Panel Report. J Allergy Clin Immunol. 2010;126(6):1105-1118.

Brandtzaeg, P. Development and basic mechanisms of human gut immunity. Nutr Rev. 1998;56(1 Pt 2):S5-18.

Brandtzaeg, P. Function of mucosa-associated lymphoid tissue in antibody formation. Immunol Invest. 2010;39(4-5):303-355.

Brandtzaeg, P., & Pabst, R. Let's go mucosal: communication on slippery ground. Trends in Immunology. 2004;25(11):570-577.

Brandtzaeg, P. E. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. Ann N Y Acad Sci. 2002;964:13-45.

Brkic, T., Banic, M., Anic, B., Grabarevic, Z., Rotkvic, I., Artukovic, B., . . . Hernandez, D. E. A model of inflammatory bowel disease induced by 2,4-dinitrofluorobenzene in previously sensitized BALB-c mice. Scand J Gastroenterol. 1992;27(3):184-188.

Clayburgh, D. R., Shen, L., & Turner, J. R. A porous defense: the leaky epithelial barrier in intestinal disease. Lab Invest. 2004;84(3):282-291.

Cohen, L. E., Nanau, R. M., Delzor, F., & Neuman, M. G. Biologic therapies in inflammatory bowel disease. Transl Res. 2014.

Comalada, M., & Peppelenbosch, M. P. Impaired innate immunity in Crohn's disease. Trends Mol Med. 2006;12(9):397-399.

Conrad, M. L., Yildirim, A. O., Sonar, S. S., Kilic, A., Sudowe, S., Lunow, M., . . . Garn, H. Comparison of adjuvant and adjuvant-free murine experimental asthma models. Clin Exp Allergy. 2009;39(8):1246-1254.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y., & Powrie, F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757-1764.

Corbin, B. D., Seeley, E. H., Raab, A., Feldmann, J., Miller, M. R., Torres, V. J., . . . Skaar, E. P. Metal Chelation and Inhibition of Bacterial Growth in Tissue Abscesses. Science. 2008;319(5865):962-965.

Corr, S. C., Gahan, C. C., & Hill, C. M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol. 2008;52(1):2-12.

Craig, S. W., & Cebra, J. J. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J Exp Med. 1971;134(1):188-200.

Crespo, J. F., & Rodriguez, J. Food allergy in adulthood. Allergy. 2003;58(2):98-113.

Chase, M. W. Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. Proc Soc Exp Biol Med. 1946;61:257-259.

Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science. 1994;265(5176):1237-1240.

Cheroutre, H. Starting at the beginning: new perspectives on the biology of mucosal T cells. Annu Rev Immunol. 2004;22:217-246.

Cheroutre, H., Lambolez, F., & Mucida, D. The light and dark sides of intestinal intraepithelial lymphocytes. Nat Rev Immunol. 2011;11(7):445-456.

Denning, T. L., Granger, S. W., Mucida, D., Graddy, R., Leclercq, G., Zhang, W., . . . Kronenberg, M. Mouse TCRalphabeta+CD8alphaalpha intraepithelial lymphocytes express genes that down-regulate their antigen reactivity and suppress immune responses. J Immunol. 2007;178(7):4230-4239.

Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811-818.

Di Sabatino, A., Biancheri, P., Rovedatti, L., MacDonald, T. T., & Corazza, G. R. New pathogenic paradigms in inflammatory bowel disease. Inflamm Bowel Dis. 2012;18(2):368-371.

Dohi, T., & Fujihashi, K. Type 1 and 2 T helper cell-mediated colitis. Curr Opin Gastroenterol. 2006;22(6):651-657.

Duerkop, B. A., Vaishnava, S., & Hooper, L. V. Immune responses to the microbiota at the intestinal mucosal surface. Immunity. 2009;31(3):368-376.

Duerr, R. H., Taylor, K. D., Brant, S. R., Rioux, J. D., Silverberg, M. S., Daly, M. J., . . . Cho, J. H. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science. 2006;314(5804):1461-1463.

Fagarasan, S., Kawamoto, S., Kanagawa, O., & Suzuki, K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol. 2010;28:243-273.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R., & Neurath, M. F. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol. 2004;172(9):5149-5153.

Faria, A. M., & Weiner, H. L. Oral tolerance. Immunol Rev. 2005;206:232-259.

Fasano, A. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. Physiol Rev. 2011;91(1):151-175.

Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4(4):330-336.

Garn, H., Neves, J. F., Blumberg, R. S., & Renz, H. Effect of barrier microbes on organ-based inflammation. J Allergy Clin Immunol. 2013;131(6):1465-1478.

Ghilardi, N., & Ouyang, W. Targeting the development and effector functions of TH17 cells. Semin Immunol. 2007;19(6):383-393.

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., . . . Nelson, K. E. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-1359.

Golias, J., Schwarzer, M., Wallner, M., Kverka, M., Kozakova, H., Srutkova, D., . . . Tuckova, L. Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. PLoS One. 2012;7(5):e37156.

Goto, Y., & Kiyono, H. Epithelial barrier: an interface for the cross-communication between gut flora and immune system. Immunol Rev. 2012;245(1):147-163.

Griffiths, C. E., Dearman, R. J., Cumberbatch, M., & Kimber, I. Cytokines and Langerhans cell mobilisation in mouse and man. Cytokine. 2005;32(2):67-70.

Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H. S., McGuirk, P., van Eden, W., . . . Rook, G. A. Mechanisms of disease: the hygiene hypothesis revisited. Nat Clin Pract Gastroenterol Hepatol. 2006;3(5):275-284.

Gustafsson, D., Sjoberg, O., & Foucard, T. Development of allergies and asthma in infants and young children with atopic dermatitis--a prospective follow-up to 7 years of age. Allergy. 2000;55(3):240-245.

Gutgemann, I., Fahrer, A. M., Altman, J. D., Davis, M. M., & Chien, Y. H. Induction of rapid T cell activation and tolerance by systemic presentation of an orally administered antigen. Immunity. 1998;8(6):667-673.

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., . . . Pabst, O. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity. 2011;34(2):237-246.

Hase, K., Kawano, K., Nochi, T., Pontes, G. S., Fukuda, S., Ebisawa, M., . . . Ohno, H. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. Nature. 2009;462(7270):226-230.

Hedin, C. R., Stagg, A. J., Whelan, K., & Lindsay, J. O. Family studies in Crohn's disease: new horizons in understanding disease pathogenesis, risk and prevention. Gut. 2012;61(2):311-318.

Helm, R. M. Food allergy animal models: an overview. Ann N Y Acad Sci. 2002;964:139-150.

Helm, R. M., Ermel, R. W., & Frick, O. L. Nonmurine animal models of food allergy. Environ Health Perspect. 2003;111(2):239-244.

Hering, N. A., Fromm, M., & Schulzke, J. D. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. J Physiol. 2012;590(Pt 5):1035-1044.

Heyman, M. Gut barrier dysfunction in food allergy. Eur J Gastroenterol Hepatol. 2005;17(12):1279-1285.

Hou, J. K., El-Serag, H., & Thirumurthi, S. Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: a systematic review. Am J Gastroenterol. 2009;104(8):2100-2109.

Husby, S., Jensenius, J. C., & Svehag, S. E. Passage of undegraded dietary antigen into the blood of healthy adults. Quantification, estimation of size distribution, and relation of uptake to levels of specific antibodies. Scand J Immunol. 1985;22(1):83-92.

Jankowski, J. A., Goodlad, R. A., & Wright, N. A. Maintenance of normal intestinal mucosa: function, structure, and adaptation. Gut. 1994;35(1 Suppl):S1-S4.

Jiang, W., Wang, X., Zeng, B., Liu, L., Tardivel, A., Wei, H., . . . Zhou, R. Recognition of gut microbiota by NOD2 is essential for the homeostasis of intestinal intraepithelial lymphocytes. J Exp Med. 2013;210(11):2465-2476.

Jin, Y., Lin, Y., Lin, L., & Zheng, C. IL-17/IFN-gamma interactions regulate intestinal inflammation in TNBS-induced acute colitis. J Interferon Cytokine Res. 2012;32(11):548-556.

Johansson, M., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J., Ermund, A., . . . Hansson, G. Composition and functional role of the mucus layers in the intestine. Cellular and Molecular Life Sciences. 2011;68(22):3635-3641.

Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A. 2008;105(39):15064-15069.

Jurjus, A. R., Khoury, N. N., & Reimund, J. M. Animal models of inflammatory bowel disease. J Pharmacol Toxicol Methods. 2004;50(2):81-92.

Jyonouchi, H. Non-IgE Mediated Food Allergy - Update of Recent Progress in Mucosal Immunity. Inflamm Allergy Drug Targets. 2012;11(5):382-396.

Kalliomaki, M., Salminen, S., Poussa, T., Arvilommi, H., & Isolauri, E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet. 2003;361(9372):1869-1871.

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., . . . Hibi, T. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. J Clin Invest. 2008;118(6):2269-2280.

Kanazawa, H., Ishiguro, Y., Munakata, A., & Morita, T. Multiple accumulation of Vdelta2+ gammadelta T-cell clonotypes in intestinal mucosa from patients with Crohn's disease. Dig Dis Sci. 2001;46(2):410-416.

Kaplan, D. H. In vivo function of Langerhans cells and dermal dendritic cells. Trends Immunol. 2010;31(12):446-451.

Kolaczkowska, E., & Kubes, P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159-175.

Komano, H., Fujiura, Y., Kawaguchi, M., Matsumoto, S., Hashimoto, Y., Obana, S., . . . et al. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A. 1995;92(13):6147-6151.

Kronenberg, M., & Rudensky, A. Regulation of immunity by self-reactive T cells. Nature. 2005;435(7042):598-604.

Kuchroo, V. K., Das, M. P., Brown, J. A., Ranger, A. M., Zamvil, S. S., Sobel, R. A., . . . Glimcher, L. H. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell. 1995;80(5):707-718.

Laiping So, A., Pelton-Henrion, K., Small, G., Becker, K., Oei, E., Tyorkin, M., . . . Mayer, L. Antigen uptake and trafficking in human intestinal epithelial cells. Dig Dis Sci. 2000;45(7):1451-1461.

Land, M. H., & Burks, A. W. Future of immunotherapy for food allergy. Immunotherapy. 2012;4(1):13-15.

Lara-Villoslada, F., Olivares, M., Jimenez, J., Boza, J., & Xaus, J. Goat milk is less immunogenic than cow milk in a murine model of atopy. J Pediatr Gastroenterol Nutr. 2004;39(4):354-360.

Li, X. M., Schofield, B. H., Huang, C. K., Kleiner, G. I., & Sampson, H. A. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol. 1999;103(2 Pt 1):206-214.

Lin, J., & Hackam, D. J. Worms, flies and four-legged friends: the applicability of biological models to the understanding of intestinal inflammatory diseases. Dis Model Mech. 2011;4(4):447-456.

Liu, Z. Q., & Yang, P. C. Hapten may play an important role in food allergen-related intestinal immune inflammation. N Am J Med Sci. 2011;3(3):103-106.

Locke, N. R., Stankovic, S., Funda, D. P., & Harrison, L. C. TCR gamma delta intraepithelial lymphocytes are required for self-tolerance. J Immunol. 2006;176(11):6553-6559.

Macdonald, T. T., & Monteleone, G. Immunity, inflammation, and allergy in the gut. Science. 2005;307(5717):1920-1925.

Macpherson, A. J., & Uhr, T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004;303(5664):1662-1665.

Madara, J. L., & Stafford, J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J Clin Invest. 1989;83(2):724-727.

Male, D. K. (2006). *Immunology* (7th ed.). [Philadelphia, Pa.?]: Mosby Elsevier.

Manzano, M., Abadia-Molina, A. C., Garcia-Olivares, E., Gil, A., & Rueda, R. Absolute counts and distribution of lymphocyte subsets in small intestine of BALB/c mice change during weaning. J Nutr. 2002;132(9):2757-2762.

Martin, R., Nauta, A. J., Ben Amor, K., Knippels, L. M., Knol, J., & Garssen, J. Early life: gut microbiota and immune development in infancy. Benef Microbes. 2010;1(4):367-382.

Masuda, K., Nakamura, K., Yoshioka, S., Fukaya, R., Sakai, N., & Ayabe, T. Regulation of microbiota by antimicrobial peptides in the gut. Adv Otorhinolaryngol. 2011;72:97-99.

Matsuzaki, H., Maeda, M., Lee, S., Nishimura, Y., Kumagai-Takei, N., Hayashi, H., . . . Otsuki, T. Asbestos-induced cellular and molecular alteration of immunocompetent cells and their relationship with chronic inflammation and carcinogenesis. J Biomed Biotechnol. 2012;2012:492608.

Mavroudi, A., & Xinias, I. Dietary interventions for primary allergy prevention in infants. Hippokratia. 2011;15(3):216-222.

Melamed, D., & Friedman, A. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. Eur J Immunol. 1993;23(4):935-942.

Melgar, S., Karlsson, A., & Michaelsson, E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. Am J Physiol Gastrointest Liver Physiol. 2005;288(6):G1328-1338.

Mizoguchi, A. Animal models of inflammatory bowel disease. Prog Mol Biol Transl Sci. 2012;105:263-320.

Mizoguchi, A., & Mizoguchi, E. Animal models of IBD: linkage to human disease. Curr Opin Pharmacol. 2010;10(5):578-587.

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., . . . Kaplan, G. G. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;142(1):46-54 e42; quiz e30.

Montero Vega, M. T. New aspects on inflammation in allergic diseases. Allergol Immunopathol (Madr). 2006;34(4):156-170.

Moon, I. J., Kim, D. Y., Rhee, C. S., Lee, C. H., & Min, Y. G. Role of angiogenic factors in airway remodeling in an allergic rhinitis murine model. Allergy Asthma Immunol Res. 2012;4(1):37-45.

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42:165-190.

Mowat, A. M., & Bain, C. C. Mucosal macrophages in intestinal homeostasis and inflammation. J Innate Immun. 2011;3(6):550-564.

Mowat, C., Cole, A., Windsor, A., Ahmad, T., Arnott, I., Driscoll, R., . . . Gastroenterology, I. B. D. S. o. t. B. S. o. Guidelines for the management of inflammatory bowel disease in adults. Gut. 2011;60(5):571-607.

Mueller, C., & Macpherson, A. J. Layers of mutualism with commensal bacteria protect us from intestinal inflammation. Gut. 2006;55(2):276-284.

Muller, S., Buhler-Jungo, M., & Mueller, C. Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. J Immunol. 2000;164(4):1986-1994.

Mullin, J. M., Laughlin, K. V., Marano, C. W., Russo, L. M., & Soler, A. P. Modulation of tumor necrosis factor-induced increase in renal (LLC-PK1) transepithelial permeability. Am J Physiol. 1992;263(5 Pt 2):F915-924.

Nagler-Anderson, C. Tolerance and immunity in the intestinal immune system. Crit Rev Immunol. 2000;20(2):103-120.

Niess, J. H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B. A., . . . Reinecker, H. C. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science. 2005;307(5707):254-258.

Nowak-Wegrzyn, A., & Sampson, H. A. Future therapies for food allergies. J Allergy Clin Immunol. 2011;127(3):558-573; quiz 574-555.

Nurtanio, N., & Yang, P. C. Role of TIM-4 in innate or adaptive immune response. N Am J Med Sci. 2011;3(5):217-221.

Pabst, O., Herbrand, H., Friedrichsen, M., Velaga, S., Dorsch, M., Berhardt, G., . . . Forster, R. Adaptation of solitary intestinal lymphoid tissue in response to microbiota and chemokine receptor CCR7 signaling. J Immunol. 2006;177(10):6824-6832.

Pabst, O., & Mowat, A. M. Oral tolerance to food protein. Mucosal Immunol. 2012;5(3):232-239.

Paganelli, R., Scala, E., Di Gioacchino, M., Bellioni, B., & Stefanini, G. F. Prophylaxis and treatment of food allergy with disodium cromoglycate. Monogr Allergy. 1996;32:246-252.

Perrier, C., & Corthesy, B. Gut permeability and food allergies. Clin Exp Allergy. 2011;41(1):20-28.

Perrier, C., Thierry, A. C., Mercenier, A., & Corthesy, B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. Clin Exp Allergy. 2010;40(1):153-162.

Perse, M., & Cerar, A. Dextran sodium sulphate colitis mouse model: traps and tricks. J Biomed Biotechnol. 2012;2012:718617.

Peterson, L. W., & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol. 2014;14(3):141-153.

Peterson, R. A. Regulatory T-cells: diverse phenotypes integral to immune homeostasis and suppression. Toxicol Pathol. 2012;40(2):186-204.

Phalipon, A., & Corthesy, B. Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins. Trends Immunol. 2003;24(2):55-58.

Platt, A. M., & Mowat, A. M. Mucosal macrophages and the regulation of immune responses in the intestine. Immunol Lett. 2008;119(1-2):22-31.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., . . . Wang, J. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.

Rafi, A., Do, L. T., Katz, R., Sheinkopf, L. E., Simons, C. W., & Klaustermeyer, W. Effects of omalizumab in patients with food allergy. Allergy Asthma Proc. 2010;31(1):76-83.

Rance, F., Boguniewicz, M., & Lau, S. New visions for atopic eczema: an iPAC summary and future trends. Pediatr Allergy Immunol. 2008;19 Suppl 19:17-25.

Rao, P. E., Petrone, A. L., & Ponath, P. D. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-{beta}. J Immunol. 2005;174(3):1446-1455.

Reis, B. S., & Mucida, D. The role of the intestinal context in the generation of tolerance and inflammation. Clin Dev Immunol. 2012;2012:157948.

Renegar, K. B., Small, P. A., Jr., Boykins, L. G., & Wright, P. F. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. J Immunol. 2004;173(3):1978-1986.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., . . . Ricciardi-Castagnoli, P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001;2(4):361-367.

Rieder, F., & Fiocchi, C. Intestinal fibrosis in IBD--a dynamic, multifactorial process. Nat Rev Gastroenterol Hepatol. 2009;6(4):228-235.

Rijnierse, A., Koster, A. S., Nijkamp, F. P., & Kraneveld, A. D. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. J Immunol. 2006;176(7):4375-4384.

Rivollier, A., He, J., Kole, A., Valatas, V., & Kelsall, B. L. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. J Exp Med. 2012;209(1):139-155.

Rona, R. J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., . . . Madsen, C. The prevalence of food allergy: a meta-analysis. J Allergy Clin Immunol. 2007;120(3):638-646.

Round, J. L., & Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9(5):313-323.

Saleh, M., & Elson, C. O. Experimental inflammatory bowel disease: insights into the host-microbiota dialog. Immunity. 2011;34(3):293-302.

Salzman, N. H., Hung, K., Haribhai, D., Chu, H., Karlsson-Sjoberg, J., Amir, E., . . . Bos, N. A. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010;11(1):76-82.

Sandborn, W. J. The future of inflammatory bowel disease therapy: where do we go from here? Dig Dis. 2012;30 Suppl 3:140-144.

Scurlock, A. M., Burks, A. W., & Jones, S. M. Oral immunotherapy for food allergy. Curr Allergy Asthma Rep. 2009;9(3):186-193.

Schmetterer, K. G., Neunkirchner, A., & Pickl, W. F. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. FASEB J. 2012;26(6):2253-2276.

Schroder, N. W., & Maurer, M. The role of innate immunity in asthma: leads and lessons from mouse models. Allergy. 2007;62(6):579-590.

Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W., & Pabst, O. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med. 2009;206(13):3101-3114.

Schwartz, R. H. T cell anergy. Annu Rev Immunol. 2003;21:305-334.

Sheridan, B. S., & Lefrancois, L. Intraepithelial lymphocytes: to serve and protect. Curr Gastroenterol Rep. 2010;12(6):513-521.

Sherwood, E. R., & Toliver-Kinsky, T. Mechanisms of the inflammatory response. Best Pract Res Clin Anaesthesiol. 2004;18(3):385-405.

Shibolet, O., & Podolsky, D. K. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol. 2007;292(6):G1469-1473.

Shin, Y. S., Takeda, K., & Gelfand, E. W. Understanding asthma using animal models. Allergy Asthma Immunol Res. 2009;1(1):10-18.

Shires, J., Theodoridis, E., & Hayday, A. C. Biological insights into TCRgammadelta+ and TCRalphabeta+ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). Immunity. 2001;15(3):419-434.

Sicherer, S. H. Epidemiology of food allergy. J Allergy Clin Immunol. 2011;127(3):594-602.

Slack, E., Hapfelmeier, S., Stecher, B., Velykoredko, Y., Stoel, M., Lawson, M. A., . . . Macpherson, A. J. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science. 2009;325(5940):617-620.

Stange, E. F., Travis, S. P., Vermeire, S., Reinisch, W., Geboes, K., Barakauskiene, A., . . . Colitis, O. European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis. J Crohns Colitis. 2008;2(1):1-23.

Stefanini, G. F., Saggioro, A., Alvisi, V., Angelini, G., Capurso, L., di Lorenzo, G., . . . et al. Oral cromolyn sodium in comparison with elimination diet in the irritable bowel syndrome, diarrheic type. Multicenter study of 428 patients. Scand J Gastroenterol. 1995;30(6):535-541.

Stone, K. D., Prussin, C., & Metcalfe, D. D. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S73-80.

Takeda, K., & Gelfand, E. W. Mouse models of allergic diseases. Curr Opin Immunol. 2009;21(6):660-665.

Tsuji, N. M., Mizumachi, K., & Kurisaki, J. Interleukin-10-secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance. Immunology. 2001;103(4):458-464.

Tulic, M. K., Hodder, M., Forsberg, A., McCarthy, S., Richman, T., D'Vaz, N., . . . Prescott, S. L. Differences in innate immune function between allergic and nonallergic children: new insights into immune ontogeny. J Allergy Clin Immunol. 2011;127(2):470-478 e471.

Van Assche, G., Dignass, A., Reinisch, W., van der Woude, C. J., Sturm, A., De Vos, M., . . . Colitis, O. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Special situations. J Crohns Colitis. 2010;4(1):63-101.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., . . . Jung, S. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity. 2009;31(3):502-512.

Veenbergen, S., & Samsom, J. N. Maintenance of small intestinal and colonic tolerance by IL-10-producing regulatory T cell subsets. Curr Opin Immunol. 2012;24(3):269-276.

Virkud, Y. V., & Vickery, B. P. Advances in immunotherapy for food allergy. Discov Med. 2012;14(76):159-165.

von Boehmer, H. Mechanisms of suppression by suppressor T cells. Nat Immunol. 2005;6(4):338-344.

Wang, J., Patil, S. P., Yang, N., Ko, J., Lee, J., Noone, S., . . . Li, X. M. Safety, tolerability, and immunologic effects of a food allergy herbal formula in food allergic individuals: a randomized, double-blinded, placebo-controlled, dose escalation, phase 1 study. Ann Allergy Asthma Immunol. 2010;105(1):75-84.

Weidinger, S., Willis-Owen, S. A., Kamatani, Y., Baurecht, H., Morar, N., Liang, L., . . . Moffatt, M. F. A genome-wide association study of atopic dermatitis identifies loci with overlapping effects on asthma and psoriasis. Hum Mol Genet. 2013;22(23):4841-4856.

Whitacre, C. C., Gienapp, I. E., Orosz, C. G., & Bitar, D. M. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J Immunol. 1991;147(7):2155-2163.

Wijburg, O. L., Uren, T. K., Simpfendorfer, K., Johansen, F. E., Brandtzaeg, P., & Strugnell, R. A. Innate secretory antibodies protect against natural Salmonella typhimurium infection. J Exp Med. 2006;203(1):21-26.

Wyatt, J., Vogelsang, H., Hubl, W., Waldhoer, T., & Lochs, H. Intestinal permeability and the prediction of relapse in Crohn's disease. Lancet. 1993;341(8858):1437-1439.

Xiao, W. B., & Liu, Y. L. Changes of CD8+CD28- T regulatory cells in rat model of colitis induced by 2,4-dinitrofluorobenzene. World J Gastroenterol. 2003;9(11):2528-2532.

Yamashita, M., & Nakayama, T. Progress in allergy signal research on mast cells: regulation of allergic airway inflammation through toll-like receptor 4-mediated modification of mast cell function. J Pharmacol Sci. 2008;106(3):332-335.

Yan, F., & Polk, D. B. Characterization of a probiotic-derived soluble protein which reveals a mechanism of preventive and treatment effects of probiotics on intestinal inflammatory diseases. Gut Microbes. 2012;3(1).

Zenewicz, L. A., Antov, A., & Flavell, R. A. CD4 T-cell differentiation and inflammatory bowel disease. Trends Mol Med. 2009;15(5):199-207.

Zhu, M. Y., Lu, Y. M., Ou, Y. X., Zhang, H. Z., & Chen, W. X. Dynamic progress of 2,4,6-trinitrobenzene sulfonic acid induced chronic colitis and fibrosis in rat model. J Dig Dis. 2012;13(8):421-429.

Chapter 2

Objectives

CHAPTER 2: OBJECTIVES

Cow's milk protein allergy (CMPA) affects 2-6% of children under 2 years. The importance of this condition is not due to its high incidence or to the unleashed symptoms, in exceptional cases it may even lead to death from anaphylactic shock, but because it is often the beginning of the "atopic career". This has been associated with an increased incidence of other diseases in older stages such as asthma, possibly due to changes in the immune system and increased intestinal permeability which occur during the development of the CMPA. However, it is unknown whether these alterations may pose a higher risk of developing others immunological diseases in adulthood such as inflammatory bowel disease, diabetes, etc.

The main objective of this thesis is to study the possible association between food allergy to cow's milk protein and inflammatory bowel disease. The use of animal models is considered a good alternative for testing the complex relationships and mechanisms of action of various human pathologies. But the first thing we must do to try to answer these questions is to optimize animal models of various diseases and especially to optimize various parameters which will interest us to analyse the overall aim of the project.

To perform the main objective, we have contemplated the following specific objectives:

OBJECTIVE I: Selection of the experimental models to study:

A) Development of a CMPA mouse model based on oral sensitization, shorter and more specific than the currently models.

B) Optimization and analysis of DNFB/DNS mouse model as an intestinal inflammation model.

OBJECTIVE II: Analysis of the possible influence of intestinal inflammation on allergic individuals. Thus, the effect of the DNFB/DNS model on the incidence and severity of OVA allergy will be analysed.

OBJECTIVE III: Evaluation of the modulatory effect of CMPA on others intestinal pathologies with an immune component. Therefore, the effect of CMPA on the incidence and severity of diseases such as inflammatory bowel disease will be discussed using various models of intestinal inflammation.

An overview of the main findings will allow future potential both nutritional and pharmacological approaches to reduce the risk of suffering these alterations in case of having suffered APLV or intestinal inflammation.

Chapter 3

DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease (IF:5.1; 11/74)

ORIGINAL ARTICLE

DNFB-DNS Hapten-induced Colitis in Mice Should Not Be Considered a Model of Inflammatory Bowel Disease

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Background: The dinitrofluorobenzene/dinitrosulfonic acid (DNFB/DNS) model was originally described as an experimental model of intestinal inflammation resembling human ulcerative colitis (UC). Due to the absence of acceptable UC experimental models for pharmacological preclinical assays, here we examine the immune response induced in this model.

Methods: Balb/c mice were sensitized by skin application of DNFB on day 1, followed by an intrarectal challenge with DNS on day 5. We further expanded this model by administering a second DNS challenge on day 15. The features of colonic inflammation and immune response were evaluated.

Results: The changes observed in colonic tissue corresponded, in comparison to the trinitrobenzene sulfonic acid (TNBS) colitis model, to a mild mucosal effect in the colon, which spontaneously resolved in less than 5 days. Furthermore, the second hapten challenge did not exacerbate the inflammatory response. In contrast to other studies, we did not observe any clear involvement of tumor necrosis factor alpha (TNF-α) or other Th1 cytokines during the initial inflammatory response; however, we found that a more Th2-humoral response appeared to mediate the first contact with the hapten. An increased humoral response was detected during the second challenge, although an increased Th1/Th17-cytokine expression profile was also simultaneously observed.

Conclusions: On the basis of these results, although the DNFB/DNS model can display some features found in human UC, it should be considered as a model for the study of the intestinal hypersensitivity seen, for example, during food allergy or irritable bowel syndrome but not intestinal inflammation per se.

(Inflamm Bowel Dis 2011;17:2087–2101)

Key Words: intestinal inflammation, irritable bowel syndrome, contact hypersensitivity, food allergy, TNBS colitis model

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic human inflammatory bowel diseases (IBDs) characterized by severe abdominal pain, diarrhea, weight loss, and rectal bleeding.[1] The exact etiology and pathophysiology of IBD are unknown, but an inadequate or prolonged activation of the intestinal immune system appears to play a crucial role in their development. In fact, the increased presence of inflammatory cells and inflammatory mediators is a feature of these intestinal conditions.[2]

In this regard, the CD4+ T cells that react to enteric microflora are considered effector cells in IBD.[3] Several immunomodulatory agents currently used for the management of human IBD, such as azathioprine, methotrexate, cyclosporin A, or tacrolimus, act by inhibiting T-cell activation.[4] Biological therapies, including the anti-tumor necrosis factor alpha (TNF-α) antibodies infliximab or adalimumab, have been successfully developed for the treatment of IBD patients.[5,6] In addition, anti-interleukin (IL)-12, anti-interferon gamma (IFN-γ), anti-IL-6 receptor, and toxin-conjugated anti-IL-7 receptor are also currently being studied for this purpose (reviewed[6]). However, differences in the T-cell response between these two related IBD pathologies have been reported. Thus, human CD promotes mainly a Th1 cytokine response associated with excessive IFN-γ/TNF-α/IL-12 secretion, whereas UC is mediated mainly by an atypical Th1/Th2 inflammation pattern, in which an increase in Th2 cytokines (without affecting IL-4 levels) is associated with the production of several Th1 proinflammatory markers (TNF-α, IL-1β).[7]

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Although recent studies have described a more complex scenario for IBD that involves other T-cell populations and inflammatory mediators (Th17, Th3, etc.),[7,8] the most significant advances in the understanding of the cause and mechanism of IBD have been achieved from the study of animal models. These allow not only experiments that are not feasible in humans but also the study of the progression of inflammation over time. Several models have been developed for this purpose in rodents.[9,10] However, most experimental murine models are for Th1-biased T-cell colitis, such as trinitrobenzene sulfonic acid (TNBS)-induced colitis model,[11,12] and there are only two well-known models for Th2 T-cell colitis: oxazolone colitis and spontaneous colitis produced in TCR-α-chain knockout mice.[13,14] Consequently, the discovery and development of new animal models for the study of Th2-biased colitis is a current unmet need for this pathology.

Rijinierse et al[15,16] developed and partly characterized a new chemically induced immunological murine model of colitis. In this model, the inflammatory reaction in the colon is evoked by skin sensitization of mice with the low-molecular-weight compound 2,4-dinitrofluorobenzene (DNFB), followed by a local intrarectal challenge with the hapten dinitrosulfonic acid (DNS). The main features of this model are diarrhea, hypertrophy of lymphoid structures, recruitment of inflammatory cells, as well as infiltration and activation of mast cells in the colon,[15] thus resembling a potential Th2-biased colitis model. Although an increased number of mast cells have been found in the mucosa of inflamed and noninflamed areas in IBD patients,[17,18] the contribution of these cells to IBD is controversial. Moreover, the critical role of TNF-α in this model[15] suggests to us the potential involvement of other cell types and Th1 cytokines.

Here we evaluated the feasibility of this DNFB/DNS-induced colitis model as a new potential experimental tool for the study of UC and examined the immune response after the first and subsequent contacts with the hapten in sensitized animals.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical (Madrid, Spain) unless otherwise stated.

Animals

Animals were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) and housed on a temperature- (22°C) and light-controlled (12 hours) cycle. This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC), in compliance with the Helsinki Declaration.

Induction of Colitis and Sample Collection

After a 7-day acclimation period, male Balb/c mice (25 g) were weighed and randomly distributed in experimental groups ($n = 8$ per group). In some sets of experiments mice were rendered colitic by the method originally described.[15] On day 0, mice were sensitized by an application of either DNFB (0.6% in acetone-olive oil, 4:1) or vehicle (acetone-olive oil, 4:1) epicutaneously on the shaved abdomen (50 μL) and on paws (50 μL divided over four paws). On day 1 mice received a boost of DNFB or vehicle on the abdomen only (50 μL). All animals were challenged intrarectally with 0.6% DNS dissolved in 10% ethanol on day 5. Briefly, the challenge was conducted under light inhalation anesthesia (3% halothane). During the experiment, animal body weight and occurrence of diarrhea were recorded daily. Mice were killed on days 1, 2, 3, 5, and 7 after the DNS challenge.

Control noncolitic animals in the study corresponded to non-DNFB-sensitized but intrarectal DNS-challenged animals. Other controls, such as healthy (nontreated) animals, intrarectal 10% ethanol instilled control animals or animals sensitized only with DNFB but not challenged with DNS, were not used in this study in order to reduce the number of experimental animals required and because of previous nonpublished results of our group that demonstrated that the effects observed in these animals do not differ from those found in the noncolitic group used as controls in this study (data not shown).

In a second group of experiments, and to evaluate the effect of a second DNS instillation, six groups of animals ($n = 8$) were sensitized (except the healthy noncolitic control group) on day 0 and 1 and then again challenged with DNS on day 5, as previously described. Four groups of mice were killed on days 6, 8, or 15 (control mice and 24 hours, 72 hours and 10 days after the first challenge). The remaining two groups were challenged again with a second instillation of DNS at day 15 and then killed 24 or 72 hours later.

Finally, in a third group of experiments colitis was induced in animals by intrarectal TNBS instillation (0.1 mg TNBS per gram of bodyweight in 50 μL of 50% ethanol) as previously described.[19] Animals were sacrificed at 5, 7, and 9 days after TNBS administration. In these studies, control animals correspond to animals where a 50% ethanol solution was administered intrarectally.

All mice were killed by means of an overdose of sodium pentobarbital. Before death, blood was collected by cardiac puncture and the plasma fraction was obtained by centrifugation and stored at −80°C until analysis.

Once the mice were killed the spleen and the colon were removed. Spleen was reserved for splenocyte cell cultures, as previously described,[20] while colon samples were placed on an ice-cold plate, cleaned of fat and mesentery, and then blotted on filter paper.[21] Each colon specimen was weighed and the length was measured under a constant load

(2 g). Afterwards, the colon was opened longitudinally and scored for macroscopically visible damage and the number of colonic patches, which appear as bulges in the tissue, were counted with the naked eye.

Representative intestinal sections were taken from a region of the inflamed colon corresponding to the segment of colonic patches or adjacent to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for histological analysis. Equivalent colonic segments were also obtained from the noncolitic group. The colon samples were subsequently sectioned in various longitudinal fragments for later use in biochemical determinations and RNA isolation.[22]

Immunohistological Analysis

The immunolabeling was performed on formalin-fixed samples embedded in paraffin using the streptavidin-biotin peroxidase complex method and a high temperature pretreatment as antigen unmasking protocols. After pretreatment, sections were incubated for 15 minutes with 1% hydrogen peroxidase to block endogenous peroxidase activity and then preincubated with protein blocking sera for 30 minutes to reduce nonspecific reactions. The indirect immunofluorescence methods were performed following established procedures. The primary mouse monoclonal antibodies used were anti-human T cell CD3 Peptide (Sigma), anti-CD20 (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and antimouse CD68 (clone FA-11) (AbD Serotec, Dusseldorf, Germany). Primary antibodies were diluted 1:40 in TBS (Tris-buffered saline) and incubated in a humidity chamber overnight at 4°C. Samples were later incubated with appropriate FITC-conjugated secondary antibodies (Dako, Glostrup, Denmark) diluted 1:30 at 37°C. Sections were then mounted with Aquatex (Merck, Whitehouse Station, NJ).

Biochemical and Immunological Parameters

Plasma and colon homogenates were centrifuged and the supernatant was frozen until cytokine and histamine measurement by enzyme-linked immunosorbent assay (ELISA). Cytokine ELISAs were purchased from R&D Systems (Minneapolis, MN) (TNF-α, IL-1β, IL-6, IL-10, IL-5, IL-17) and Invitrogen (La Jolla, CA) (IFN-γ, IL-4, and IL-12). All the analyses were performed following the manufacturer's instructions. Plasma and colonic immunoglobulin (Ig) determinations were performed using ELISAs from Bethyl Laboratories (Montgomery, TX). The dilutions performed were 1:10,000 for the determination of total IgG, IgG1, IgG2a, IgA, IgM in plasma and 1:2 for IgE. In the colon determinations, dilutions used were 1:1000 (IgG1), 1:500 (IgG total, IgG2a), 1:5000 (IgA) and no dilution for IgE. The histamine determination kit was obtained from Spi Bio (Montigny le Bretonneux, France) and measurements made following the manufacturer's protocol.

Myeloperoxidase (MPO) activity was measured following the technique described[23]; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25°C.

Inducible nitric oxide synthase (iNOS) expression was analyzed by western blot in colon samples as previously described.[24]

Reverse-transcription Polymerase Chain Reaction (RT-PCR) Analysis of Cytokines and mouse Mast Cell Protease-1 (mMCP-1) Expression in Colonic and Spleen Samples

Total RNA from colonic samples was isolated using TRIzol Reagent (Gibco-BRL, Gaithersburg, MD) following the manufacturer's protocol. cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham, Biosciences, Arlington Heights, IL). The primer sequences and PCR conditions are described in Table 1 and RT-PCR was performed as previously described.[22]

Statistics

All results are expressed as the mean ± standard error of the mean (SEM). Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post-hoc least significance tests. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Rockville, MD), with statistical significance set at $P < 0.05$.

RESULTS

DNS Instillation in DNFB-sensitized Mice Induces Mild and Transitory Intestinal Inflammation

The first objective of this study was to characterize the course of the inflammatory process induced in mice by the sensitization/challenge with DNFB/DNS. These mice did not show any significant alteration in body weight evolution or food consumption during the 7-day experiment compared with untreated mice (not shown). However, an intestinal effect was suspected on the basis of the presence of mild diarrhea, which was detected in DNS-treated animals just after 24 hours. This effect gradually increased during the first 72 hours after DNS challenge but disappeared at day 5. Moreover, delayed-type hypersensitivity (DTH) wounds appeared on the abdominal sensitization point. These wounds were visible at day 4 and reached their maximum size at day 7.

The intestinal effects of the hapten-challenged groups were evidenced macroscopically by a significant increase in the colonic weight/length ratio in comparison with noncolitic mice during the first 72 hours after colitis induction, although statistical differences were obtained only at 24–48 hours (Fig. 1A). Moreover, DNS-challenged mice recovered values similar to those observed in healthy animals after 5 and 7 days of hapten administration (not shown). The

TABLE 1. PCR Conditions and Primer Sequences

Gene	Primer	Sequence	Annealing Temperature	Cycle Number
β-actin	Forward	AATCGTGCGTGACATCAAAG	55°C	20
	Reverse	ATGCCACAGGATTCCATACC		
IFNγ	Forward	TGGAGGAACTGGCAAAAGGATGGT	60°C	35
	Reverse	TTGGGACAATCTCTTCCCCAC		
TNFα	Forward	AACTAGTGGTGCCAGCCGAT	56°C	30
	Reverse	CTTCACAGAGCAATGACTCC		
IL-2	Forward	TGATGGACCTACAGGAGCTCCTGA	60°C	35
	Reverse	GAGTCAAATCCACAACATGCCGCA		
IL-4	Forward	ACGAGGTCACAGGAGAAGGGAC	60°C	35
	Reverse	GGAGCAGCTTATCGATGAATCC		
IL-5	Forward	GCACAGTGGTGAAAGAGACC	60°C	35
	Reverse	TAATCCAGGAACTGCCTCGT		
IL-6	Forward	GTGACAACCACGGCCTTCCCTACT	56°C	35
	Reverse	GGTAGCTATGGTACTCCA		
IL-10	Forward	TCCTTAATGCAGGACTTTAAGGG	55°C	32
	Reverse	GGTCTTGGAGCTTATTAAAAT		
IL-12	Forward	CTGGTGCAAAGAAACATGGA	55°C	35
	Reverse	TGGTTTGATGATGTCCCTGA		
IL-13	Forward	GCCAGCCCACAGTTCTACAGC	62°C	35
	Reverse	GTGATGTTGCTCAGCTCCTCA		
IL-17	Forward	CCTGGGTGAGCCGACAGAAGC	62°C	26
	Reverse	CCACTCCTGGAACCTAAGCAC		

transient course of the intestinal inflammation induced by the DNFB/DNS protocol was corroborated in all measures performed in this study. Thus, in order to facilitate understanding, no data corresponding to the 5 and 7 day timepoints were included in the study.

Intestinal inflammation can also be measured as alterations in colonic patch morphology. Colonic patches are small lymphoid follicles, consisting predominantly of B-cell zones[16] that appear on the mucosal site of the colon. An increase in the number of visible colonic patches is indicative of hypertrophy of these structures.[16,25] In this regard, macroscopic examination of the colonic specimens after DNS challenge in DNFB-sensitized mice showed an increased number of colonic patches at all timepoints analyzed during the first 72 hours in comparison with noncolitic mice (Fig. 1B).

No other significant differences in the size or morphology of any of the internal organs analyzed were observed, although the size of the spleen tended to increase 24 and 48 hours after colitis induction (not shown).

Colonic inflammatory infiltrate was indirectly measured as colonic MPO activity, which increased by ≈20%–30% in the DNFB/DNS group, although only at 48 hours after DNS colitis induction reached significance (Fig. 1C). Accordingly, the histological examination of the colonic sections showed some inflammatory changes, including hyperemia, edema of the submucosa associated with a slight ulceration, loss of normal crypt architecture with necrosis, crypt abscesses, as well as mixed cell infiltration, largely composed by granulocytes and mononuclear cells (mainly lymphocytes and plasmatic cells without showing an increase of macrophage population) (Fig. 1D).

However, despite the evident inflammatory state, in comparison with other experimental IBD mouse models, such as the TNBS colitis (Fig. 2), the intestinal lesions observed in the DNFB/DNS model were only mild and spontaneously reverted very fast. In contrast, TNBS administration was fatal for 30%–40% of the animals (5–7 days after TNBS administration) and reduced almost 20% the body weight of the survivors at day 5 (data not shown), starting their recuperation after this point. Accordingly, intestinal inflammation measured at colon weight/length also peaked at day 5 and returned to basal levels 9 days after colitis induction (Fig. 2A). Moreover, macroscopically visible ulceration and necrotic lesions in the mice colon were clearly visible, similar to what has been described in TNBS-induced colitis model in rats.[24] No necrotic or macroscopically visible intestinal ulceration areas were seen in

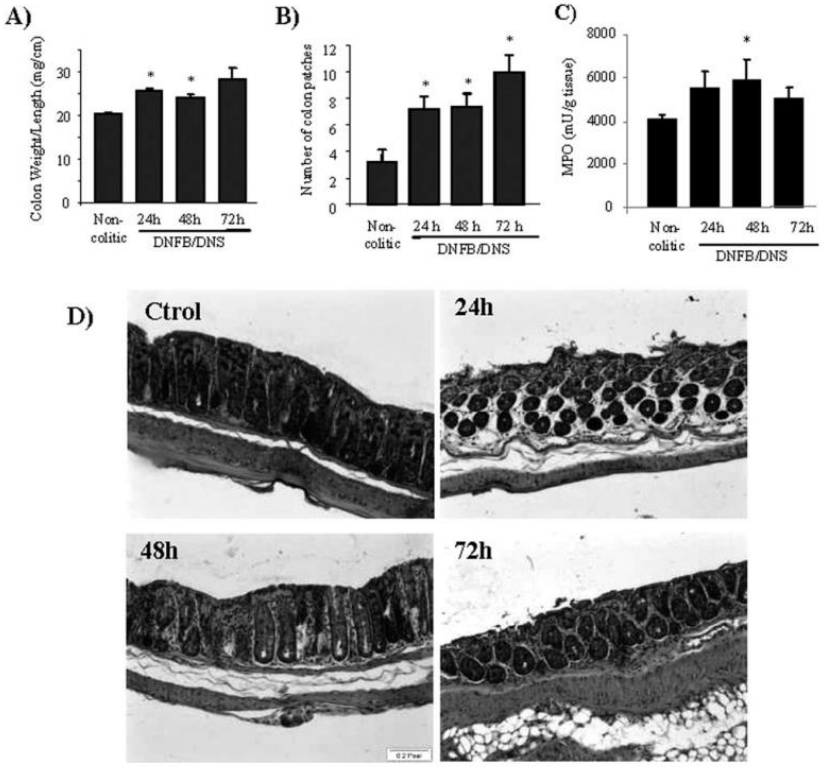

FIGURE 1. DNFB/DNS protocol induces a mild colonic inflammatory process. (A) After killing the animals, we measured and weighed each colon specimen. Colon weight/length ratios at a range of timepoints after DNS challenge are presented as the mean ± SEM and compared to those of noncolitic animals. (B) For each colon sample and previous to its sectioning, colon patches were counted. (C) MPO activity in colon homogenates 24, 48, and 72 hours after DNS challenge. The results are shown as means ± SEM. *$P < 0.05$ vs. control noncolitic group. (D) Histological sections of colonic mucosa stained with hematoxylin and eosin were obtained from control noncolitic animals (Ctrol) and compared with the inflammatory process induced 24 (24h), 48 (48h), and 72 (72h) hours after DNS challenge. Images shown are representative of the alterations observed in each treatment group. (original magnification ×100).

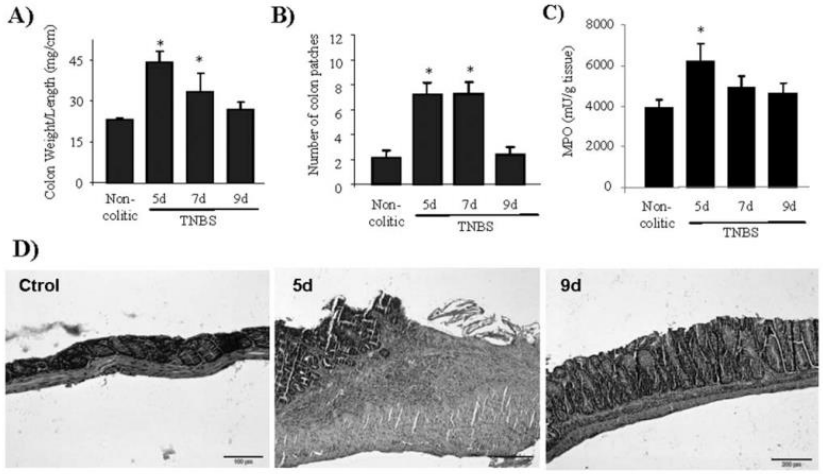

FIGURE 2. TNBS protocol induces a more severe colonic inflammation. (A) Colon weight/length ratios at 5, 7, and 9 days after TNBS administration were measured and compared to those of noncolitic animals (intrarectally treated with 50% ethanol). (B) For each colon sample and previous to its sectioning, colon patches were counted. (C) MPO activity in colon homogenates after 5, 7, and 9 days after TNBS challenge. All results are shown as means ± SEM. *$P < 0.05$ vs. control noncolitic group. (D) Histological sections of colonic mucosa stained with hematoxylin and eosin were obtained from control noncolitic animals (Ctrol) and colitic animals at different timepoints. Images shown are representative of the alterations observed in each treatment group. (original magnification ×100).

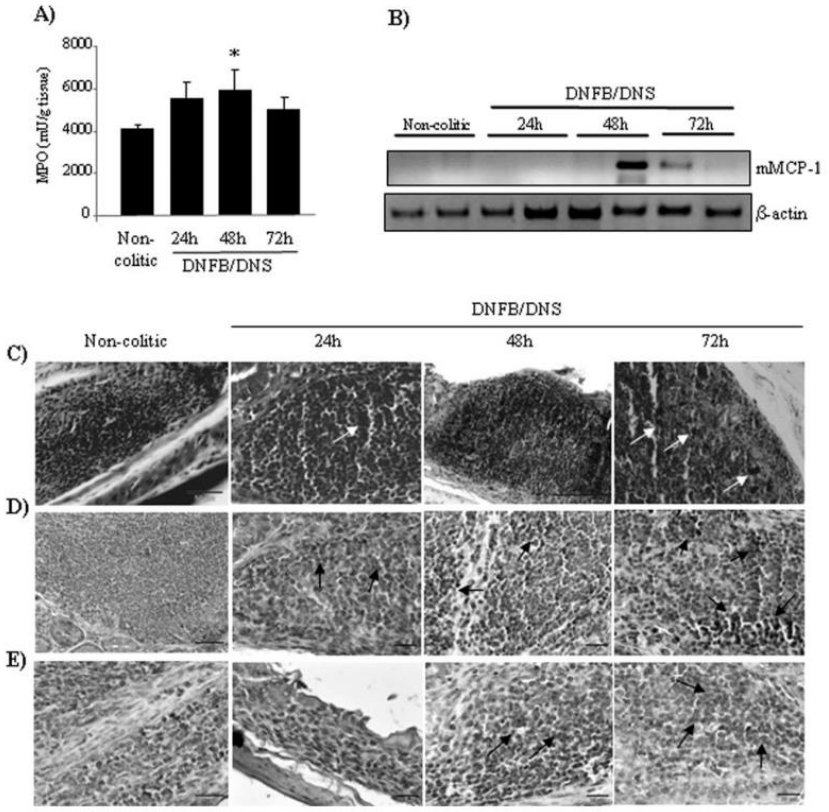

FIGURE 3. Lymphocytes are the main immune cell type involved in the colonic inflammatory infiltrate. (A) Analysis of mMCP-1 expression in colonic samples by RT-PCR. β-Actin expression was used as control. The gel shown is representative of all colonic samples per group ($n = 8$). (B) Colonic segments were incubated with Toluidine blue, which stains mast cells. (C,D) Immunofluorescence of T-lymphocytes (C) and B-lymphocytes (D) was performed in histological sections of colonic mucosa from colitic mice with antihuman T cell CD3 peptide and anti-CD20 (M-20), respectively. Scale bars = 20 μm in non-colitic panels and 50 μm in DNFB/DNS panels.

the DNFB/DNS model, but an increased reddish coloration was appreciable (data not shown). Besides, an increased number of colon patches was also observed in the TNBS mice model (Fig. 2B), although in this case the number and the size of the patches were lower than that observed in the DNFB/DNS model.

Histological assessment of colonic samples from the TNBS group revealed severe transmural disruption of the normal architecture of the colon, extensive ulceration, and inflammation involving all the intestinal layers of the colon (Fig. 2D). Most of the animals showed epithelial ulceration of the mucosa affecting over 50% of the surface and the inflammatory process was associated with crypt hyperplasia and dilation. Finally, colonic samples were also characterized by severe edema, interstitial microhemorrhages, and diffuse and granulomatous leukocyte infiltration, mainly composed of neutrophils and macrophages in the mucosa layer, which correlates with the increased MPO expression observed in the colon (Fig. 2C).

Colonic Inflammation Induced by DNFB/DNS Colitis Is Driven Mainly by Lymphocytes and Mast Cells

To further characterize the cellular nature of the inflammatory infiltrate observed in the colon, immune-staining of the colonic samples was performed. Mucosal mast cell involvement in the DNS-induced colitis was analyzed by RT-PCR detection of mMCP-1 (Fig. 3A) and by Toluidine blue staining of the colonic samples (Fig. 3B). An increase in mast cell number, especially 48–72 hours after DNS challenge, was observed throughout the colon. The presence of these cells was more evident in the distal colon, where clusters of mast cells were located around colonic patches, and also in crypt abscesses and on the

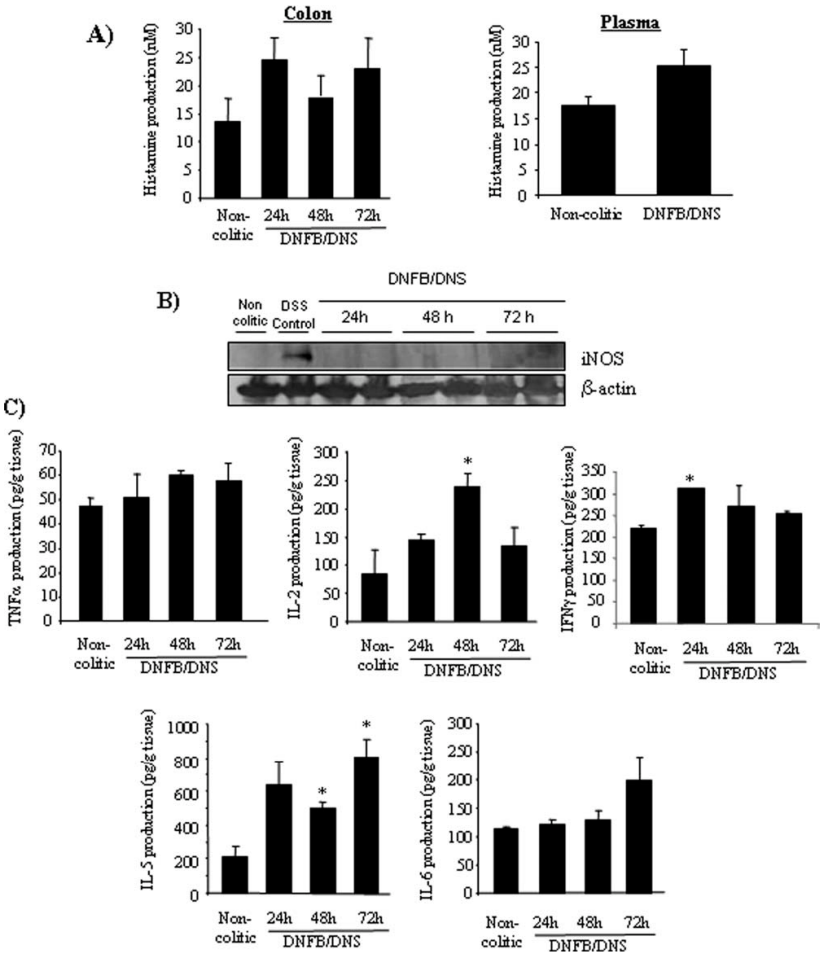

FIGURE 4. IL-5 appears to be the main cytokine involved in colonic inflammatory response. (A) Histamine levels in colonic and plasma samples 24, 48, and 72 hours after DNS challenge. The results of plasma samples are shown only at 48 hours and are presented as the mean ± SEM and compared to noncolitic animals. (B) iNOS expression is evaluated for Western blot. β-Actin expression was used as a control of loading and transfer, and the gel shown is representative of all colonic samples per group ($n = 8$). Moreover, a sample from day 5 of dextran sodium sulphate (DSS) administration was also included for comparison (positive control). (C) TNF-α, IL-2, IFN-γ, IL-5, and IL-6 expression in colitic DNFB/DNS groups. The concentrations of cytokines in colonic homogenates were analyzed by ELISA following the manufacturer's protocol. All data are expressed as mean ± SEM. *$P < 0.05$ vs. control noncolitic group.

surface of ulcerated epithelium (Fig. 3B). Similarly, an increased expression of mMCP-1 in mast cell granules peaked at 48 hours after colitis induction, although the magnitude of this gene expression was highly variable between colonic samples (Fig. 3A).

In healthy intestinal mucosa from noncolitic mice, minimal immunofluorescence for T (anti-CD3) (Fig. 3C) and B lymphocytes (anti-CD20) (Fig. 3D) was observed. However, immunofluorescence staining of colonic samples from the DNFB/DNS groups revealed lymphocytes as the predominant cells in the inflammatory infiltrates of the intestine. These cells were located mainly in the lamina propria and the submucosa-adjoining regions of colonic patches, as also shown by hematoxylin and eosin staining of the samples.

Despite the clear inflammatory infiltrate found in the colon, no statistically significant differences were observed in the expression of common inflammatory markers described to participate in other models of IBD. Histamine levels were only slightly increased in colon samples and in

plasma (Fig. 4A,B), thus suggesting that the activation and degranulation of mast cells was only moderate. Similarly, no statistical differences were observed in other proinflammatory mediators related to macrophage activation, namely, iNOS (Fig. 4B) and cyclooxygenase-2 (COX-2) (not shown). These observations confirmed findings from the histological analysis, which revealed a negligible number of infiltrated macrophages in the colonic samples of the DNFB/DNS animals. In this regard, although colonic TNF-α production tended to increase in colitic mice, these animals did not show a significant difference at any timepoint tested (Fig. 4C). To analyze the possible bias of the lymphocyte response, we also evaluated the colonic expression and production of several lymphokines implicated in intestinal inflammation (Fig. 4C). Surprisingly, the expression of cytokines induced in colonic samples was lower than that theoretically expected on the basis of the considerable lymphocyte infiltration observed in the colonic samples in the histological studies. The main difference observed between colitic and healthy animals was a transient increased expression of IFN-γ and IL-2. However, only the expression of the Th2 cytokine IL-5 was constantly overexpressed during the course of the inflammatory process (Fig. 4C). No differences were observed in the plasma levels in any of the cytokines tested, thus demonstrating that the inflammatory effect observed was restricted mainly to the gut.

We also analyzed B cell activation and the subsequent Ig secretion in colonic samples and plasma. In contrast to our observations with cellular inflammatory cytokines, the secretion of Ig was clearly affected by the DNS challenge. In this regard, the colitis induced by DNS in DNFB-sensitized mice increased IgE, IgA, and IgG1 expression significantly in the colon (Fig. 5), while it did not modify or even slightly reduced IgG2a expression. Similarly, in this case the same responses observed at the inflammatory focus (except in the case of IgA) were translated to the blood compartment, where the expression of atopic Ig, IgE and IgG1, increased while a reduction of IgG2a and IgA was seen.

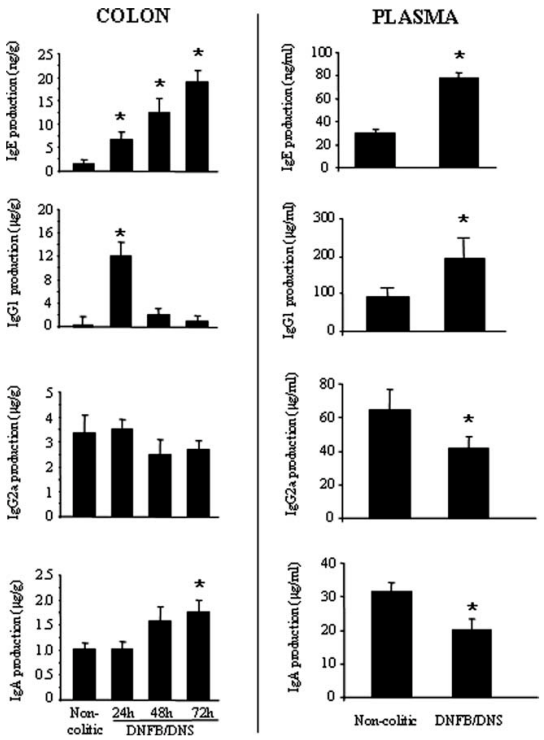

FIGURE 5. The DNFB/DNS protocol increases IgE and IgG1 expression. IgE, IgG1, IgG2a, and IgA expression in colonic homogenates and plasma samples 24, 48, and 72 hours after DNS challenge. The results of plasma samples are shown only at 48 hours. Immunoglobulin concentrations were analyzed by ELISA following the manufacturer's protocol. All data are expressed as mean ± SEM. *$P < 0.05$ vs. control noncolitic group.

Colonic Inflammation Induced by DNFB/DNS Colitis Is Not Exacerbated After a Second DNS Challenge

So far our results suggest that the main driver of the colitis induced in the DNFB/DNS model is a humoral response mediated by B cells. However, the experimental protocol used (7 days) did not favor the results observed. Thus, we checked the experimental model again and forced it further by inducing an exacerbation of the inflammatory process by a second challenge with the hapten 2 weeks after the first.

Macroscopically, the second challenge showed a similar profile to the first, with the presence of mild diarrhea and a reactivation of the DTH wounds in the abdomen (Fig. 6A). Similarly, no further increase was observed in colonic weight/length ratio or in the number of colon patches 24 and 72 hours after the second challenge in comparison with the values obtained in the first one (data not shown). These observations suggest that no exacerbated or faster process occurred after the second DNS challenge in DNFB-sensitized animals. In concordance with these macroscopic results, the levels of colonic histamine after the second challenge were lower than those detected after the initial one (Fig. 6B).

To understand the magnitude and bias of the immune response during the second reactivation of colitis, we analyzed colonic gene expression (Table 2) and colonic cytokine secretion (Fig. 7A). In this regard, IL-10, IL-12, and IL-13 gene expression decreased 24 hours after the stimuli. Although it was not statistically significant, a reduction

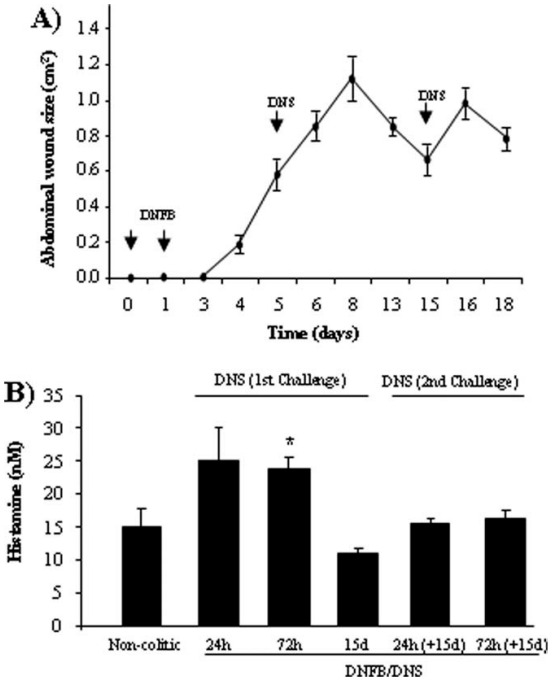

FIGURE 6. A second DNS challenge does not exacerbate colonic inflammation. (A) After DNFB administration, mice showed a typical DTH wound at the abdominal sensitization point. The wound was measured (height and width) at a range of times. (B) Histamine levels in colonic homogenates were analyzed by ELISA following the manufacturer's protocol. All the results are presented as the mean ± SEM and compared to noncolitic animals. *$P < 0.05$ vs. control noncolitic group.

was also detected for IFN-γ, IL-4, and TNF-α. Three days after the initial challenge, the expression of IL-2 was increased while that of TNF-α was significantly reduced. Remarkably, 2 weeks after the induction of colitis most of the cytokines analyzed were downregulated and only IL-13 was overexpressed when compared with healthy animals. Despite the second DNS challenge on day 15, the expression of most of the genes remained downregulated during the first 24 hours, except IL-13, which remained high, and TNF-α and IL-4, which were clearly induced in response to the second challenge. Three days after the second challenge, colonic cytokine gene expression returned to the basal levels observed in healthy animals.

Analysis of colonic cytokine production showed a clear increase in IL-5 expression after the first challenge. This increase is possibly responsible for driving the altered immune response in this experimental setting, while no major differences were observed in TNF-α (Figs. 4C, 7A) or other Th1 cytokines. In addition, an increase in the colonic expression of IL-17 at 72 hours was also detected after

TABLE 2. PCR Analysis of Cytokine Expression in Colon Samples

Colon	Healthy (Noncolitic)	Colitic Animals				
		24 h	72 h	15 d	15d+24 h	15d+72 h
IFNγ/β-actin	0.86 ± 0.1	0.74 ± 0.03 (−13.7%)	0.75 ± 0.04 (−12.8%)	0.76 ± 0.04	0.72 ± 0.02 (−5.1%)	0.71 ± 0.05 (−6.4%)
TNF-α/β-actin	0.83 ± 0.11	0.75 ± 0.02 (−10.1%)	0.64 ± 0.08* (−23%)	0.62 ± 0.09*	0.86 ± 0.06† (+38.4%)	0.87 ± 0.06† (+40.1%)
IL-2/β-actin	1.18 ± 0.07	1.22 ± 0.12 (+3.7%)	1.35 ± 0.05* (+14.9%)	1.07 ± 0.08	1.07 ± 0.06 (+0.3%)	1.05 ± 0.05 (−6.4%)
IL-4/β-actin	0.80 ± 0.06	0.73 ± 0.03 (−8.6%)	0.84 ± 0.03 (+4.7%)	0.74 ± 0.03	0.89 ± 0.01† (+20.1%)	0.82 ± 0.07 (+10.7%)
IL-5/β-actin	0.87 ± 0.13	0.83 ± 0.11 (−4.8%)	0.93 ± 0.12 (+7.2%)	0.56 ± 0.03*	0.56 ± 0.1* (+0.8%)	0.66 ± 0.03† (+18.1%)
IL-6/β-actin	0.89 ± 0.06	0.88 ± 0.08 (−1.2%)	0.80 ± 0.05 (−10%)	0.64 ± 0.02*	0.62 ± 0.01* (−3.1%)	0.67 ± 0.04* (+4.3%)
IL-10/β-actin	0.71 ± 0.04	0.61 ± 0.01* (−14.7%)	0.67 ± 0.03 (−6.7%)	0.61 ± 0.03*	0.62 ± 0.02* (+3.0%)	0.68 ± 0.05 (+12.7%)
IL-12/β-actin	1.27 ± 0.1	1.03 ± 0.06* (−18.8%)	1.26 ± 0.04 (−0.2%)	1.09 ± 0.05	1.01 ± 0.07* (−7.9%)	1.1 ± 0.1(+0.5%)
IL-13/β-actin	1.47 ± 0.17	1.19 ± 0.09* (−18.8%)	1.31 ± 0.08 (−11.1%)	1.81 ± 0.17*	1.98 ± 0.19* (+9.5%)	2.38 ± 0.2*,† (+31.9%)
IL-17/β-actin	0.84 ± 0.05	0.81 ± 0.01 (−3.6%)	0.81 ± 0.01 (−3.3%)	0.66 ± 0.01*	0.67 ± 0.01* (+2.2%)	0.67 ± 0.02* (+1.5%)

Values indicate the densitometry analysis of the PCR bands of each gene referred to the expression of the control gene β-actin. PCR conditions for each gene are specified in the Materials and Methods. Values are represented as the mean of the group ± SEM (n=10). The value in parentheses corresponds to the percentage (%) of variation in comparison to the basal value before DNS challenge, meaning healthy (noncolitic) values for groups 24 h and 72 h, and group 15 d values for groups 15d+24h and 15d+72h.
*$P < 0.05$ vs. healthy values;
$^{\dagger}P < 0.05$ vs. 15d values.

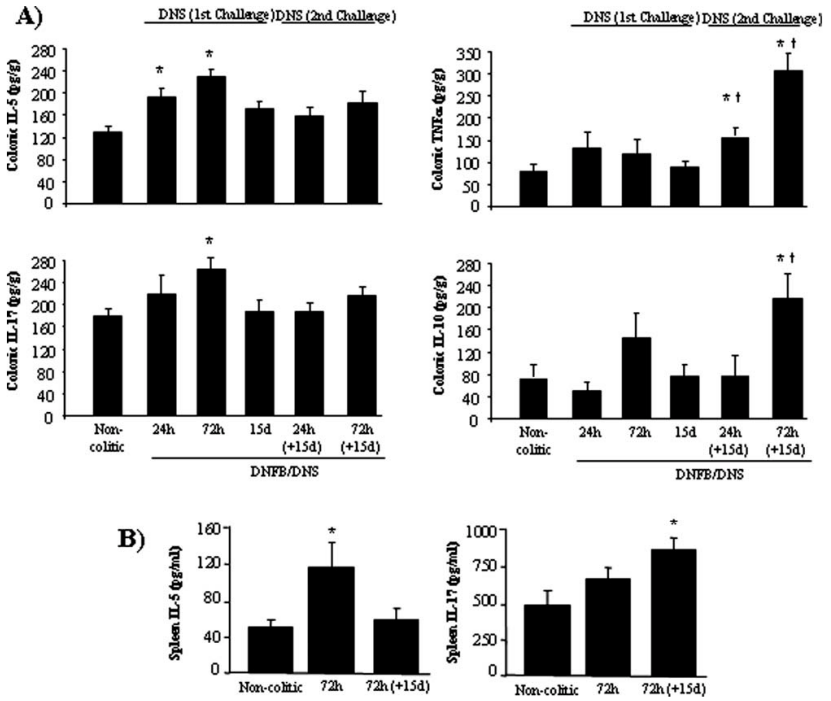

FIGURE 7. A second DNS challenge modifies the initial Th1/Th2 cytokine bias. (A) IL-5, TNF-α, IL-17, and IL-10 expression in the colitic DNFB/DNS groups. Cytokine concentrations in colonic homogenates were analyzed by ELISA following the manufacturer's protocol. All data are expressed as mean ± SEM. *$P < 0.05$ vs. control noncolitic group. †$P < 0.05$ vs. 15d DNFB/DNS group. (B) IL-5 and IL-17 production in ConA-activated splenocytes. Splenocytes were obtained from the noncolitic, 72-hour (+15 days) groups and incubated with ConA for 48 hours. Cytokine concentrations (means ± SEM) in the culture supernatants were analyzed by ELISA. The results of a representative experiment of four independent trials are shown. *$P < 0.05$ vs. control noncolitic group.

the first challenge. Moreover, the Th3 cytokine IL-10 tended to increase. However, after the second challenge the increased colonic expression of IL-5 and IL-17 reverted and the main immune response was driven mainly by the expression of TNF-α and IL-10 (Fig. 7A). Again, no significant differences were observed in cytokine expression in plasma, but the absence of IL-5 expression during the second challenge was evidenced also by the immune response of concanavalin A (ConA)-stimulated lymphocytes obtained from the spleen of these animals (Fig. 7B). In contrast to colon samples, IL-17 secretion was increased in splenocytes of animals that received a second challenge.

Ig expression was also evaluated after the second hapten challenge in this experimental model. As previously seen (Fig. 5), the main effect induced by DNS administration on plasma Ig was a clear reduction on IgA, IgM, and IgG2a, which was evident from the first 24 hours after the challenge (Table 3). A marked increase in IgG1 (50%) was observed after 24 hours, although it did not reach statistical significance because of the high variance among animals. Similarly, an increase in IgE expression was observed 72 hours after the challenge. Ten days after the initial challenge (day 15), we observed a general increase in IgG and IgE expression, while IgM and IgA expression in plasma returned to initial values (Table 3). After a second DNS challenge, the expression of most of the Igs was not modified and they maintained the increased amounts found just before the second challenge (day 15) except for IgG1, which was still increased and reached values 3 times higher than those found in healthy animals (Table 3).

At the inflammatory foci, the effect of the first DNS challenge on colonic Ig, the main change was a high and rapid increase in IgG1 and IgE expression, which increased more than 10 times in comparison with the values found in noncolitic animals. Three days later, an increase in colonic IgA expression was also seen. As observed in plasma, 2 weeks after the induction of colitis, a general increase in all Ig isotypes was still evident. This would explain why a second DNS challenge was only capable of slightly increase IgG1 expression (Table 3).

Finally, after the first challenge, the IgG1/IgG2a and IgE/IgG2a ratios in colon and plasma were clearly biased

TABLE 3. Immunoglobulin Levels in Plasma and Colon Homogenates

	Healthy (Noncolitic)	Colitic Animals				
		24 h	72 h	15 d	15d+24 h	15d+72 h
Plasma						
Total IgG (μg/ml)	116.8 ± 9.2	86.7 ± 22.8 (−25.8%)	88.0 ± 14.2 (−24.7%)	172.6 ± 12.8*	174.8 ± 41.1 (+1.2%)	185.2 ± 17.6* (+7.3%)
IgG1 (μg/ml)	129.9 ± 14.4	195.9 ± 67.7 (+50.9%)	119.6 ± 20.2 (−7.8%)	288.4 ± 20.5*	396.8 ± 100.9* (+37%)	400.8 ± 59.3* (+38.9%)
IgG2a (μg/ml)	62.4 ± 8.9	35.1 ± 13.7 (−43.8%)	30.9 ± 11.9* (−50.6%)	129.4 ± 10.1*	103.6 ± 25.7 (−19.9%)	122.7 ± 16.7* (−5.2%)
IgA (μg/ml)	33.8 ± 3.8	11.8 ± 1.9* (−65%)	15.1 ± 2.5* (−63.4%)	29.9 ± 1.5	21.8 ± 3.1*,† (−27.3%)	28.7 ± 3.0 (−4.3%)
IgE (ng/ml)	34.2 ± 3.6	32.5 ± 5.7 (−4.9%)	76.1 ± 7.3* (+122.5%)	51.9 ± 4.4*	50.7 ± 3.9* (−2.3%)	57.0 ± 5.0* (+9.8%)
IgM (μg/ml)	58.0 ± 7.3	19.7 ± 3.0* (−67%)	22.2 ± 5.3* (−61.8%)	44.9 ± 5.5	49.7 ± 10.7 (+10.8%)	109 ± 35.9 (+143.5%)
Colon						
Total IgG (μg/g)	5.5 ± 3.4	92.6 ± 58.4 (+1500%)	6.5 ± 4.0 (+16.6%)	188.8 ± 48.6*	211.9 ± 78.9* (+12.3%)	297.2 ± 50* (+57.4%)
IgG1 (μg/g)	1.15 ± 0.35	10.7 ± 4.3* (+828%)	1.54 ± 0.8 (+33.5%)	8.1 ± 2.5*	19.1 ± 10.3* (+135.8%)	17.6 ± 3.2*,† (+117.6%)
IgG2a (μg/g)	3.3 ± 1.5	3.4 ± 1.3 (+3.3%)	2.6 ± 0.5 (−20.3%)	10.6 ± 1.4*	9.3 ± 1.5* (−12.3%)	11.1 ± 1.0* (+4.6%)
IgA (mg/g)	1.03 ± 0.14	1.08 ± 0.18 (+4.7%)	1.69 ± 0.17* (+64.5%)	1.64 ± 0.12*	1.30 ± 0.11† (−20.7%)	1.88 ± 0.25* (+14.4%)
IgE (ng/g)	nd	7.7 ± 3.4*	17.5 ± 2.5*	24.9 ± 5.3*	15.6 ± 3.5* (−47.4%)	17.9 ± 4.1* (−28%)

Values are represented as the mean of the group ± SEM (n=10). The value in parentheses corresponds to the percentage (%) of variation in comparison to the basal value before DNS challenge, meaning healthy (noncolitic) values for groups 24 h and 72 h, and group 15 d values for groups 15d+24h and 15d+72h.
*$P < 0.05$ vs. healthy values;
†$P < 0.05$ vs. 15d values; nd, not detected.

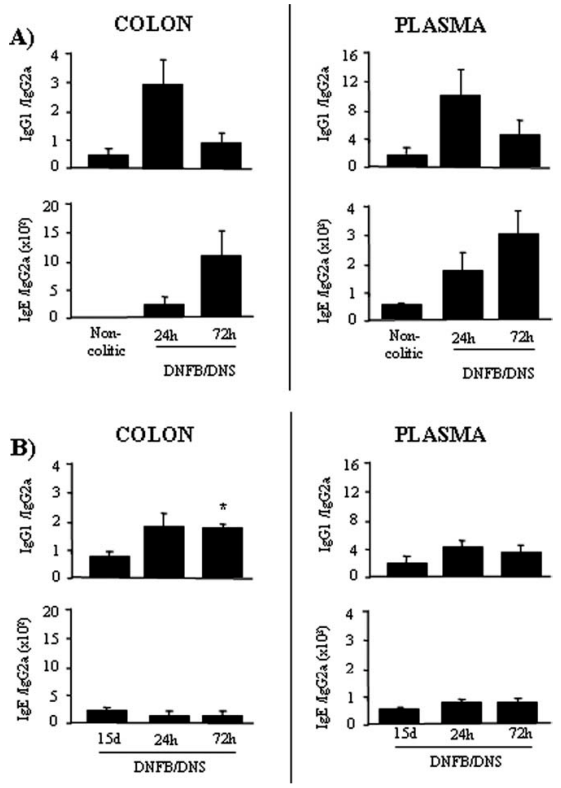

FIGURE 8. A second DNS challenge modifies the initial Ig bias. IgG1/IgG2a and IgE/IgG2a ratios in colon and plasma 24 and 72 hours after the first DNS challenge (A) or the second DNS challenge (B). All the results are presented as the mean ± SEM and compared to noncolitic animals.

to an atopic response due to the high expression of IL-5, IgG1 being the main Ig involved in the allergic response in these animals (Fig. 8A). Curiously, the Ig profile during the second challenge differed completely. Although a higher humoral response was observed, no clear biased immune response could be defined on the basis of the Ig ratios (Fig. 8B).

DISCUSSION

Although current experimental animal models for IBD do not perfectly match the patho-physiological features observed in humans, they have contributed to improving our knowledge and to recent therapeutic advances in these intestinal conditions.[9,10] In this regard, most rodent models support a central role for T-cell activation in chronic intestinal inflammation,[3,7] which is associated mainly with a Th1 phenotype. This finding is in agreement with clinical observations of CD, but differs from that observed in UC.[7,26] Thus, the development of new animal models resembling UC inflammatory milieu continues to be one of the unmet needs in this pathology.

To our knowledge, there are two main animal hapten-induced models for Th2-biased colitis response, both of which share some of the features of human UC, namely, the oxazolone[14,27] and DNFB[15,28] models. Although they present various characteristics that are useful for some aspects of this pathology in general, the main problem presented by these models is the difficulty to obtain a homogeneous response between experiments. In some circumstances a very mild and superficial inflammation of the mucosa is observed, while in similar circumstances a rapid onset of inflammation peaks within a few days of hapten administration and leads to wasting and bloody diarrhea, which results in either death of the mouse or in complete recovery after 5 days.[14,28]

Recently, Rijnierse et al[15,16] reported a chemically induced immunological model for colonic colitis related to the DNFB model, in which the intrarectal hapten challenge was changed to DNS. This model has the advantage that it allows more consistent and homogeneous results thanks to a reduced onset of action and more progressive inflammation. Moreover, this model is associated with mast cell activation without macrophage participation, and demonstrates a central role for mast cell-derived TNF-α expression.[15] Given the scarce data regarding the DNFB/DNS model compared to others, such as DSS or TNBS, here we further explored the immune response elicited in this model and its usefulness as an experimental model with UC features.

For this purpose, we designed various experiments following the protocol originally described.[15] As reported, consistent colonic inflammation was observed in the treated animals; however, this process was mild and peaked after 3 days and totally disappeared at day 5. No major macroscopic differences were observed, according with the fact that the severity of the inflammation was lower than expected based on previously published data.[15,16]

In comparison, the TNBS-induced colitis model used in this study showed an increased severity, with clear macroscopic intestinal damage including necrotic areas. Moreover, the intestinal effect was visible for almost 1 week and progressively resolved thanks to intestinal epithelium regeneration. Nevertheless, similar to what has been described in the intrarectal administration of DNFB without previously sensitization,[28] the onset of the inflammatory process was poorly homogeneous, being nonexistent in 10% of the animals while lethal in 30%–40% of them. Although more robust TNBS colitis models in mice with previous sensitization have been described in a very limited number of publications,[29,30] we were unsuccessful in reproducing these models at concentrations lower than that used for colitis induction without sensitization.

The microscopic analysis of the inflammatory intestinal infiltrate confirmed the scarce involvement of macrophages in the DNFB/DNS model and suggested a more relevant role for B and T lymphocytes. The key role of lymphocytes in this model is suggested by the increased number of colon patches observed in comparison to that observed in the TNBS model and confirmed by the immunostaining of the inflammatory infiltrate. In contrast to the critical involvement of mast cells previously described in the DNFB/DNS model,[16] and although we were able to detect this cell type by RT-PCR and immune-staining, the contribution of these cells to intestinal damage was modest. This finding is consistent with the moderate increase in colonic and plasmatic histamine levels observed during the inflammatory episode. The contribution of mast cells to IBD is controversial in humans[31,32] and especially in animal models.[16,33,34] However, and in contrast to what occurs in other mammalian species, including rat, sheep, and human, the presence of mast cells in the mouse is rare unless the animal has recently harbored an intestinal nematode infection.[35,36] This observation would explain the low number of mast cells observed in the colonic specimens evaluated in the present study.

Regarding the cytokine milieu induced after DNS administration, we found a totally different scenario to that initially described by Rijnierse et al.[15] In our study a negligible colonic TNF-α concentration was observed after the first challenge with DNS, while the concentrations of IL-2 and especially IL-5 were markedly higher. This finding is not surprising since it is known that serum levels of TNF-α are not consistently correlated with disease severity in either humans or IBD experimental models.[37] Moreover, a scarce representation of macrophages, the main TNF-α-producing cells, at the inflammatory foci was observed in this study. We do not have an explanation for the differences observed since almost identical protocols were used. However, we propose that the expression of the cytokines observed in our experiments is consistent with the cell population detected in the intestinal infiltrate.

Although it has been described that mast cells from patients with IBD could release IL-5, we consider that the high IL-5 expression detected in the colon samples of the DNS-colitic animals has a lymphocyte origin since it has been reported that mast cells, at least those from the nasal mucosa and bronchus, contain very low amounts of this cytokine.[38,39] The role of IL-5 in IBD requires further research, but early studies suggested additional activities of this cytokine in the regulation of mouse B cells and antibody production in some colitis models.[40] In IL-5 knockout mice, IL-5 has been implicated in eosinophil recruitment in acute colonic inflammation, although deficiency of this cytokine does not affect the course of the disease or the number of immunoglobulin-producing cells in the mucosa.[40]

Furthermore, given that the DNFB/DNS model has been reported to be a Th2-related colitic model, in these initial experiments we also evaluated the humoral component of the immune response of this model. In the original studies, the only statement regarding humoral response was the description of the model as non-IgE-related.[15,16] On the basis of the results obtained in the present study, this statement could be attributable to the absence of anaphylactic processes, in accordance with that generally described by contact hypersensitivity and DTH responses. However, a clear increase in the IgE response in colon and plasma was observed during the DNS-induced inflammatory process. Moreover, we noted that the expression of IgG1 was highly restricted in time together with a progressive, but delayed, increased expression of colonic IgA.

On the basis of our findings and reports that several patho-physiological features of IBD resemble hypersensitivity-like responses in the gastrointestinal tract,[41,42] we propose that the DNFB/DNS mice model should not be considered a model for the study of IBD. This recommendation is supported by the finding that the mild inflammation induced by DNS and the lack of a clear disruption of mucosal integrity did not allow the infiltration of luminal antigens and therefore did not favor the involvement of innate immunity. These observations are relevant since the recruitment of local macrophages would act as professional antigen-presenting cells to lymphocytes, thereby allowing the disruption of the intestinal immune tolerance.[43] Thus, the inflammation observed in the DNFB/DNS model resolves spontaneously in less than 5 days and despite the initial humoral response induced, this response does not have a fast onset or increased response after a second contact with the antigen.

In general, based on our expertise and the published works, it seems that murine models of hapten-induced colitis are less reliable than those used in rats. In this sense, mice models not only show a high variability in the onset of inflammation, but also a higher complexity and less biased immune responses were observed in these models in comparison to their rat equivalents. However, the availability of knockout or transgenic mice and a higher number of immune tools for mice than rats require improvement of the current mice colitis models and probably the discovery and description of new ones, especially in intestinal pathologies.

Although the DNFB/DNS mice model is not suitable for IBD research, it shows some characteristics that other experimental models do not present and that should be taken into consideration and further explored. In this regard, the inflammation induced by DNS is more similar to a contact DTH response, with the advantage that both T

and B cell responses are involved and the humoral response plays a pivotal role. Moreover, a clearly non-biased cytokine response was induced after challenge with DNS, especially after a second contact with the hapten. Recently, the involvement of B cells and humoral Th2 response has also been described in cutaneous DTH responses induced by DNFB,[44,45] in contrast to that initially postulated, which favored the unique role of T cells and Th1 response in contact hypersensitivity reactions.[46]

Therefore, the intestinal manifestation and the complexity of the immune response observed in the DNFB/DNS mice model more than UC manifestations resemble the immune response detected in some food-hypersensitive patients where a mixture of IgE and non-IgE responses are observed,[47,48] and the response observed during some cases of irritable bowel syndrome,[49,50] a pathology that also requires experimental models for the evaluation of new pharmacological treatments. Further studies, especially those directed to evaluate the electrophysiology of the intestine, will offer new clues to the usefulness of this model for the study of complex pathologies such as irritable bowel syndrome.

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REFERENCES

1. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol*. 2003;3:521–533.
2. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol*. 2010;28:573–621.
3. Maynard CL, Weaver CT. Intestinal effector T cells in health and disease. *Immunity*. 2009;31:389–400.
4. Blumberg RS. Inflammation in the intestinal tract: pathogenesis and treatment. *Dig Dis*. 2009;27:455–464.
5. D'Haens GR. Top-down therapy for IBD: rationale and requisite evidence. *Nat Rev Gastroenterol Hepatol*. 2001;7:86–92.
6. Rutgeerts P, Vermeire S, Van Assche G. Biological therapies for inflammatory bowel diseases. *Gastroenterology*. 2009;136:1182–1197.
7. Zenewicz LA, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med*. 2009;15:199–207.
8. Liu ZJ, Yadav PK, Su JL, et al. Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol*. 2009;15:5784–5788.
9. Galvez J. Experimental models of inflammatory bowel disease in rodents. In: Peppelenbosch M, Comalada M, eds. Preclinical Research into Crohn's Disease: A Practical Guide. Kerala, India: Transworld Research Network; 2009:153–171.
10. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol*. 2002;20:495–549.
11. Bailón E, Camuesco D, Nieto A, et al. The intestinal anti-inflammatory effects of the novel agent UR-1505 in the TNBS model of rat colitis are mediated by T-lymphocyte inhibition. *Biochem Pharmacol*. 2007;74:1496–506.
12. Comalada M, Camuesco D, Sierra S, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur J Immunol*. 2005;35:584–592.
13. Dianda L, Hanby AM, Wright NA, et al. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol*. 1997;150:91–97.
14. Kojima R, Kuroda S, Ohkishi T, et al. Oxazolone-induced colitis in BALB/C mice: a new method to evaluate the efficacy of therapeutic agents for ulcerative colitis. *J Pharmacol Sci*. 2004;96:307–313.
15. Rijnierse A, Koster AS, Nijkamp FP, Kraneveld AD. TNF-alpha is crucial for the development of mast cell-dependent colitis in mice. *Am J Physiol Gastrointest Liver Physiol*. 2006;291:G969–G976.
16. Rijnierse A, Koster AS, Nijkamp FP, Kraneveld AD. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J Immunol*. 2006;176:4375–4384.
17. Lilja I, Gustafson-Svärd C, Franzén L, et al. Tumor necrosis factor-alpha in ileal mast cells in patients with Crohn's disease. *Digestion*. 2000;61:68–76.
18. Raithel M, Winterkamp S, Pacurar A, et al. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. *Scand J Gastroenterol*. 2001;36:174–179.
19. Daniel C, Sartory N, Zahn N, et al. FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4+CD25+ regulatory T cells. *J Immunol*. 2007;178:2458–2468.
20. Bailón E, Cueto-Sola M, Utrilla P, et al. Butyrate in vitro immune-modulatory effects might be mediated through a proliferation-related induction of apoptosis. *Immunobiology*. 2010;215:863–873.
21. Peran L, Camuesco D, Comalada M, et al. A comparative study of the preventative effects exerted by three probiotics, *Bifidobacterium lactis, Lactobacillus casei* and *Lactobacillus acidophilus*, in the TNBS model of rat colitis. *J Appl Microbiol*. 2007;103:836–844.
22. Arribas B, Rodríguez-Cabezas ME, Comalada M, et al. Evaluation of the preventative effects exerted by Lactobacillus fermentum in an experimental model of septic shock induced in mice. *Br J Nutr*. 2009;101:51–58.
23. Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology*. 1984;87:1344–1350.
24. Camuesco D, Comalada M, Rodríguez-Cabezas ME, et al. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br J Pharmacol*. 2004;143:908–918.
25. Dohi T, Fujihashi K, Rennert PD, et al. Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. *J Exp Med*. 1999;189:1169–1180.
26. Brown SJ, Mayer L. The immune response in inflammatory bowel disease. *Am J Gastroenterol*. 2007;102:2058–2069.
27. Wang X, Ouyang Q, Luo WJ. Oxazolone-induced murine model of ulcerative colitis. *Chin J Dig Dis*. 2004;5:165–168.
28. Brkic T, Banic M, Anic B, et al. A model of inflammatory bowel disease induced by 2,4-dinitrofluorobenzene in previously sensitized BALB-c mice. *Scand J Gastroenterol*. 1992;27:184–188.
29. Eri R, Kodumuni KN, Summerlin DJ, et al. Supression of colon inflammation by CD80 blockade: evaluation in two models of inflammatory bowel disease. *Inflamm Bowel Dis*. 2008;14:458–470.
30. Shen C, Dillissen E, Kasran, A, et al. Anti-inflammatory activity of a pteridine derivative (4AZA2096) alleviates TNBS-induced colitis in mice. *J Interferon Cytokine Res*. 2006;26:575–582.
31. Nolte H, Spjeldnaes N, Kruse A, et al. Histamine release from gut mast cells from patients with inflammatory bowel diseases. *Gut*. 1990;31:791–794.
32. Xie H, He SH. Roles of histamine and its receptors in allergic and inflammatory bowel diseases. *World J Gastroenterol*. 2005;11:2851–2857.
33. Chin KW, Barrett KE. Mast cells are not essential to inflammation in murine model of colitis. *Dig Dis Sci*. 1994;39:513–525.
34. Xu X, Weksler-Zangen S, Pikarsky A, et al. Mast cells involvement in the inflammation and fibrosis development of the TNBS-induced rat model of colitis. *Scand J Gastroenterol*. 2002;37:330–337.
35. Friend DS, Ghildyal N, Austen KF, et al. Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J Cell Biol*. 1996;135:279–290.

36. Miller HR, Huntley JF, Newlands GF, et al. Granule proteinases define mast cell heterogeneity in the serosa and the gastrointestinal mucosa of the mouse. *Immunology*. 1988;65:559–566.
37. Fiocchi C. Cytokines and intestinal inflammation. *Transplat Proc*. 1996;28:2442–2443.
38. Bradding P, Walls AF, Church MK. Role of mast cells and basophils in inflammatory responses. In: Holgate ST, ed. Immunopharmacology of the Respiratory System. New York: Harcourt Brace; Academic Press; 1995:53–84.
39. He SH. Key role of mast cells and their major secretory products in inflammatory bowel disease. *World J Gastroenterol*. 2004;10: 309–318.
40. Stevceva L, Pavli P, Husband A, et al. Eosinophilia is attenuated in experimental colitis induced in IL-5 deficient mice. *Genes Immun*. 2000;1:213–218.
41. Kagnoff MF. Immunology of the intestinal tract. *Gastroenterology*. 1993;105:1275–1280.
42. Kraneveld AD, Buckley TL, van Heuven-Nolsen D, et al. Delayed-type hypersensitivity-induced increase in vascular permeability in the mouse small intestine: inhibition by depletion of sensory neuropeptides and NK1 receptor blockade. *Br J Pharmacol*. 1995;114: 1483–1489.
43. Kamada N, Hisamatsu T, Honda H, et al. Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J Immunol*. 2009;183:1724–1731.
44. Larsen JM, Geisler C, Nielsen MW, et al. Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis*. 2007;57:300–308.
45. Zhang EY, Chen AY, Zhu BT. Mechanism of dinitrochlorobenzene-induced dermatitis in mice: role of specific antibodies in pathogenesis. *PLoS One*. 2009;4:e7703.
46. Grabbe S, Schwarz T. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today*. 1998;19:37–44.
47. Crittenden RG, Bennett LE. Cow's milk allergy: a complex disorder. *J Am Coll Nutr*. 2005;24:582S–591S.
48. Lied GA. Gastrointestinal food hypersensitivity: symptoms, diagnosis and provocation tests. *Turk J Gastroenterol*. 2007;18:5–13.
49. Chadwick VS, Chen W, Shu D, et al. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology*. 2002; 122:1778–1783.
50. Gwee KA, Leong YL, Graham C, et al. The role of psychological and biological factors in postinfective gut dysfunction. *Gut*. 1999;44: 400–406.

Chapter 4

A shorter and more specific oral sensitization-based experimental model of food allergy in mice (IF:2.2; 96/137)

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Research paper

A shorter and more specific oral sensitization-based experimental model of food allergy in mice

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ABSTRACT

Cow's milk protein allergy (CMPA) is one of the most prevalent human food-borne allergies, particularly in children. Experimental animal models have become critical tools with which to perform research on new therapeutic approaches and on the molecular mechanisms involved. However, oral food allergen sensitization in mice requires several weeks and is usually associated with unspecific immune responses. To overcome these inconveniences, we have developed a new food allergy model that takes only two weeks while retaining the main characters of allergic response to food antigens.

The new model is characterized by oral sensitization of weaned Balb/c mice with 5 doses of purified cow's milk protein (CMP) plus cholera toxin (CT) for only two weeks and posterior challenge with an intraperitoneal administration of the allergen at the end of the sensitization period. In parallel, we studied a conventional protocol that lasts for seven weeks, and also the non-specific effects exerted by CT in both protocols.

The shorter protocol achieves a similar clinical score as the original food allergy model without macroscopically affecting gut morphology or physiology. Moreover, the shorter protocol caused an increased IL-4 production and a more selective antigen-specific IgG1 response. Finally, the extended CT administration during the sensitization period of the conventional protocol is responsible for the exacerbated immune response observed in that model.

Therefore, the new model presented here allows a reduction not only in experimental time but also in the number of animals required per experiment while maintaining the features of conventional allergy models. We propose that the new protocol reported will contribute to advancing allergy research.

1. Introduction

Cow's milk protein allergy (CMPA) is one of the major causes of food hypersensitivity in children. Approximately 2 to 6% of infants exhibit cow's milk hypersensitivity in the first year of life (Sampson, 2004). CMPA is associated with a broad spectrum of IgE-mediated and non-IgE-mediated hypersensitivity disorders. The clinical features of allergy to cow's milk proteins (CMP) involving IgE-mediated reactions are expressed mostly as immediate symptoms, which may affect the skin (e.g., urticaria and eczema), respiratory tract (e.g., asthma and rhinoconjunctivitis), gastrointestinal tract (e.g., vomiting, diarrhea and colic) or systemic anaphylactic shock (Sampson, 1997; 2004). Although most patients outgrow CMPA by 3 years of age, patients with IgE-mediated hypersensitivity have a

Abbreviations: CMP, cow's milk protein;CMPA, cow's milk protein allergy;CT, cholera toxin;IP, intraperitoneal

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lower rate of outgrowing CMPA and higher susceptibility to develop allergic reactions to other foods than patients with non-IgE-mediated disorders.

The incidence of CMPA is rising in the western world (Bjorksten, 2001). This increase may not be an isolated phenomenon but may herald the beginning of an "atopic career". Infants and children with CMPA may also develop additional atopic diseases such as other food allergies, rhinitis, asthma and early atopic dermatitis, the latter affecting up to 15–20% of children (Broberg et al., 2000; Oehling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007).

Significant advances in our understanding of the mechanism behind food allergy and its aetiology have been achieved from the study of animal models. These models allow not only experiments that are not feasible in humans but also the study of the molecular mechanisms involved in atopy and the development and testing of potential new treatments. For this purpose, several rodent models have been developed, these using distinct sensitization routes, such as intraperitoneal (Hsieh et al., 2003; Thang et al., 2011) or subcutaneous (Gonipeta et al., 2010; Strid and Strobel, 2005; Valeur et al., 2009) injection of allergen or genetically modified bacteria that express food antigens (Adel-Patient et al., 2005). However, these models have several limitations that significantly diminish their utility and they do not reflect the pathogenic mechanism that leads to food allergy, and thus could underestimate the involvement of the mucosal immune system. In this regard, the desired model should mimic human food allergy by provoking food hypersensitivity by oral ingestion. To accomplish this prerequisite, it is necessary to bypass the tendency of mice to develop oral tolerance and to ensure a Th2 response after antigen administration. For this reason, weaned Balb/c mice have commonly been used (Gonipeta et al., 2010; Lara-Villoslada et al., 2004, 2005; Thang et al., 2011) because this strain of mice is more susceptible to developing Th2 responses than other strains (Sun et al., 2001). Moreover, toxins such as cholera toxin (CT) are required to stimulate a Th2 response and the production of IgG1 antibodies (Marinaro et al., 1995; Snider et al., 1994). Recently, other bacterial toxins, such as staphylococcal enterotoxin B (Ganeshan et al., 2009) or approaches based on anti-acid treatment (Diesner et al., 2008; Untersmayr et al., 2010) have been described. Moreover, a desirable food allergy model should provoke systemic reactions with a specific antigen response that is not observable in some allergy models (Ito et al., 1997).

Despite the variety of models currently available, they are flawed by two main common drawbacks. First, they require long periods of sensitization, which extend for 6 to 8 weeks to obtain a sustained and measurable systemic immune response. And second, the adjuvants of bacterial origin or anti-acid treatments used to ensure disruption of the tolerogenic potential of the oral route for such a long period could mislead the results obtained as a result of the immune-stimulatory properties of adjuvants and also the lack of specificity of the approach, which allows the generation of allergic response to other food components of the diet not related to the antigen of interest.

With the goal to solve these two main drawbacks of current allergy models, here we present a shorter murine allergy model consisting of only 2 weeks of oral sensitization but requiring intraperitoneal challenge to achieve a consistent immune response and compare it with a conventional model of milk allergy consisting of 6 weeks of oral sensitization, which has previously been described (Lara-Villoslada et al., 2004, 2005; Li et al., 1999). Both models used CT as adjuvant, but the immune stimulatory effects induced by this bacterial toxin were abolished in the shorter protocol, thereby allowing a more selective and specific response than that observed in the conventional model.

2. Materials and methods

2.1. Reagents and preparation of CMP

CT was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for ELISA were purchased from Bethyl Laboratories (Montgomery, TX) for immunoglobulins (Igs) or from Biosource (Camarillo, CA) for cytokines. All other products were of the highest grade available and were purchased from Sigma Chemical Co.

To obtain CMP, homogenized fat free cow's milk was obtained from Puleva Food SA (Granada, Spain) and centrifuged at 5000 ×*g* for 10 min at 4 °C and the upper layer of fat was discarded to obtain skimmed milk, which was stored at −20 °C in 1-ml aliquots and defrosted immediately before use. Protein content in cow's milk was measured by the Kjeldahl method, as previously described (Lara-Villoslada et al., 2004).

2.2. Animals

Female Balb/c mice (3-wk-old, immediately after weaning) were purchased from Harlan (Barcelona, Spain) and housed under a temperature (22 °C) and light-controlled (12 h) cycle. Animals ($n = 10$ per group) were maintained on a plant-based chow diet (Harlan Laboratories, Indianapolis, IN) under specific-pathogen-free conditions. These studies were carried out following the European Union Directive (86/609/EEC) for the protection of vertebrates used for experimental and other scientific purposes, in compliance with the Helsinki Declaration.

2.3. Sensitization and challenge

2.3.1. Original protocol

Mice were sensitized intragastrically with CMP (1 mg of CMP per gram of body weight) plus CT (0.3 μg/g) as an adjuvant in a total volume of 200 μL of PBS, and boosted 7 times at weekly intervals (Fig. 1A). Oral gavage was performed using a polyvinyl chloride tube-feeding needle purchased from Vygon (Ecoue, France). To analyze the effect of the adjuvant *per se*, a group of mice received the same dosage of CT in PBS (CT 6w control). Moreover, a control of non-sensitized animals (sensitized with only PBS and challenged with PBS and/or CMP) was also used (non-sensitized). Seven weeks after the first oral gavage, all groups were fasted overnight and challenged intragastrically with a high dose of CMP (35 mg/mouse).

2.3.2. Shorter protocol

Mice were also sensitized intragastrically with CMP plus CT at the same doses but boosted only 5 times at three to four

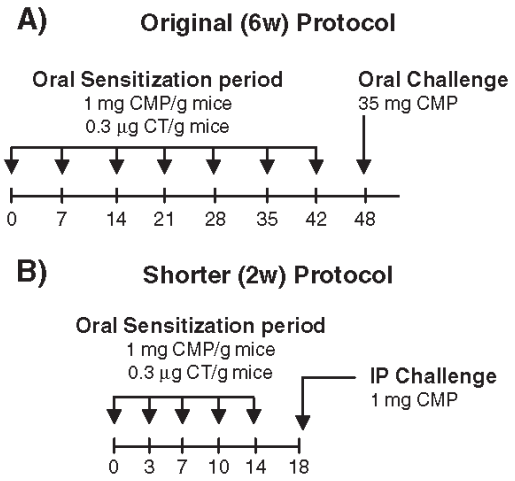

Fig. 1. Protocol diagram. A) Original protocol adapted from Li et al., 1999, and consisting of oral sensitization and challenge. B) Shorter protocol, comprising oral sensitization but intraperitoneal challenge.

day intervals (Fig. 1B). Equivalent control groups as in the original protocol were also used. Eighteen days after the first oral gavage, mice were challenged intraperitoneally with a 1 mg dose of CMP (100 µl).

In both protocols, 30 min after the last challenge, mice were killed by IP administration of sodium pentothal (50 mg/kg), and blood was collected by cardiac puncture in tubes containing EDTA. Spleen and intestine were removed from each mouse and weighed.

2.4. Evaluation of symptoms

Immediately after challenge, hypersensitivity responses were evaluated using a scoring system modified slightly from previous reports (Lara-Villoslada et al., 2004, 2005; Li et al., 1999) and scored as shown in Table 1. An extra half point for each score was obtained when the symptom became visible at 10 min or less after the challenge. Symptom evaluation was performed by 2 independent researchers who were blinded to the study treatments.

Gastrointestinal symptoms were evaluated as diarrhea and small intestine and colon inflammation, as previously described (Bailon et al., 2011). Animals that suffered from diarrhea on at least 2 consecutive occasions were considered positive cases. To evaluate intestinal inflammation, intestinal samples were placed on ice-cold plates, cleaned of fat and mesentery, and then blotted on filter paper. Each small intestine or colon specimen was weighed and the length was measured under a constant load (2 g).

Table 1
Score system to evaluate systemic hypersensitivity response (modified from Li et al., 1999).

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth and pilar erecti
3	Decreased activity with increased respiratory rate
4	Wheezing and labored respiration
5	Cyanosis around the mouth and the tail
6	Death

Symptoms were evaluated by two independent researchers who were blinded to the study treatments. An extra half-point for each score was assigned to mice when symptoms appeared in the first 10 min after the CMP challenge.

2.5. Determination of histamine and cytokine levels

Blood was centrifuged (3500×*g*) for 10 min at 4 °C, and plasma aliquots were collected and frozen at −80 °C. Colon homogenates were obtained as previously described (Garrido-Mesa et al., 2011).

Plasma histamine levels were determined using an enzyme immunoassay kit (IBL Laboratories, Hamburg, Germany) following the manufacturer recommendations. IL-4 and Ig levels were measured in plasma and colon homogenates by ELISA, following the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). The dilutions performed were 1:10,000 for the determination of total IgG1, IgG2a and IgA in plasma and at 1:2 for IgE. In the colon determinations, dilutions used were 1:1000 (IgG1), 1:500 (IgG2a), 1:5000 (IgA) or no dilution for IgE determination.

Levels of specific IgG1 to CMP were measured as previously described (Li et al., 1999) in non-diluted plasma. Briefly, 96-well plates were coated with 20 µg/mL of CMP in coating buffer (0.5 M Na_2CO_3). After overnight incubation at 4 °C, plates were washed 3 times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) and blocked with a solution (50 mM Tris, 0.14 M NaCl, 1% BSA). Plasma samples were then added to the plates and incubated for 1 h at room temperature (25 °C). Plates were washed 3 times, and 100 µL of goat antimouse IgG1 antibody conjugated with peroxidase (Bethyl Laboratories) was added for 1 h at 25 °C. Staining was performed with 3,3′–5,5′-tetramethyl-benzidine (TMB) (Sigma Chemical Co.) for 30 min at 25 °C in the dark, stopped with 0.1 N H_2SO_4, and plates were then read at 450 nm. All analyses were performed in triplicate.

2.6. Statistical analysis

Statistical significance was calculated by the Student's *t*-test in the case of parametric parameters, such as the ELISA results. All tests were performed with one tail following the 2-sample equal variance model (homoscedastic). For non-parametric parameters (score and number of mice with diarrhea), the Mann–Whitney *U*-test and Fisher's exact test were used to determine statistical significance. In all cases data are represented as the mean ± SEM and figures correspond to representative data from one of at least three independent experiments.

3. Results

Two CMPA protocols were tested in this study with the objective to evaluate the non-selective effect of the adjuvant

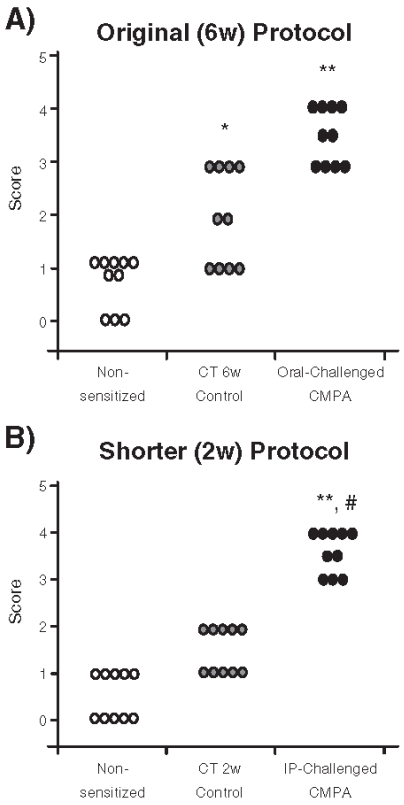

Fig. 2. Score of hypersensitivity symptoms. Mice ($n=10$ per group) were challenged with cow's milk. During the first 30 min, the symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 6 (death) as described in Table 1. Black circles indicate individual mice from one independent and representative experiment. A) Original protocol, where non-sensitized animals correspond to animals oral challenged with CMP; CT 6w Control, correspond to those receiving only CT during sensitization (7 doses in weekly intervals during 6 weeks) and orally challenged with CMP; while oral-challenged CMPA animals correspond to those receiving CMP and CT during oral sensitization (6 weeks) and orally challenged with CMP, as indicated in the Materials and methods section. B) Shorter protocol, where non-sensitized animals correspond to those only IP challenged with CMP; CT 2w Control correspond to animals receiving only CT during sensitization (5 doses in 2 weeks) and IP challenged with CMP; and finally, IP-challenged CMPA corresponds to animals orally sensitized with CT and CMP during 2 weeks and IP challenged with CMP. Mann–Whitney *U*-test was performed to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared with non-sensitized animals; # ($p<0.05$), compared with CT control.

(CT) and the effect of the length of sensitization. The two protocols used are summarized in Fig. 1. Although the shorter protocol consists of two fewer sensitizing doses containing CT, the main difference between them is that in the shorter one the 5 sensitizing doses are administered in only two weeks while 6 weeks are required in the original protocol. Nevertheless, to obtain a measurable response, the shorter protocol requires that the challenge be made by IP administration since no allergic response was obtained after oral challenge (data not shown).

In order to examine the non-selectivity of CT effects, the study included three types of control groups per protocol. However, no differences were observed between the control group of animals in which both sensitization and challenge were performed only with PBS and with the second control group corresponding to animals sensitized with only PBS but challenged with CMP. For this reason, the data corresponding to these two non-sensitized groups were analyzed together (as a unique group) and referred as "non-sensitized" group in order to simplify the understanding of the work. The third control performed corresponds to the adjuvant treatment, where a group of mice were sensitized only with CT in the absence of CMP during 6 weeks (CT 6w Control) or 2 weeks (CT 2w Control) and challenged with CMP. All these groups were compared with the oral- and IP-Challenged CMPA groups.

Hypersensitivity symptoms became evident in most cases within 15 to 30 min after challenge. The severity of the reaction was scored as indicated in Table 1 and shown in Fig. 2. As expected, no major allergic symptoms were observed in non-sensitized animals. The most severe allergic reactions were observed in both protocols (oral and IP) of CMPA-challenged mice (Fig. 2). However, although no significant differences on the allergy symptoms in CMPA-challenged animals were observed between both protocols, a significant difference from each particular Control group was only observable in the shorter protocol, since the CT 2w Control animals showed a more moderate symptomatology than CT 6w Controls.

Moreover, the number of animals with diarrhea was higher in the original protocol, especially in oral-challenged CMPA animals but clearly observable also in CT 6w Control animals (Table 2). Nevertheless, diarrhea in the two protocols was never as severe as to cause a significant decrease in body weight or in food intake (data not shown). Thirty minutes after the challenge, mice were killed under anesthesia and the intestine of each mouse was removed and weighed. In concordance with the incidence of diarrhea, alterations in gut morphology, consisting of modifications in

Table 2
Intestinal and immunological macroscopic symptoms of atopy.

		Original (6w) protocol		Shorter (2w) protocol	
	Non-sensitized	CT 6w Control	Oral-challenged CMPA	CT 2w Control	IP-challenged CMPA
Diarrhea	0/10	4/10 *	7/10 **, #	1/10	2/10 ††
Small intestine (weight/length) (mg/cm)	17.66 ± 1.67	14.88 ± 1.08 *	20.88 ± 1.71 #	16.33 ± 1.53	17.96 ± 1.83
Colon (weight/length) (mg/cm)	19.08 ± 1.81	26.32 ± 0.92 **	27.78 ± 2.19 **	18.28 ± 1.34 ††	17.18 ± 1.96 ††
Spleen (mg/g mice)	3.94 ± 0.42	4.65 ± 0.08 *	5.21 ± 0.1 **,#	4.86 ± 0.17 *	5.97 ± 0.4 **,#

Values represent the mean ± SEM of a representative experiment. Animals that suffered from diarrhea on at least 2 consecutive occasions were considered positive cases. Fisher's exact test was used for the statistical analysis of diarrhea. All the other parameters were evaluated using Student's *t*-test with one tail following the 2-sample equal variance model. * ($p<0.05$), ** ($p<0.01$) compared with non-sensitized animals; # ($p<0.05$), ## ($p<0.01$) compared with each particular CT control; † ($p<0.05$), †† ($p<0.01$) compared with similar group (CMPA or CT control) between both protocols.

the weight/length ratio of small or large intestine was found only in groups using the original protocol. Again, similar values than those observed in non-sensitized animals were obtained in CT 2w Control animals and in the IP-challenged CMPA group (Table 2). Taken together, these results suggest that CT administration for 6 weeks, but not for 2, has an impact on gut physiology and this effect is not exacerbated by the presence of CMP during the sensitization period.

The allergy process with systemic effects was confirmed not only for the symptoms observed in whole animals but by the increased size of the spleen (Table 2). In this case, although sensitization with CT alone enlarged the spleen in both protocols, a significant exacerbation was observed in CMPA groups of both protocols, thereby demonstrating that the addition of the target protein during sensitization increased the immune response observed after challenge with the same protein.

These results were further supported by histamine and IL-4 levels (Fig. 3). In this regard, a significant increase in plasma histamine was observed in CMPA groups of the two protocols in comparison with non-sensitized and CT Control groups. Nevertheless, the amounts of histamine found in groups belonging to the original protocol were significantly higher than those obtained in the shorter protocol. This difference was due to the effect of CT treatment, since no differences in plasma histamine levels were observed between non-sensitized animals and CT 2w Controls, while a four-fold increase was detected in the CT 6w Control group (Fig. 3A). Similarly, in the CT groups, plasma and colonic IL-4 levels were increased only in the CT 6w Controls in comparison with non-sensitized animals. However, in this case, the levels of IL-4 were exacerbated only in the IP-challenged CMPA group and no further increase was detected in the oral-challenged CMPA group (Fig. 3B–C).

Allergy response was further characterized by measuring Ig production in plasma and colon. Although CMPA is an IgE-mediated hypersensibility; an increase in IgE response is not easily observable in mouse allergy models while an increase in IgG1 is more frequently detected (Lara-Villoslada et al., 2004, 2005; Thang et al., 2011). Despite this consideration, total IgG1 and IgE levels increased in the oral-challenged CMPA group but not in the IP-challenged CMPA one (Fig. 4A-B). However, when the CMP-specific IgG1 response was analyzed, a similar increased response was detected in the two CMPA groups, independently of the protocol used (Fig. 4C). This observation demonstrates that a specific Ig response was induced in both allergy models and suggests that the immune response detected in the shorter protocol is more antigen-specific than that induced by the original protocol. Regarding other plasma Ig levels, the amount of IgG2a (more indicative of a Th1 response) was maintained or slightly increased in the original protocol but reduced in the IP-challenged CMPA group (Fig. 4D). In contrast, an increase in IgA production was observed only in plasma samples from the oral-challenged CMPA mice (Fig. 4E).

Regarding colon Ig levels, the two groups of the original protocol showed an increased production of IgE and IgG1 compared with non-sensitized animals while only an increase in IgG1 was observed in groups subjected to the shorter protocol. No difference between CT Control group and the CMPA group was observed in any protocol

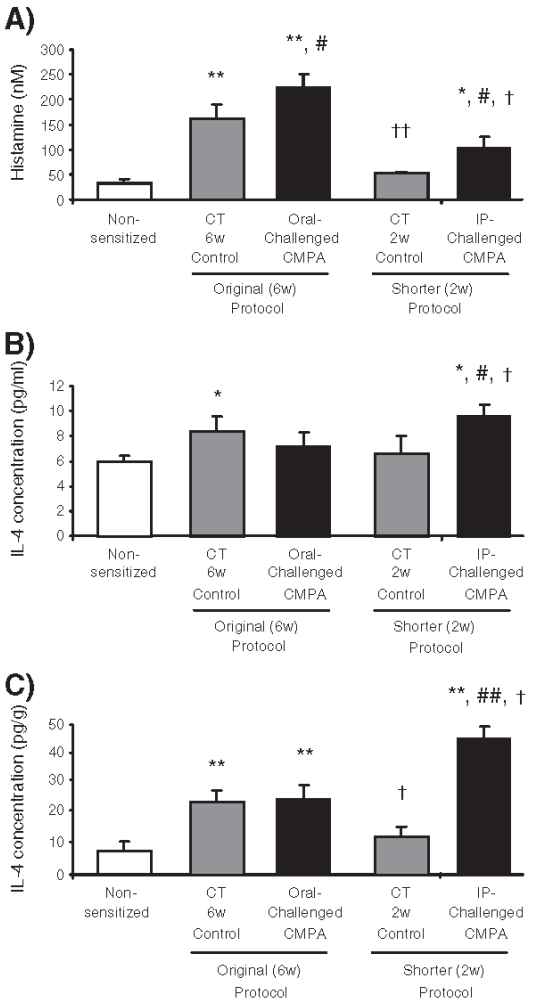

Fig. 3. Histamine and IL-4 levels. Plasma and colon homogenates from all mice ($n=10$ per CT or CMPA groups of each protocol, and 20 animals in non-sensitized group) were collected after sacrifice. A) Histamine concentration was determined by enzyme immunoassay kit and represented as plasma concentration (nM). IL-4 concentration measured by ELISA in plasma (B) or colon homogenates (C) was also measured in samples from all animals. All results are expressed as the median ± SEM. Student's *t*-test was used to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared with non-sensitized animals; # ($p<0.05$), ## ($p<0.01$) compared with each particular CT control; † ($p<0.05$), †† ($p<0.01$) compared with similar group (CMPA or CT control) between both protocols.

(Fig. 5A–B). In contrast to observations in plasma, colon IgG2a was increased in the CT 6w Control group and further exacerbated in the oral-challenged CMPA animals, while no differences were observed in groups sensitized for only two weeks (Fig. 5C). Similarly, colonic IgA was increased in the oral-challenged CMPA group but decreased in IP-challenged CMPA mice (Fig. 5D). This observation is consistent with the macroscopic manifestations of the gut previously described (Table 2).

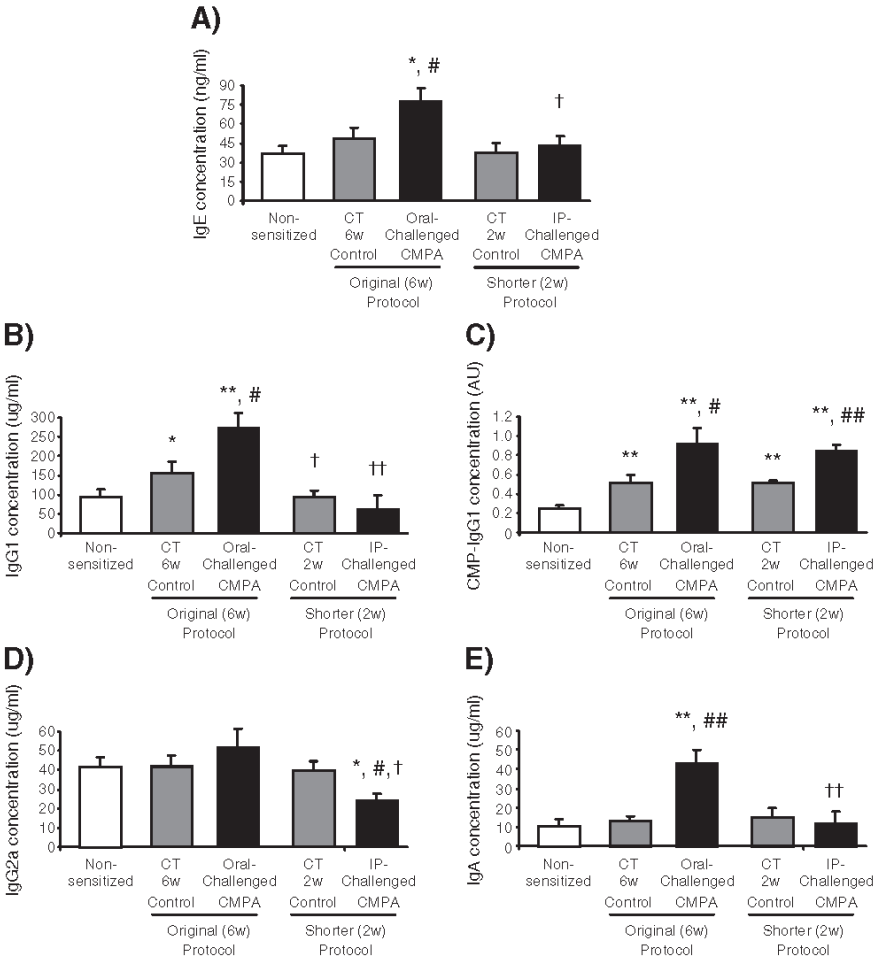

Fig. 4. Plasma Ig levels. Plasma from all mice was collected after sacrifice and used to measure IgE (A), IgG1 (B) CMP-specific IgG1 (C), IgG2a (D) and IgA (E) by ELISA as described in the Materials and methods section. Results are expressed as the mean plasma concentration ± SEM except for C) were CMP-specific IgG1 levels were represented as Absorbance units (AU) ± SEM. Student's *t*-test was used to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared with non-sensitized animals; # ($p<0.05$), ## ($p<0.01$) compared with each particular CT control; † ($p<0.05$), †† ($p<0.01$) compared with similar group (CMPA or CT control) between both protocols.

4. Discussion

Research into human pathology has been widely supported by laboratory data thanks to the use of diverse experimental animal models. Mouse and rat models have been used satisfactorily in studies of various allergic diseases, such as asthma (Bates et al., 2009; Kucharewicz et al., 2008; Stevenson and Birrell, 2011). However, no fully acceptable mouse model of CMPA or other food allergies has been established. As a result, studies use distinct animal models, thereby hindering the comparison of results. An experimental model of food allergy should be characterized by the robustness and reproducibility of the symptoms, the selectivity and antigen-specificity of the response, the involvement of the mucosal immune system but with systemic dispersion of the effects, and the easiness of the protocol, both regarding time and resources.

Several models of food allergy have been described, these differing in the route of sensitization and challenge, the strain of mouse used, the use of adjuvant or co-administered drugs, the type of antigen or the doses required and the duration of the sensitization period (Diesner et al., 2008; Ganeshan et al., 2009; Gonipeta et al., 2010; Hsieh et al., 2003; Strid and Strobel, 2005; Thang et al., 2011; Untersmayr et al., 2010; Valeur et al., 2009). However, for all these models the most significant obstacle to oral sensitization is the strong innate tendency to develop oral tolerance to ingested antigens. Consequently, long sensitization periods and the introduction of adjuvants such as CT are common practice (Lara-Villoslada et al., 2004, 2005; Li et al., 1999; Schouten et al., 2009).

Here we tested the immune consequences of this sensitization approach and compared it with the allergy response obtained with a novel shorter CMPA protocol, requiring only two weeks of sensitization. Our results suggest

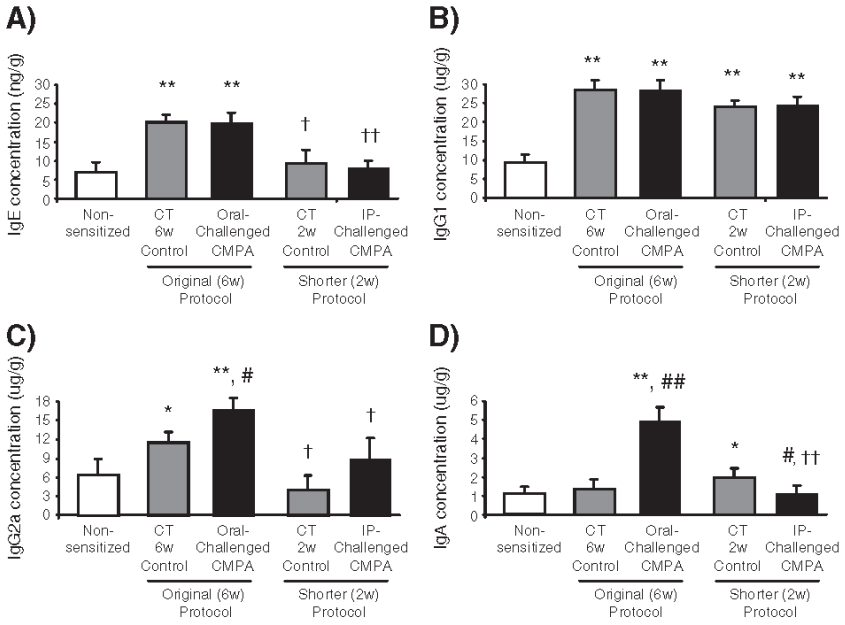

Fig. 5. Igs levels in colonic samples. Colon homogenates obtained from all mice were obtained after sacrifice and used to measure IgE (A), IgG1 (B), IgG2a (C) and IgA (D) by ELISA as described in the Materials and methods section. Results are expressed as the mean colonic concentration per gram of colon specimen ± SEM. Student's *t*-test was used to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared with non-sensitized animals; # ($p<0.05$), ## ($p<0.01$) compared with each particular CT control; † ($p<0.05$), †† ($p<0.01$) compared with similar group (CMPA or CT control) between the two protocols.

that the long-term use of CT rather than its dosage seems to be responsible for the unselective immune response induced by this adjuvant. In this regard, weekly administration of CT for 6 weeks without administration of CMP during sensitization led to a significant increase in several allergy response markers, such as diarrhea, colon edema, spleen enlargement, histamine levels, plasma and colon IL-4, plasma and colonic IgG1 or colonic IgE or IgG2a. Although CT stimulates a Th2 response and the production of IgG1 antibodies (reviewed by Berin and Shreffler, 2008), the mechanisms through which this adjuvant promotes immune responses remain controversial (Cox et al., 2006). Indeed, allergy-suppressing rather than stimulating effects have been described in mice through the induction of secretory IgA (Smits et al., 2009) or Th1-associated IgG2a responses (Grdic et al., 1999; Kroghsbo et al., 2003; Snider et al., 1994). Although CT treatment clearly increased IgG2a levels in colon, in no case was this related to a suppressive effect on CMPA, an effect previously observed in respiratory mucosa (Smits et al., 2009).

The reduced effect on the same parameters induced by 5 doses of CT administered in only 2 weeks (CT 2w Control in shorter protocol) suggests that the immune-enhancing effects of CT are not direct but related to the induction of an allergic response to other food antigens present in the diet, a process that would probably require more than 2 weeks of treatment to become evident. In this regard, oral challenge with CMP after 2 weeks of sensitization was not sufficient to induce an allergic response (unpublished data) and at least 5 weeks was required (Lara-Villoslada et al., 2004, 2005). Unfortunately, no samples were obtained to evaluate the immune status before CMP challenge nor was a third control group consisting of CT sensitization without challenge included in this study. Consequently, a potential effect of the oral challenge cannot be discarded on the final response observed in CT Control groups despite the fact that no effect was observed in non-sensitized animals. The reason we cannot discard this possibility is that oral challenge was performed using a considerable amount of protein and volume of administration that could induce mucosal distension (as observed in small intestine specimens from original protocol samples), thereby facilitating the extravasation of intact food-borne proteins in the gut that are not related to CMP. Moreover, the high amount of CMP during challenge could induce cross-reaction with other food antigens. In this regard, cross-reactions between caseins and soy-derived proteins have been reported in allergic individuals (Chandra et al., 1989; Hill et al., 1984; Perkkio et al., 1981). This cross-reactivity could explain the presence of a higher plasma level of CMP-specific IgG1 antibodies in 6w CT Control animals after challenge despite the fact that these mice did not ingest this antigen through diet.

The strong immune response observed in CT 6w Control animals does not preclude the fact that CMPA was successfully induced using the original protocol, as demonstrated by the fact that a trend to increase clinical score was observed in oral-challenged CMPA group and a significant increase was observed in parameters such as spleen size, plasma histamine and plasma Igs such as IgE, IgG1, IgA and CMP-specific IgG1 or colonic IgG2a and IgA. However, of note, the differences observed between the CT 6w Control group and the oral-challenged CMPA animals as a result of the immune-

enhancing potential of CT were reduced. Therefore, an increase in sample size of the oral-challenged CMPA animals is required to demonstrate significance. Moreover, the CT effect explains why no differences were observed in critical allergy markers such as the clinical score or IL-4 production in plasma and colon. Finally, our results suggest that the extended use of CT also induces some alterations – such as those detected in the intestine – that may modify the final outcome of CMPA. In this regard, we have previously described that the alternation in intestinal permeability induced during an active intestinal inflammation exacerbates the immune response against ingested food antigens (Cueto-Solà et al., 2010).

Our data demonstrate that the novel protocol has several advantages over published methods. In summary, it reduces the experimental time required by two thirds, CT Control groups do not interfere with the data interpretation, thereby allowing significance to be reached in key allergy parameters such as the clinical score or IL-4 levels, and the response obtained is more allergen-specific than that achieved with the original protocol, since it does not increase the total amount of Igs in plasma or colon, while clearly increasing with similar potency the amount of CMP-specific IgG1, a common occurrence in allergic response (Lara-Villoslada et al., 2004, 2005).

Despite these benefits, the short CMPA protocol described here also has some drawbacks, the first regarding the IP challenge required to induce a measurable response. Although the CMPA protocol could be considered a non-physiological route of antigen contact, it is the best option to internalize minimal amounts of antigen, thereby allowing the maintenance of a specific and selective response to the target antigen. In order to ensure internalization of minimal amounts of intact protein sufficient to induce the allergy response, oral challenge with huge amounts of protein is required (Lara-Villoslada et al., 2004; 2005; Thang et al., 2011). Alternatively, the use of inhibitors of acid secretion in the gut with the same objective (Diesner et al., 2008; Untersmayr et al., 2010) presents other drawbacks, such as the loss of selectivity in response to other food antigens. Moreover, no detectable increase in IgE levels in plasma or colon is detected, this Ig being a hallmark of allergy. However, the lack of IgE response against CMP and other food-borne antigens has been reported (Lara-Villoslada et al., 2004, 2005; Thang et al., 2011). In this regard, serum concentrations of allergen-induced IgE are highly dependent on the type of allergen administered and cow milk caseins do not appear to be effective at inducing IgE (Thang et al., 2011). This observation also suggests that the increase in IgE induced in the original protocol could be triggered by food antigens other than CMP. Finally, the short duration of the modified protocol and the IP challenge probably explain why no IgA response was induced in this protocol despite targeting intestinal mucosa. Nevertheless, the lack of IgA response could also be an advantage since the blocking effect of the Igs (Smits et al., 2009) is avoided in our model.

5. Conclusions

Current available experimental models of food allergy are highly time-consuming and the robustness and sensitivity of the effects induced by the food-borne antigen are sometimes diminished due to the immune-enhancing properties of the adjuvant required to overcome oral tolerance in mice. Here we present a novel CMPA model in mice that requires only 2 weeks of sensitization and that allows a more robust, specific and selective immune response to the target antigen. These characteristics thus favor the consistency of the results and a reduction in the number of animals required.

Acknowledgments

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References

Adel-Patient, K., Ah-Leung, S., Creminon, C., et al., 2005. Oral administration of recombinant *Lactococcus lactis* expressing bovine beta-lactoglobulin partially prevents mice from sensitization. Clin. Exp. Allergy 35, 539.
Bailon, E., Cueto-Sola, M., Utrilla, P., et al., 2011. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm. Bowel Dis. 17, 2087.
Bates, J.H., Rincon, M., Irvin, C.G., 2009. Animal models of asthma. Am. J. Physiol. Lung Cell. Mol. Physiol. 297, 401.
Berin, M.C., Shreffler, W.G., 2008. TH2 adjuvants: implications for food allergy. J. Allergy Clin. Immunol. 121, 1311.
Bjorksten, B., 2001. The epidemiology of food allergy. Curr. Opin. Allergy Clin. Immunol. 1, 225.
Broberg, A., Svensson, A., Borres, M.P., et al., 2000. Atopic dermatitis in 5–6-year-old Swedish children: cumulative incidence, point prevalence and severity scoring. Allergy 55, 1025.
Chandra, R.K., Puri, S., Hamed, A., 1989. Influence of maternal diet during lactation and use of formula feeds on development of atopic eczema in high risk infants. Br. J. Med. 299, 228.
Cox, E., Verdonck, F., Vanrompay, D., Goddeeris, B., 2006. Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa. Vet. Res. 37, 511.
Cueto-Solà, M., Bailón, E., Garrido-Mesa, N., et al., 2010. Active colitis exacerbates immune response to food antigens. Gut 59, A105s3.
Diesner, S.C., Knittelfelder, R., Krishnamurthy, D., et al., 2008. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. Immunol. Lett. 121, 45.
Ganeshan, K., Neilsen, C.V., Hadsaitong, A., et al., 2009. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. J. Allergy Clin. Immunol. 123, 231.
Garrido-Mesa, N., Camuesco, D., Arribas, B., et al., 2011. The intestinal anti-inflammatory effect of minocycline in experimental colitis involves both its immunomodulatory and antimicrobial properties. Pharmacol. Res. 63, 308.
Gonipeta, B., Parvataneni, S., Paruchuri, P., Gangur, V., 2010. Long-term characteristics of hazelnut allergy in an adjuvant-free mouse model. Int. Arch. Allergy Immunol. 152, 219.
Grdic, D., Smith, R., Donachie, A., et al., 1999. The mucosal adjuvant effects of cholera toxin and immune-stimulating complexes differ in their requirement for IL-12, indicating different pathways of action. Eur. J. Immunol. 29, 1774.
Hill, D.J., Ford, R.P., Shelton, M.J., et al., 1984. A study of 100 infants and young children with cow's milk allergy. Clin. Res. Allergy 2, 125.
Hsieh, K.Y., Hsu, C.I., Lin, J.Y., et al., 2003. Oral administration of an edible-mushroom-derived protein inhibits the development of food allergic reactions in mice. Clin. Exp. Allergy 33, 1595.
Ito, K., Inagaki-Ohara, K., Murosaki, S., et al., 1997. Murine model of IgE production with a predominant Th2-response by feeding protein antigen without adjuvant. Eur. J. Immunol. 27, 3427.
Kroghsbo, S., Christensen, H.R., Frokiaer, H., 2003. Experimental parameters differentially affects the humoral response of the cholera-toxin-based murine model of food allergy. Int. Arch. Allergy Immunol. 131, 256.
Kucharewicz, I., Bodzenta-Lukaszyk, A., Buczko, W., 2008. Experimental asthma in rats. Pharmacol. Rep. 60, 783.

Lara-Villoslada, F., Olivares, M., Jimenez, J., et al., 2004. Goat milk is less immunogenic than cow milk in a murine model of atopy. J. Pediatr. Gastroenterol. Nutr. 39, 354.
Lara-Villoslada, F., Olivares, M., Xaus, J., 2005. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J. Dairy Sci. 88, 1654.
Li, X.M., Schofield, B.H., Huang, C.K., et al., 1999. A murine model of IgE-mediated cow's milk hypersensitivity. J. Allergy Clin. Immunol. 103, 206.
Marinaro, M., Staats, H.F., Hiroi, T., et al., 1995. Mucosal adjuvant effects of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J. Immunol. 155, 4621.
Oehling, A., Resano, A., Sanz, M.L., et al., 1998. Importance of food allergy in atopic dermatitis. Allergy 53, 139.
Ozol, D., Mete, E., 2008. Asthma and food allergy. Curr. Opin. Pulm. Med. 14, 9.
Perkkio, M., Savilahti, E., Kuitunen, P., 1981. Morphometric and immunohistochemical study of jejunal biopsies from children with intestinal soy allergy. Eur. J. Pediatr. 137, 63.
Sampson, H.A., 1997. Food allergy. JAMA 278, 1888.
Sampson, H.A., 2004. Update on food allergy. J. Allergy Clin. Immunol. 113, 805.
Schouten, B., Van Esch, B.C.A.M., Hofman, G.A., et al., 2009. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. J. Nutr. 139, 1398.
Skripak, J.M., Matsui, E.C., Mudd, K., Wood, R.A., 2007. The natural history of IgE-mediated cow's milk allergy. J. Allergy Clin. Immunol. 120, 1172.
Smits, H.H., Gloudemans, A.K., Van Nierwegen, M., et al., 2009. Cholera toxin B suppresses allergic inflammation through the induction of secretory IgA. Mucosal Immunol. 2, 331.
Snider, D.P., Marshall, J.S., Perdue, M.H., Liang, H., 1994. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. J. Immunol. 153, 647.
Stevenson, C.S., Birrell, M.A., 2011. Moving towards a new generation of animal models for asthma and COPD with improved clinical relevance. Pharmacol. Ther. 130, 93.
Strid, J., Strobel, S., 2005. Skin barrier dysfunction and systemic sensitization to allergens through the skin. Curr. Drug Targets Inflamm. Allergy 4, 531.
Sun, S., Winship, T., Kuchan, M.J., 2001. Dietary ribonucleotides modulate type 1 and type 2 responses against OVA in young Balb/c mice. J. Nutr. 131, 1165.
Thang, C.L., Baurhoo, B., Boye, J.I., et al., 2011. Effects of *Lactobacillus rhamnosus* GG supplementation on cow's milk allergy in a mouse model. Allergy Asthma Clin. Immunol. 7, 20.
Untersmayr, E., Diesner, S.C., Oostingh, G.J., et al., 2010. Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. PLoS One 5, 14210.
Valeur, J., Lappalainen, J., Rita, H., et al., 2009. Food allergy alters jejunal circular muscle contractility and induces local inflammatory cytokine expression in a mouse model. BMC Gastroenterol. 9, 33.

Chapter 5

Active colitis exacerbates immune response to internalized food antigens in mice (IF:2.2; 95/137)

Original Paper

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Active Colitis Exacerbates Immune Response to Internalized Food Antigens in Mice

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Key Words
Intestinal inflammation · Colitis · Food allergy · Sensitization · Mucosal immunology

Abstract
Background: Previous studies have indicated that colitis increases intestinal permeability to food antigens. This condition also generates an immunoreactive milieu in the gut, which may exacerbate or counteract allergy reactions. This, along with the fact that both colitis and allergy are being co-diagnosed more frequently, means the scientific interest on the immune relation between these pathologies is increasing. We evaluated the immune response to an internalized food antigen that was initiated during a concomitant active intestinal inflammatory response. ***Methods:*** An ovalbumin (OVA)-induced immune response was analyzed in healthy mice and in mice suffering from colitis induced by the administration of dinitrofluorobenzene/dinitrosulfonic acid (DNFB/DNS) at the moment of OVA challenge. The OVA-induced clinical score and allergy response both in plasma and in splenocyte cultures from these animals were compared. ***Results:*** Although no differences were observed in the allergy clinical score, the concomitant active colitis led to an increase in the immune response to OVA antigen, as shown by increased spleen size and OVA-induced splenocyte proliferation, exacerbated expression of total and OVA-specific IgG1 levels, increased colonic IL-4 expression and OVA-induced IL-4 and IL-5 cytokine expression in spleen cells. ***Conclusions:*** Our results indicate that animals with active colitis undergo an exacerbated immune response to an internalized antigen. This finding could be relevant for the allergy management of patients presenting simultaneously with chronic colitis.

Introduction

Abnormal immunologic reactions against endogenous or exogenous antigens, infectious agents, intestinal microbiota or environmental factors contribute to the pathogenesis of several intestinal conditions, including inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) [1, 2]. Since 1961, when Truelove [3] proposed that ulcerative colitis (UC) was due to an abnormal response towards food allergens such as milk, the hypothesis that allergic reactions to diet antigens are

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involved in the etiology and pathogenesis of IBD has remained controversial [4–9]. In fact, practices based on the elimination of a particular food or the use of elemental diets are still common among many IBD and IBS patients, especially those with Crohn's disease (CD) [8–11].

Concerning these initial proposals, a strong association between allergy and UC has been consistently shown in several studies, despite the fact that a causative relation between food allergy and IBD has not been fully demonstrated to date. In this regard, the incidence of cow's milk protein allergy in IBD patients is higher than that observed in a healthy population (20.9% in UC and 8.5% in CD patients compared to 2.8% in the normal population) [12] or even higher and reaching 50% of prevalence in UC patients when referring to general allergic diseases not limited to cow's milk protein allergy [13].

The involvement of allergy as a cause or consequence of UC was further analyzed by D'Arienzo et al. [13]. They found a low rate of sensitization in UC patients before the appearance of clinical symptoms was demonstrated. This observation, together with the finding that the first appearance of intestinal symptoms in most UC patients occurs simultaneously with or precedes allergic symptoms [13], suggests that UC facilitates the onset of allergic symptoms in subjects sensitized to environmental antigens. Moreover, many patients with celiac disease are sensitized to many other food proteins. This observation may reflect either a hypersensitive immune status or an increase in sensitization because of enhanced gut permeability caused by the celiac disease [14]. Finally, another potential explanation for the priming capacity of colitis to cause food allergy could be related to bias in the immune response involved in these two pathologies. In this regard, UC and atopy share a Th2 profile [15, 16]. However, a Th1 component has been extensively described in intestinal inflammation, even in UC patients [17, 18]. This component would exert an opposite action, inhibiting the initiation of the humoral Th2 response induced by allergens.

Of concern is the fact that not only is the incidence of food allergies and colitis rising in the Western world [19, 20], but the age of onset is getting younger [21]. Thus the potential interaction and exacerbation of these two pathologies is of increasing interest. Food allergy is one of the major causes of hypersensitivity in children, affecting approximately 2% of the general population [22]. Allergenic food deprivation is currently the only approach available to combat such responses. This medical practice is especially relevant in patients codiagnosed with IBD, where a nutritional deficit of particular food components could be deleterious.

Experimental animal models are commonly used to simplify and study complex human pathologies such as allergy or colitis [23–33], and they allow the discovery of interactions and common elements of particular signaling events and molecular pathways. With this aim, we examined the exacerbating or inhibitory potential of active intestinal colitis on the allergic response to an internalized food antigen, using the dinitrofluorobenzene/dinitrosulfonic acid (DNFB/DNS) model of colitis [27] and a well-established model of allergy based on ovalbumin (OVA) intraperitoneal (IP) sensitization and challenge. The DNFB/DNS model in mice induces a transient colitis that extends for 3 days and is associated with a mild intestinal inflammation. The altered immune response induced in this model could be considered an unconventional Th2-biased response with an increase on IL-5, IL-10 and humoral response but also Th1 cytokines such as IFNγ, TNFα or IL-17 after subsequent DNS expositions, similarly to what have been described by other UC models such as dextran sodium sulfate-induced colitis in mice [27].

Our findings indicate that the altered immune response induced by DNFB/DNS during colitis is sufficient to exacerbate the allergic response generated against an internalized food antigen. This finding thus supports the notion that patients with colitis may have a more severe response to antigens to which they have previously been sensitized.

Material and Methods

Reagents

All chemicals were purchased from Sigma Chemical Co. (Madrid, Spain) unless otherwise stated. Antibodies for ELISA were purchased from Bethyl Laboratories (Montgomery, Tex., USA: immunoglobulins) or from Biosource (Camarillo, Calif., USA: cytokines). Bovine casein ELISA kit was obtained from Crystal Chem Inc. (Downers Grove, Ill., USA).

Animals

Female 8-week-old BALB/c mice were purchased from Harlan (Barcelona, Spain) and housed at 22°C with a 12-hour-light controlled cycle. They were kept on a casein-based chow diet (Harlan Laboratories, Indianapolis, Ind., USA) under specific-pathogen-free conditions. This study was carried out following the European Union Directive (86/609/EEC) for the protection of vertebrates used for experimental and other scientific purposes, in compliance with the Helsinki Declaration. Animal use was approved by the Animal Research Committee of the University of Granada (No. 2011-355-CEA).

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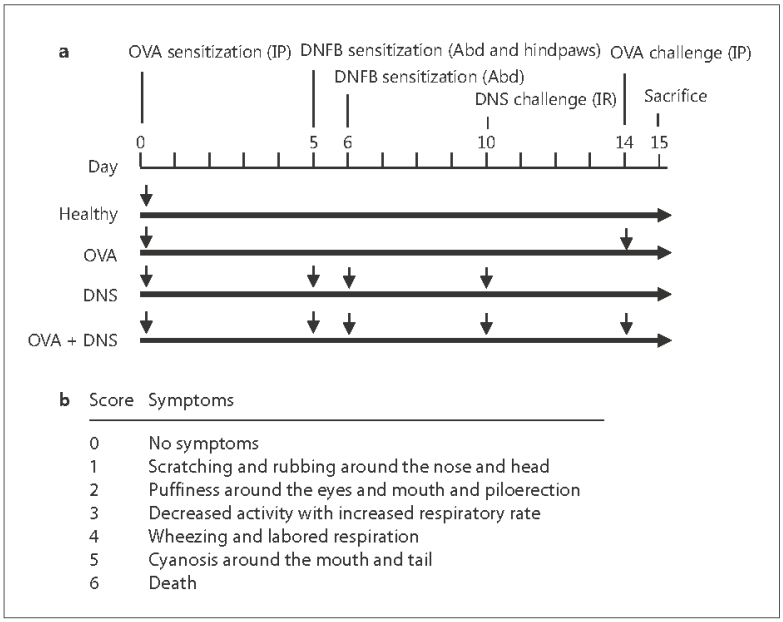

Fig. 1. a Protocol scheme. **b** Score of hypersensitivity symptoms. Thirty minutes after OVA challenge, the symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 6 (death) as shown and previously described [33]. Abd = Abdomen; IR = intrarectal administration.

Animal Models

After a 7-day acclimation period, the mice were weighed and randomly distributed into 4 experimental groups (healthy, OVA, DNS and OVA + DNS; n = 10 per group; fig. 1a).

OVA Sensitization

OVA sensitization was induced at day 0 in all groups by IP administration of 200 µl of a solution containing 1 mg of OVA II per mouse plus Imject Alum adjuvant [(40 mg/ml $Al(OH)_3$/40 mg/ml $Mg(OH)_2$); Pierce, Rockford, Ill., USA]. Control animals (i.e. the healthy group) did not receive any other treatment.

Colitis Induction (DNFB/DNS Model)

In 2 other groups (DNS and OVA + DNS), colitis was induced as previously described [27]. On day 5 (after OVA sensitization), mice were sensitized by epicutaneous administration of DNFB (0.6% in acetone-olive oil 4:1) on the shaved abdomen (50 µl) and on the paws (50 µl divided between the hindlegs). On day 6, the mice received a boost of DNFB only on the abdomen (50 µl). On day 10, animals from both groups were challenged intrarectally with 0.6% DNS dissolved in 10% ethanol. The challenge was conducted under light inhalation anesthesia (3% halothane).

OVA Challenge and Allergy Score

Finally, on day 14, the OVA and OVA + DNS groups were subjected to an IP challenge with OVA (1 mg/mouse plus Imject Alum adjuvant, as previously described).

Using a scoring system modified slightly from previous reports [28, 33], we evaluated hypersensitivity responses 30 min after OVA challenge (fig. 1b). An extra half point for each score was obtained when the symptom became visible at ≤10 min after the challenge. Symptom evaluation was performed by two independent observers who were blind to the treatments.

Blood and Tissue Sample Collection

Twenty-four hours after OVA challenge, all mice were sacrificed by means of a sodium pentobarbital overdose. Before death, the blood of each animal was collected in an EDTA tube by cardiac puncture and the plasma fraction was obtained by centrifugation and stored at –80°C until analysis. Once the mice were dead, the spleen and colon were removed. The spleen was weighed and reserved for splenocyte cell cultures, as previously described [30], while colon samples were placed on an ice-cold plate, cleaned of fat and mesentery and then blotted on filter paper [31]. Each colon specimen was weighed, and the length was measured under a constant load (2 g). Afterwards, the colon was opened longitudinally and evaluated for visible damage and the number of colonic patches, which appear as bulges in the tissue, was counted with the naked eye.

Myeloperoxidase Determination

Myeloperoxidase (MPO) activity in colon homogenates was measured following the technique described by Krawisz et al. [34]. The results were expressed as MPO units per gram of wet tissue. One unit of MPO activity was defined as that degrading 1 µmol of hydrogen peroxide per minute at 25°C.

Splenocyte Cultures

To obtain primary splenocytes, the removed spleens were homogenized in DMEM plus 1% penicillin/streptomycin. After centrifugation (1,500 rpm for 5 min) erythrocytes were lysed with buffer (1.7 M NH_4Cl, 0.12 M $KHCO_3$ and 9 mM EDTA) for 30 min at 4°C. Resting cells were counted by using a hemocytometer and

they were cultured in medium containing DMEM plus 10% FBS in order to perform proliferation and stimulation assays. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Splenocytes were cultured in 24-well plates and stimulated with Concanavalin A (ConA; 5 μg/ml), LPS (50 μg/ml) or OVA (100 μg/ml) for 48 or 96 h, and the supernatants were collected and frozen until ELISA analysis.

Proliferation Assay

Spleen-derived cells were cultured in 24-well plates (3×10^6 cells/ml) in 0.5 ml of media and stimulated with ConA (5 μg/ml), LPS (50 μg/ml) or OVA II (100 μg/ml) for 24 h. Cell proliferation was measured by ^{3}H-thymidine (ICN Pharmaceuticals Inc., Costa Mesa, Calif., USA) incorporation as previously described [30, 32].

Determination of Casein, Immunoglobulin and Cytokine Levels

Plasmatic and cell culture supernatant protein level measurements were performed by ELISA, following the manufacturer's recommendations. Blood was centrifuged (3,500 *g* for 10 min at 4°C), and plasma aliquots were collected and frozen at −80°C. No dilutions were required for the cytokine determinations of plasma and splenocyte culture supernatants. No plasma dilutions were performed for the analysis of casein levels. For immunoglobulin measurements, dilutions of 1:10,000 were performed for the determination of total IgG1, IgG2a and IgA in plasma, and 1:2 for IgE. Secondary antibodies (Bethyl Laboratories) were diluted 1:10,000 for total IgG1, IgG2a and IgA and 1:5,000 for IgE.

Levels of specific IgG1 to OVA were measured in nondiluted plasma. Plates (96-well) were coated with 20 μg/ml of OVA grade II in coating buffer (0.5 M Na_2CO_3). After overnight incubation at 4°C, plates were washed 3 times with wash solution (50 mM Tris, 0.14 M NaCl and 0.05% Tween 20) and blocked with 50 mM Tris, 0.14 M NaCl and 1% BSA. Plasma samples were then added to the plates and incubated for 1 h at room temperature (25°C). Plates were washed 3 times, and 100 μl of 1:1,000 diluted goat antimouse IgG1 antibody conjugated with peroxidase (Bethyl Laboratories) was added for 1 h at 25°C. Staining was performed with 3,3′-5,5′-tetramethyl-benzidine (TMB) for 30 min at 25°C in the dark and stopped with 0.1 M H_2SO_4. The plates were then read at 450 nm. All analyses were performed in triplicate.

Statistical Analysis

Statistical significance was calculated with the Student t test in the case of parametric parameters such as the ELISA results. All tests were performed with one tail following the 2-sample equal variance model (homoscedastic). For the hypersensitivity score, the Mann-Whitney U test was used to determine statistical significance. In all cases, data are represented as the mean ± SEM, and figures correspond to representative data from one of at least three independent experiments.

Results

The selection of the animal models to be used and the treatment protocol were the main concerns of the study (see Discussion). After several approaches, we selected the DNFB/DNS colitis model and the OVA-induced allergy model by IP administration. We considered these models highly suitable for the proposed goal of the study because both protocols have differentiated routes of allergen administration and an adequate duration (fig. 1a). Furthermore, both models induce only slight alterations on the physiology and behavior of the animals, while producing a clear effect on the immune system. Accordingly, no significant alterations in body weight were seen during the 10 days before colitis induction (challenge with DNS, day 10). DNS-induced colitis led to a trend to transient loss of or no gaining of body weight, this loss being more evident in concomitant allergic animals (OVA + DNS), reaching statistical significance when compared with healthy mice, despite the fact that the allergic process per se did not affect body weight in noncolitic animals (OVA) (fig. 2a). No difference in food intake or water consumption was observed among any of the groups. No differences were detected in the transient incidence of diarrhea/soft stools in either colitic group (data not shown) that could have been responsible for the body weight variations observed.

The IP challenge with OVA induced a fast and robust allergy response characterized by an initial intense scratching by the animals followed by a generally reduced activity, piloerection, wheezing and labored breathing. Moreover, almost one third of the animals showed cyanosis around the mouth and nose. Similar symptoms were observed in both allergy groups (fig. 2b), although a fast onset of the symptoms was observable in the animals suffering concomitant colitis.

The animals were sacrificed 24 h after OVA challenge (day 15), and intestinal and immunologic parameters were analyzed to evaluate the effects on colitis and allergy induced by the two hypersensitivity pathologies. As expected, major macroscopic alterations were not seen in gut parameters after single or combined DNS or OVA challenges after 5 days of the intrarectal DNS challenge (fig. 3a) nor in the number of visible Peyer's patches (0.8 ± 0.4, 1.4 ± 0.6, 2.2 ± 0.8 and 2.6 ± 1.1 in the healthy, OVA, DNS and OVA + DNS groups, respectively; not significant), except for a small intestine distension observed in groups subjected to a single challenge (fig. 3b). Moreover, no differences were observed in the colonic inflammatory infiltrate measured as MPO activity (fig. 3c) or in the gut permeability measured as detection of casein protein fragments from the diet in the plasma of animals from the different groups (7.58 ± 1.26, 9.06 ± 1.89, 8.72 ± 1.19 and 10.11 ± 2.25 pg/ml in the healthy, OVA, DNS and OVA + DNS groups, respectively; not significant).

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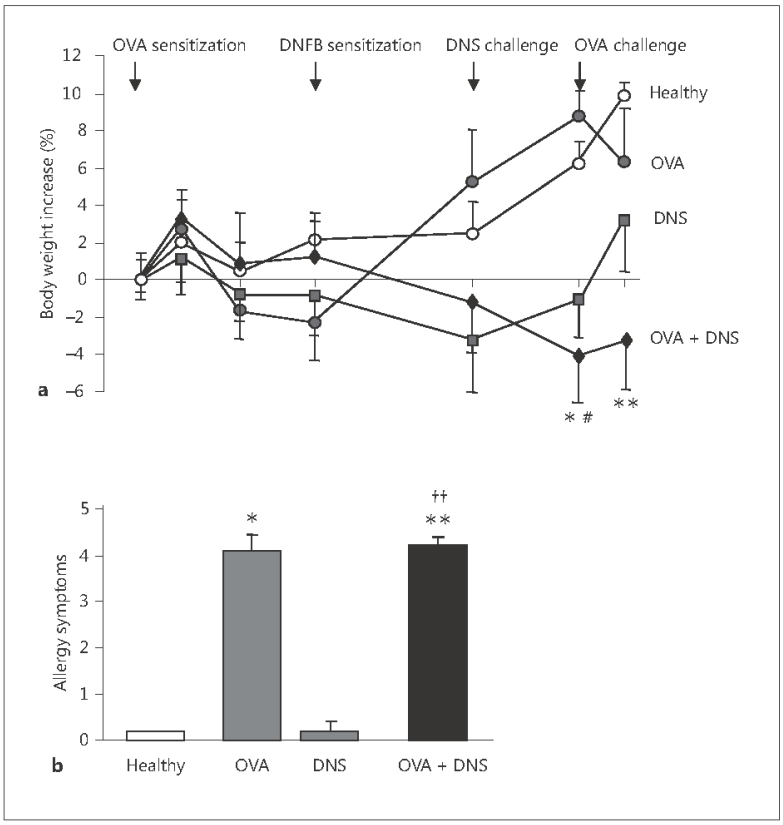

Fig. 2. No clinical exacerbation of allergy symptoms was observed in colitic or non-colitic animals. **a** Body weight modifications were recorded daily throughout the experiment and are represented as body weight increase (%) vs. animal weight at day zero. Data correspond to mean ± SEM. The Student t test was performed to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group. **b** Allergy symptoms were scored 30 min after OVA challenge following the scoring system shown in figure 1b. The Mann-Whitney U test was performed to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; †† $p < 0.01$, compared to the DNS group.

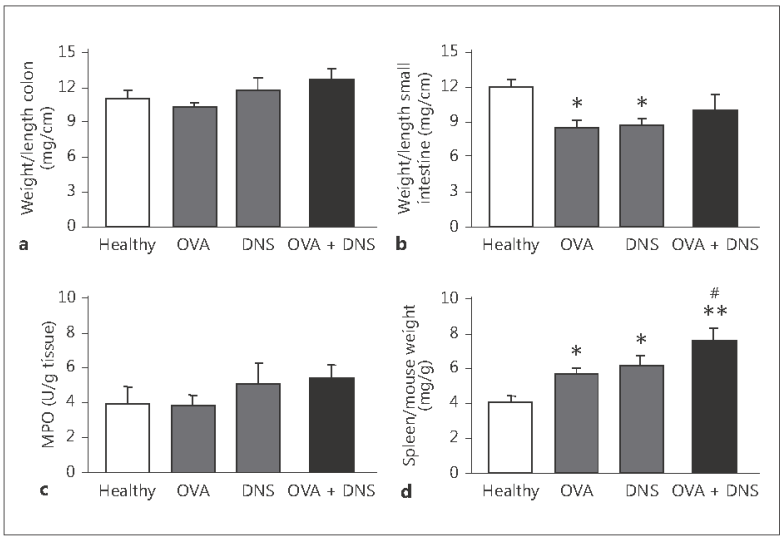

Fig. 3. Intestinal and macroscopic effects. Weight/length ratio of the colon (**a**) and small intestine (**b**), MPO activity in colon homogenates (**c**) and spleen weight (**d**) were measured 24 h after OVA challenge in all animals (as described in Material and Methods). All results are expressed as the mean ± SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group.

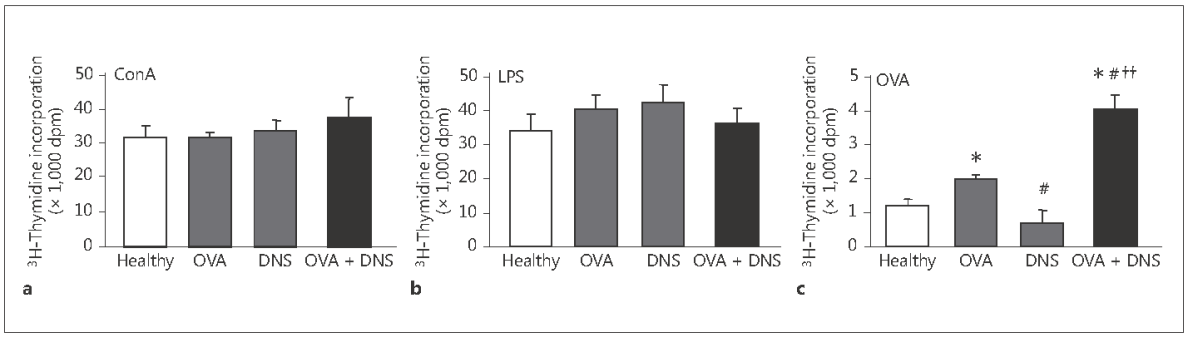

Fig. 4. OVA-induced lymphocyte proliferation was increased in allergic animals with concomitant colitis. Lymphocyte proliferation was measured by ^{3}H-thymidine incorporation in splenocytes obtained from all animal groups cultured in the presence of 5 μg/ml ConA (**a**) or 10 μg/ml LPS (**b**) for 48 h or in the presence of 100 μg/ml purified OVA grade II (Sigma) (**c**) for 96 h. Results are expressed as 1/1,000 of the mean dpm ± SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, compared to healthy animals; # $p < 0.05$, compared to the OVA group; †† $p < 0.01$, compared to the DNS group.

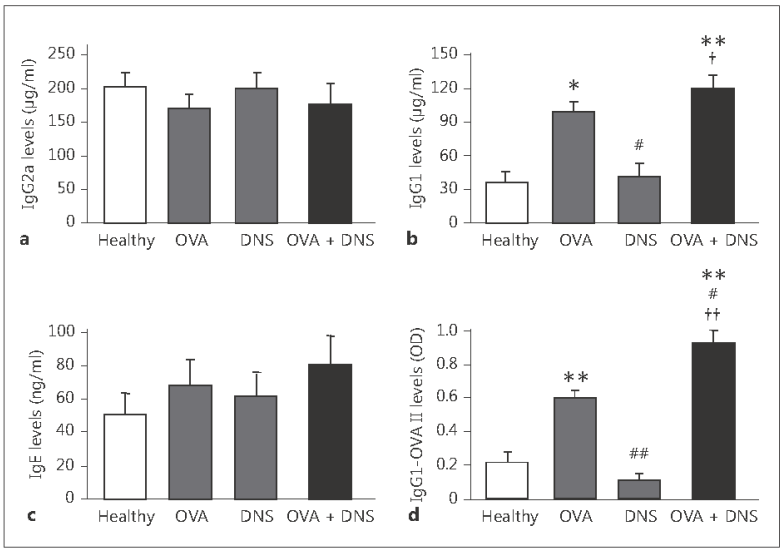

Fig. 5. Active colitis increased the plasma levels of total and OVA-specific IgG1 induced by OVA challenge. Plasma from all mice was collected after sacrifice and used to measure total IgG2a (**a**), total IgG1 (**b**), total IgE (**c**) or OVA-specific IgG1 (**d**) levels by ELISA (as described in Material and Methods). Results are expressed as the mean plasma concentration (**a–c**) or as total absorbance units (**d**) ± SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, ## $p < 0.01$, compared to the OVA group; † $p < 0.05$, †† $p < 0.01$, compared to the DNS group.

Despite the slight intestinal effects at the analyzed time point, the incidence of the two pathologies was observable thanks to alterations in the immune system. Accordingly, a significant enlargement of the spleen was observed after OVA or DNS challenge and was exacerbated by the concomitant pathologies (fig. 3d). This spleen enlargement was associated with an increase in the number of splenocytes (7.98 ± 1.1, 11.5 ± 0.9, 12.2 ± 0.9 and 11.1 ± 0.5 million cells/ml in the healthy, OVA, DNS and OVA + DNS groups, respectively). To analyze the immunoreactivity of the splenocytes, cell proliferation was evaluated by ^{3}H-thymidine incorporation after stimulation of cells with ConA (T cell stimulus), LPS (B cell stimulus) or OVA (antigen-specific lymphocytes) (fig. 4). The data obtained pointed to the high antigen selectivity of the OVA-induced allergy model, since no increased splenocyte proliferation was observed after general lymphocyte activators such as ConA or LPS (fig. 4a, b), while a sig-

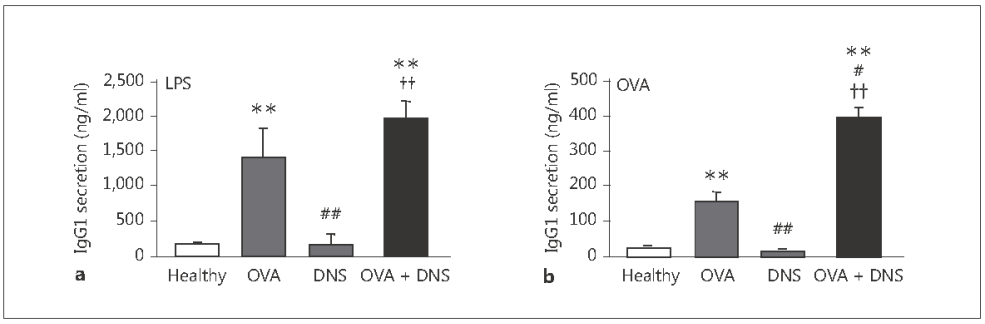

Fig. 6. Active colitis increased the secretion of total and OVA-specific IgG1 in splenocytes from concomitantly allergic animals. Lymphocyte immunoglobulin secretion was measured in splenocytes obtained from all animal groups cultured in presence of 10 μg/ml LPS (**a**) or 100 μg/ml purified OVA grade II (Sigma) for 96 h (**b**). Results are expressed as the mean concentration ± SEM. The Student t test was used to determine statistical significance. ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, ## $p < 0.01$, compared to the OVA group; †† $p < 0.01$, compared to the DNS group.

nificant increase in response was observed in the OVA group after the ex vivo stimulation with the OVA allergen (fig. 4c). Moreover, although no increased proliferation was seen in the DNS group subjected to any of the stimuli used, the colitic process induced by DNS duplicated the in vitro splenocyte response induced by OVA stimulation in colitic animals (DNS + OVA) (fig. 4c).

The exacerbation of the specific allergy response to OVA in animals suffering concomitant colitis was also observed when analyzing the humoral response. Accordingly, total IgG2a plasma levels of all treated groups resembled those of healthy animals (fig. 5a). Only differences in total IgG1 levels (fig. 5b), the main immunoglobulin that drives allergy in mouse models [28, 33], but not IgE (fig. 5c), were observed between treated groups, where increased levels were detected in both OVA-induced allergy groups compared to the 2 nonallergic groups (healthy and DNS-colitic). Again, the specificity of the response was demonstrated through the analysis of the OVA-specific IgG1 plasma levels, which increased 3-fold in the OVA group and even more in OVA + DNS animals (fig. 5d). Consistent with the immunoglobulin plasma levels, IgG1 secretion induced by LPS- or OVA-activated splenocytes was also increased in the allergy group and was exacerbated in allergic animals that also had colitis (fig. 6).

Finally, to further analyze the immune response induced, the cytokine response exerted in the two pathologies was also measured in the plasma and splenocytes. Although significant differences in plasma levels were not observed in any of the treatment groups (not shown) or in the supernatants of cultured splenocytes activated with ConA (table 1), a clear Th2-biased increase in cytokine response was detected in spleen T cells activated with OVA in the allergy groups. This response increased in magnitude in allergy animals with concomitant colitis (fig. 7). In this regard, the allergic process induced by OVA challenge in colitic animals significantly increased the secretion of IL-2, IL-4 and IL-5 in splenocytes. This observation suggests an increase in OVA-reactive lymphocyte clones in the spleen of these animals, a phenomenon that was not significant (although a trend was noted) in noncolitic allergy animals (fig. 7), which achieved significance only for IL-5 secretion.

Discussion

Intestinal hypersensitivity conditions such as IBD, IBS, celiac disease or food allergy are the result of interactions between immunologic, genetic and environmental factors. The coexistence of IBD and allergy has become more frequent in recent decades, and many studies indicate a connection between these two pathologies [4–13, 20–22]. At present, we do not know whether food sensitization is a risk for or a result of intestinal inflammation. However, what is clear is that almost one third of unselected patients with gastrointestinal inflammatory diseases report adverse reactions to food as a cause of their symptoms [35]. Food commonly identified by colitic patients as causing symptoms includes milk, peanuts, citrus fruits, wheat, eggs and fish, all of which are correlated

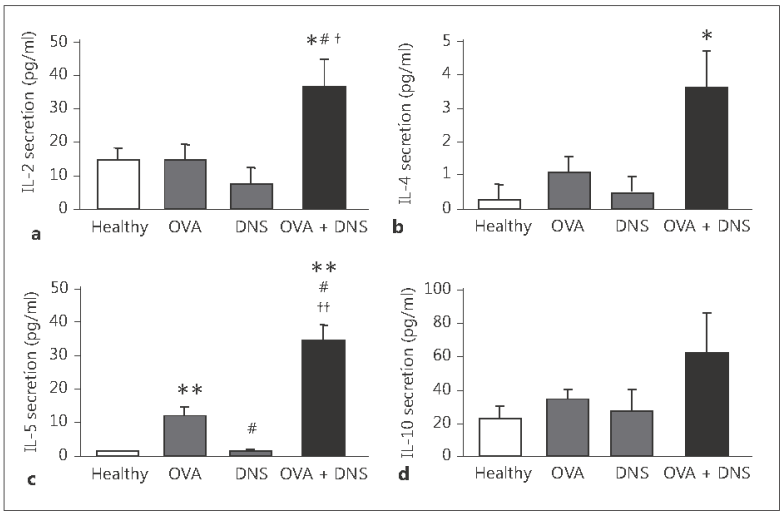

Fig. 7. Active colitis modified the cytokine profile induced in response to an internalized food allergen. Cytokine secretion was measured in splenocytes from all animal groups cultured in the presence of 100 µg/ml purified OVA grade II (Sigma) for 96 h. Cytokines were measured in the nondiluted culture supernatants by ELISA, following the manufacturer's instructions. Results are expressed as the mean concentration ± SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group; † $p < 0.05$, †† $p < 0.01$, compared to the DNS group.

Table 1. ConA-induced cytokine expression in spleen cells

	Healthy	OVA	DNS	OVA + DNS
IL-2	1,581.1±143.2	1,193.2±50.6	1,351.1±55.9	1,114.5±171.4
IL-4	594.8±137.2	570.0±90.4	493.4±101.4	299.6±38.5
IL-5	59.1±23.3	44.6±6.7	20.09±2.4	36.0±10.2
IL-10	539.9±45.9	523.9±64.7	664.9±43.4	507.1±10.8

Data correspond to cytokine levels in pg/ml from splenocyte cultures obtained from each animal (n = 10 per group) and are represented as the mean ± SEM. No statistical difference was observed in any of the comparisons.

with sensitization [9, 12, 36–38]. As a result, elimination diets or elementary diets have become commonplace for some colitic patients in recent decades [10, 39]. In this regard, more than 20% of patients in the active phase of UC [40] or CD [41] benefit from milk-free diets. Moreover, it should also be considered that food allergy may be present in patients who do not report adverse reactions to food, as they may not recognize that a certain food triggers their symptoms because the allergens are multiple or ubiquitous and therefore difficult to identify by experience [35]. Taking all these data together, there is a clear correlation between IBD and food allergy.

Since human pathological research has been widely supported by laboratory data thanks to the use of diverse experimental animal models, our objective was to analyze the relation between allergic response and colitis using simplified experimental models. For this purpose, our first and more complex task was to select the most suitable experimental models resembling the pathophysiological mechanism for the correlation between the two pathologies observed in humans. Three main interrelated mechanisms have been hypothesized that could be responsible for the association between colitis and allergy, namely increased intestinal permeability, antigen cross-reactivity and the existence of a biased immune response [4, 14, 34].

It is currently well accepted that alterations in the mucosal barrier and permeability may occur during intestinal inflammation, which could facilitate the entrance of antigens and, therefore, the onset of the hypersensitivity symptoms in subjects sensitized to environmental antigens involved in allergy and IBD, despite the fact that this hypothesis has not been confirmed [13, 14] and is even neglected [42] in some studies. However, mucosal barrier

disruption in experimental models is highly variable and leads to the entrance of several food and gut antigens that will finally cause an unspecific immune response, thus leading to a misleading evaluation of the results obtained. Therefore, to achieve the main desired characteristics for any experimental model, i.e. the robustness and reproducibility of the data obtained, we made the compromise of eliminating the altered intestinal permeability from the equation, despite being aware that by doing so we are limiting the similarities to the complex human situation. Based on this, we selected the DNFB/DNS colitis model because it induces mild inflammation that does not alter mucosal integrity [27], confirmed by the fact that no significant differences were observed in gut permeability to intact proteins. Moreover, the selection of a modified IP OVA-induced allergy model [43] allows the evaluation of an allergic response caused by a specific internalized antigen at the desired concentration, thus reducing the variability and lack of specificity of the induced response.

On the other hand, the cross-reactivity of antibodies against allergens and antibodies related to intestinal inflammation, caused by an exacerbated response to similar mucosal antigens, has also been postulated to be responsible for the association between the two pathologies. This cross-reactivity has been found between some inhalant and food antigens involved in colitis, such as Betv1, Mald1 [14, 44], and between food antigens and autoantigens involved in other autoimmune diseases such as type I diabetes [45]. In this regard, the selection of DNFB/DNS and OVA antigens in our mouse models, the controlled and casein-based diet used and the absence of alteration of intestinal integrity greatly reduce the likelihood of cross-reactivity between DNS-modified antigens and OVA. Accordingly, our results demonstrate that the observed exacerbation of the allergic response was specific for OVA and did not result from a general upregulation or hyperreactivity of the systemic immune system in the colitic animals. Due to this, no increase was observed in total immunoglobulins (except IgG1) and the increased immune response in splenocytes (increased production of IgG1, IL-4 and IL-5) was observable only after splenocyte stimulation with purified OVA but not with general stimuli.

Therefore, our data suggest that the main driver of the exacerbated allergic response observed in the colitic animals was due to the immune modulation resulting during the intestinal inflammation induced by the DNFB/DNS model. In this regard, an increased allergy response to OVA due to a biased Th2 environment induced by haptens such as TNBS has been previously described [46]. However, it should be noted that the immune exacerbated response observed was not due to an additive effect of the immune mediator levels induced during colitis to those at the time of OVA challenge, as the immune response induced by the DNFB/DNS model was mild and returned to basal levels after the first 3 days [27] – shown by the poor altered immune response observed in the DNS group. This was also a reason to select the DNFB/DNS model in Balb/c mice rather than use other haptens or mouse strains with an increased immune response that could interfere with the evaluation and interpretation of the results observed.

In this regard, the colitic process and the biased immune response originating during the DNFB/DNS sensitization/challenge period may have produced transitory immune factors or stimuli that reinforce the maintenance or perpetuation of the OVA-specific clones generated during OVA sensitization. Several factors have been described as participating in the maintenance and perpetuation of memory B cells such as IL-4, B-cell activating factor, nerve growth factor [47, 48] or even IL-18 [49], an inflammatory cytokine that also plays a critical role in mucosal repair and homeostasis. Moreover, the Th2 cytokines and mediators produced locally in high amounts during colitis, namely IL-3, IL-5 and GM-CSF (by eosinophils) and SCF or IL-4 (by mast cells), are of major importance not only for the development and recruitment of allergy effector cells but also for the enhancement of their functional activity and for increasing mediator release in response to classical triggering agents [14]. Accordingly, IgE-mediated food allergy is more frequently described in UC [13, 37, 50] than in CD [51, 52] in relation to the more Th2-biased pathomechanism of UC. In support of this notion, an increased in vitro response to OVA stimulation, but not to other stimuli, was observed in splenocytes obtained from the OVA + DNS group in comparison to all the other groups. Moreover, the involvement of the acquired immune system in the exacerbated response was also evidenced by the enlargement of the spleen and an increase in IL-2 expression.

Taken together, our results clearly demonstrate that an exacerbated immune response to internalized OVA antigen occurs in animals with active colitis; this was the main objective of the study. However, experimental models are not always fully satisfactory, and this one is not an exemption. The IP OVA model induced a robust allergy symptomatology that impeded the detection of clinical differences between the colitic and noncolitic groups. Moreover, we observed a distension of the small intestine in both the DNS and OVA groups. Although this feature did not modify the gut permeability of the animals and has

also been observed in other intestinal hypersensitivity models [27, 29], the etiology of this phenomenon remains unknown. Finally, a poor systemic immune response, i.e. no changes in levels of plasma cytokines or total IgE antibodies, was observed in our models, despite the fact that these molecules are common markers of human allergy. Nevertheless, the lack of systemic cytokine disturbances and the non-IgE IgG1-biased response are common features of several allergy models [24, 25, 27–29].

The observations presented in this work might be relevant for subjects codiagnosed with colitis and food allergy who present with a highly reactive immune response and increased gut permeability. More studies are required to evaluate the potential effect of colitis on the sensitization risk to new antigens. In this regard, an increased control of allergy reactivity and the implantation of strategies to avoid or reduce the increased risk of anaphylaxis in these subjects should be explored.

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References

1 Scharl M, Rogler G: Inflammatory bowel disease pathogenesis: what is new? Curr Opin Gastroenterol 2012;28:301–309.
2 Renz H, Brandzaeg P, Hornef M: The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol 2012;12:9–23.
3 Truelove SC: Ulcerative colitis provoked by milk. Br Med J 1961;1061:154–160.
4 Zwetchkenbaum JF, Burakoff R: Food allergy and the irritable bowel syndrome. Am J Gastroenterol 1988;83:901–904.
5 Knoflach P, Park BH, Cunningham R, Weisser MM, Albini B: Serum antibodies to cow's milk proteins in ulcerative colitis and Crohn's disease. Gastroenterol 1987;92:479–485.
6 Rosekrans PCM, Meijer CJLM, van der Wal AM, Lindeman J: Allergic proctitis, a clinical and immunological entity. Gut 1980;21:1017–1023.
7 Jenkins HR, Pincott JR, Soothill JF, Milla PJ, Harries JT: Food allergy: the major cause of infantile colitis. Arch Dis Child 1984;59:326–329.
8 Farah DA, Calder I, Benson L, Mackenzie JF: Specific food intolerance: its place as a cause of gastrointestinal symptoms. Gut 1985;26:164–168.
9 Pearson M, Teahon K, Levi AJ, Bjarnason I: Food intolerance and Crohn's disease. Gut 1993;34:783–787.
10 Rajendran N, Kumar D: Role of diet in the management of inflammatory bowel disease. World J Gastroenterol 2010;16:1442–1448.
11 Borrelli O, Cordischi L, Cirulli M, et al: Polymeric diet alone versus corticosteroids in the treatment of active paediatric Crohn's disease: a randomized controlled open-label trial. Clin Gastroenterol Hepatol 2006;4:744–753.
12 Galssman MS, Newman LJ, Berezin S, Gryboski JD: Cow's milk protein sensitivity during infancy in patients with inflammatory bowel disease. Am J Gastroenterol 1990;85:838–840.
13 D'Arienzo A, Manguso F, Astarita C, et al: Allergy and mucosal eosinophil infiltrate in ulcerative colitis. Scand J Gastroenterol 2000;6:624–631.
14 Bischoff SC, Mayer JH, Manns MP: Allergy and the gut. Int Arch Allergy Immunol 2000;121:270–283.
15 Pallone F, Monteleone G: Interleukin 12 and Th1 responses in inflammatory bowel disease. Gut 1998;48:735–736.
16 Del Prette G: Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. Allergy 1992;47:450–455.
17 Strober W, Fuss IJ: Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterol 2011;140:1756–1767.
18 Raza A, Yousaf W, Gianella R, Shata MT: Th17 cells: interactions with predisposing factors in the immunopathogenesis of inflammatory bowel disease. Expert Rev Clin Immunol 2012;8:161–168.
19 Ngo P, Furuta G, Burks W: The pathobiology of eosinophilic gastroenteritis of childhood: is it really the eosinophil, allergic mediated, or something else? Curr Gastroenterol Rep 2004;6:436–440.
20 Molodecky NA, Soon IS, Rabi DM, et al: Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterol 2012;142:46–54.
21 Ruemmele FM: Pediatric inflammatory bowel diseases: coming of age. Curr Opin Gastroenterol 2010;26:332–336.
22 Hill DJ, Hosking CS: Emerging disease profiles in infants and young children with food allergy. Pediatr Allergy Immunol 1997;10:21–28.
23 Takeda G, Gelfand EW: Mouse models of allergy diseases. Curr Opin Immunol 2009;21:660–665.
24 Dearman RJ, Kimber I: Animal models of protein allerginicity: potential benefits, pitfalls and challenges. Clin Exp Allergy 2009;39:458–468.
25 Gálvez J: Experimental models of inflammatory bowel disease in rodents; in Peppelenbosch MP, Comalada M (eds): Preclinical Research into Crohn's disease: A Practical Guide. Kerala, Transworld Research Network, 2009, pp 1–27.
26 Mizoguchi A, Mizoguchi E: Animal models of IBD: linkage to human disease. Curr Opin Pharmacol 2010;10:578–587.
27 Bailon E, Cueto-Sola M, Utrilla P, et al: DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis 2011;17:2087–2101.
28 Lara-Villoslada F, Olivares M, Jimenez J, Boza J, Xaus J: Goat milk is less immunogenic than cow milk in a murine model of atopy. J Pediatr Gastroenterol Nutr 2004;39:354–360.
29 Bailon E, Cueto-Sola M, Utrilla P, et al: A shorter and more specific oral sensitization-based experimental model of food allergy in mice. J Immunol Methods 2012;381:41–49.
30 Bailón E, Camuesco D, Nieto A, et al: The intestinal anti-inflammatory effects of the novel agent UR-1505 in the TNBS model of rat colitis are mediated by T-lymphocyte inhibition. Biochem Pharmacol 2007;74:1496–1506.
31 Camuesco D, Comalada M, Concha A, et al: Intestinal anti-inflammatory activity of combined quercitrin and dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, in rats with DSS-induced colitis. Clin Nutr 2006;25:466–476.

32 Sierra S, Lara-Villoslada F, Comalada M, Olivares M, Xaus J: Dietary fish oil n-3 fatty acids increase regulatory cytokine production and exert anti-inflammatory effects in two murine models of inflammation. Lipids 2006;41:1115–1125.
33 Lara-Villoslada F, Olivares M, Xaus J: The balance between caseins and whey proteins in cow's milk determines its allergenicity. J Dairy Sci 2005;88:1654–1660.
34 Krawisz JE, Sharon P, Stenson WF: Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. Gastroenterol 1984;87:1344–1350.
35 Bischoff SC, Herrmann A, Mayer J, Manns MP: Food allergy in patients with gastrointestinal disease. Monogr Allergy 1996;32:130–142.
36 van den Bogaerde J, Kamm MA, Knight SC: Immune sensitization to food, yeast and bacteria in Crohn's disease. Aliment Pharmacol Ther 2001;15:1647–1653.
37 Grzybowska-Chlebowczyk U, Wos H, Sieron AL, et al: Serologic investigations in children with inflammatory bowel disease and food allergy. Mediators Inflamm 2009;2009:512695.
38 Mishkin S: Dairy sensitivity, lactose malabsorption, and elimination diets in inflammatory bowel disease. Am J Clin Nutr 1997;65:567–567.
39 Jones VA, Workman E, Freeman AH, et al: Crohn's disease: maintenance of remission by diet. Lancet 1985;22:177–180.
40 Wright R, Truelove SC: A controlled therapeutic trial of various diets in ulcerative colitis. Br Med J 1965;22:138–141.
41 Greenberg GR: Nutritional management of inflammatory bowel disease. Semin Gastroenterol 1993;4:69–86.
42 Huber A, Genser D, Spitzauer S, Scheiner O, Jensen-Jarolim E: IgE/anti-IgE immune complexes in sera from patients with Crohn's disease do not contain food-specific IgE. Int Arch Allergy Immunol 1998;115:67–72.
43 Iliev ID, Tohno M, Kurosaki D, et al: Immunostimulatory oligodeoxynucleotide containing TTTCGTTT motif from *Lactobacillus rhamnosus* GG DNA potentially suppresses OVA-specific IgE production in mice. Scand J Immunol 2008;67:370–376.
44 Ebner C, Hirschwehr R, Bauer L, et al: Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v1 and Bet v2 (birch profilin). J Allergy Clin Immunol 1995;95:962–969.
45 Scott FW, Norris JM, Kolb H: Milk and type I diabetes. Diabetes Care 1996;19:379–383.
46 Liu ZQ, Zheng PY, Yang PC: Hapten facilitates food allergen-related intestinal hypersensitivity. Am J Med Sci 2013;345:375–379.
47 Mackay F, Schneider P, Rennert P, Browning J: BAFF and APRIL: a tutorial on B cell survival. Ann Rev Immunol 2002;21:231–264.
48 Linker R, Gold R, Luhder F: Function of neurotrophic factors beyond the nervous system: inflammation and autoimmune demyelination. Crit Rev Immunol 2009;29:43–68.
49 Airoldi I, Raffaghello L, Cocco C, et al: Heterogeneous expression of interleukin-18 and its receptor in B-cell lymphoproliferative disorders deriving from naïve, germinal center, and memory B lymphocytes. Clin Cancer Res 2004;10:144–154.
50 van den Bogaerde J, Cahill J, Emmanuel AV, et al: Gut mucosal response to food antigens in Crohn's disease. Alimen Pharmacol Ther 2002;16:1903–1915.
51 Frieri M, Claus M, Boris M, et al: Preliminary investigation on humoral and cellular immune responses to selected food proteins in patients with Crohn's disease. Ann Allergy 1990;64:345–351.
52 Young CA, Sonnenberg A, Burns EA: Lymphocyte proliferation response to baker's yeast in Crohn's disease. Digestion 1994;55:40–43.

Chapter 6

Atopic mice show an increased or reduced immune response during colitis depending on the animal model used

Atopic mice show an increased or reduced immune response during colitis depending on the animal model used.

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***Running title*:** Atopy modulates intestinal inflammation

Keywords: mouse experimental models; atopy; food allergy; inflammatory bowel disease; intestinal inflammation; intestinal hypersensitivity.

Abbreviations: CMP, cow's milk protein; CMPA, cow's milk protein allergy; CT, cholera toxin; DNFB, dinitrofluorobenzene; DNS, dinitrosulfonic acid; DSS, dextran sodium sulfate; IP, intraperitoneal

ABSTRACT

Colitis is one of the most common gastrointestinal ailments among those seeking health care for gastrointestinal disorders, although the potential mechanisms underlying this pathology are multiple, including inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS). In this regard, an increase on permeability and an exacerbated immune reactivity have been proposed as common potential initiator elements in both colitic processes. Food allergy shares some aspects with colitis, and therefore it has been hypothesized that an increased prevalence of colitic disorders could be observed in atopic individuals.

To experimentally test this hypothesis we have generated a mouse model of atopy and we have assayed the immune response generated in these atopic mice after induction of two models of colitis, a DNFB/DNS-colitis model more resembling IBS and a DSS-colitis model closer to human IBD. The obtained results show that the atopic condition generated in mice modulates both intestinal alterations in opposite ways. Atopy partially protects animals from DSS-induced inflammation while exacerbates the immune response induced by DNFB/DNS administration. The positive effect of atopy on DSS-colitis is mainly shown through the attenuation of the cellular component and inflammation process (disease activity index, inflammatory infiltrate or intestinal oedema) while in DNFB-DNS colitis the main altered component is the Th2 humoral response.

In conclusion, experimental models suggest that the altered immune response and intestinal permeability observed in atopic individuals could be a predisposing factor for IBS, although at the same time the Th2 biased environment observed in atopic animals could partly protect from intestinal inflammation or colitis mainly driven by a cellular or Th1 immune response.

1. Introduction

Food allergy is an immune-mediated adverse reaction to food mainly produced by IgE-mediated reactions and occurs in 5–10% of children until 2 years of age. The most common food allergy is to cow milk proteins (CMP), an allergy that affects between 2-6% of children under 2 years of age and is still increasing nowadays (Hill, 1996). The majority of children allergic to milk or egg will outgrow their food allergies, but in contrast peanut, tree nut, fish and shellfish allergies are commonly lifelong. Allergenic food deprivation is currently the only approach available to combat such responses. However, due to the previous exposure to the diet antigen a sporadic allergic process is enough to lead to the breakdown of mucosal tissue homeostasis, which can persist during all life. This fact acquires a high importance in the perinatal period in which the establishment of immune tolerance in the mucosa takes place (Custovic et al., 2013; Peters et al., 2013).

In this regard, the importance of cow's milk protein allergy (CMPA) is neither due to the high incidence nor to the symptoms. Indeed, it can cause death by anaphylactic shock. The relevance of this allergy is because it triggers the so-called "atopic career", which has been associated with an increased risk to acquire other diseases such as asthma, rhinitis or atopic dermatitis in adult age (Broberg et al., 2000; Oehlling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007). Thus, it is not strange to think the hypothesis that allergic reactions to diet antigens can be involved in the etiology and pathogenesis of different gut pathologies, such as IBD or IBS. In fact, the coexistence of intestinal pathologies and food allergy has become more frequent in recent decades, and many studies indicate a connection between these two pathologies

(Borrelli et al., 2006; D'Arienzo et al., 2000; Farah et al., 1985; Galssman et al., 1990; Jenkins et al., 1984; Knoflach et al., 1987; Pearson et al., 1993; Rajendran and Kumar, 2010; Rosekrans et al., 1980; Zwetchkenbaum and Burakoff, 1988).

In this sense, a strong association between allergy and IBD has been consistently shown in several studies, despite this fact, a causative relation between food allergy and IBD has not been fully demonstrated to date (D'Arienzo et al., 2000; Galssman et al., 1990). Similarly, most patients with IBS also believe that diet plays a significant role in inducing IBS symptoms and they desire to know what foods to avoid because they report precipitation of symptoms on food ingestion (Morcos et al., 2009). However, although patients with IBS self-report food allergies more often than the general population (Eswaran et al., 2011), there is no consistent evidence of the fact that IBS patients suffer from food allergy, or that food intolerance plays a role in IBS symptoms (El-Salhy et al., 2012). Controversial studies have emerged regarding the potential correlation between allergy and pathogenesis of both IBD and IBS, and therefore the demonstration that an increased risk to suffer colitis in atopic individuals is still an unmet medical need in order to design better preventive or prophylactic strategies in those infants that suffer from CMPA in early stages of their life.

Experimental animal models are commonly used to simplify and study complex human pathologies such allergy, IBS or IBD (Bailon et al., 2011; Dearman and Kimber, 2009; Galvez, 2009; Mizoguchi and Mizoguchi, 2010; Takeda and Gelfand, 2009) and they allow the discovery of interactions and common elements of particular signalling events and molecular pathways. Moreover, they represent the easiest way to demonstrate complicated hypothesis. For example, in previous works we have used improved animal models for food allergy and colitis to demonstrate that an active colitis

process exacerbates the immune response against an internalized antigen (Cueto-Sola et al., 2013). Although several models of food allergy are currently available (Bailon et al., 2012; Diesner et al., 2008; Ganeshan et al., 2009; Gonipeta et al., 2010; Hsieh et al., 2003; Lara-Villoslada et al., 2004; 2005; Strid and Strobel, 2005; Thang et al., 2011; Untersmayr et al., 2010; Valeur et al., 2009), to our knowledge none of them have explored the atopic condition or the alteration of the remaining immune response several weeks after an allergic episode. With this aim, and using combinations of animal colitic models, we have optimized a new protocol for atopy adulthood individuals and we have demonstrated that infant atopy can modify some immune-related pathologies during adult life. Our findings indicate that the altered immune response by CMPA in atopic mice is sufficient to reduce the DSS-induced colitis but increase the DNFB/DNS-colitis in adult mice; demonstrating once again that when using combinations of animal models, scientist must be careful in the obtained conclusions because the results can totally change regarding the animal model that have been used.

2. Materials and methods

2.1. Reagents and preparation of CMP

All chemicals were purchased from Sigma Chemical Co (Madrid, Spain) unless otherwise stated. Antibodies for immunoglobulins ELISA were purchased from Bethyl Laboratories (Montgomery, TX).

To obtain CMP, homogenized fat free cow's milk was obtained from Puleva Food SA (Granada, Spain) and centrifuged at 5000 x *g* for 10 min at 4ºC and the upper layer of fat was discarded to obtain skimmed milk, which was stored at -20ºC in 1-mL aliquots and defrosted immediately before use. Protein content in cow's milk was measured by the Kjeldahl method, as previously described (Lara-Villoslada et al., 2004).

2.2. Animals

Female Balb/c mice (3-week-old, immediately after weaning) were purchased from Harlan (Barcelona, Spain) and housed under a temperature- (22°C) and light- (12 h) controlled cycle. Animals were maintained on a plant-based chow diet (Harlan Laboratories, Indianapolis, IN) under specific-pathogen-free conditions. This study was carried out following the European Union Directive (86/609/EEC) for the protection of vertebrates used for experimental and other scientific purposes, in compliance with the Helsinki Declaration.

2.3. Induction of allergy and atopic animals to CMP

The shorter protocol of acute allergy to cow's milk protein (CMP) in mice was developed as previously described with minor modifications (Bailon et al., 2012). Mice were sensitized intragastrically with CMP (1 mg of CMP per gram of body weight) plus cholera toxin (CT) (0.3 μg/g) as an adjuvant in a total volume of 200 μL, and boosted 5 times at a three to four days interval. Oral gavage was performed using a polyvinyl chloride tube-feeding catheter purchased from Vygon (Ecoue, France). The different additional control groups were evaluated previously (Bailon et al., 2012). Eighteen days after the first oral gavage, mice were challenged intraperitoneally (IP) with a 1 mg dose of CMP (100 μl).

Immediately after last challenge, hypersensitivity responses were evaluated after 30 min of CMP challenge. Then, after 24 hours (acute allergic mice) or 16 days (atopic mice), mice were killed by IP administration of sodium pentothal (50 mg/kg) and allergic process was evaluated. The presented values of healthy animals correspond to both control animals groups sacrificed at day 19 (n=8) and 35 (n=8) after first oral gavage.

2.3.1. Evaluation of symptoms

Hypersensitivity responses (day 18) were evaluated using a scoring system slightly modified from previous reports (Lara-Villoslada et al., 2004; 2005) and scored as shown previously (Bailon et al., 2012). An extra half-point for each score was obtained when the symptom became visible at 10 min or less after the challenge. Symptom evaluation was performed by 2 independent researchers who were blinded to the study treatments.

Blood was collected by cardiac puncture in tubes containing EDTA (Ethylene diamine-tetra-acetic). The spleen, small intestine and colon were removed from each mouse. The spleen and the small intestine were weighed and discarded, while colon samples were placed on an ice-cold plate, cleaned of fat and mesentery, and then blotted on filter paper. Each colon specimen was weighed, and the length was measured under a constant load (2g). Afterwards, the colon was opened longitudinally and scored for macroscopically visible damage.The number of colonic patches, which appear as bulges in the tissue, were counted with the naked eye. Then, colonic tissue was minced and aliquoted to be used for biochemical determinations or for Ig measurement.

2.4. Induction of colitis

2.4.1. Dextran sodium sulphate (DSS) model of mouse colitis

In a second experiment, healthy or atopic female Balb/c mice were randomly assigned to two different groups: non-atopic or naïve mice (n=24) and atopic animals (n=24). In each group, colitis was induced by adding DSS (36-50 KDa, MP Biomedicals, Ontario) in the drinking water at a concentration of 1,5% (moderate DSS, n=8) or 3.5% (severe DSS, n=8) for a period of 5 days, and then DSS was removed. The non-colitic animals (control) from naïve mice or atopic mice groups (n=8) were administered tap water instead of DSS during all the experiment. Consumption of food and water, animal body weight, the presence of gross blood in the faeces, and stool consistency were recorded daily. These parameters were each assigned to a score, which was used to calculate an average daily disease activity index (DAI) for each animal as previously described (Bailon et al., 2007; Camuesco et al., 2006).

Before death, blood of each animal was collected in an EDTA tube by cardiac puncture and the plasma fraction was obtained by centrifugation and stored at -80ºC until analysis. Once the mice were dead, the spleen, small intestine and colon samples were removed and analyzed as mentioned before.

2.4.2. DNFB/DNS hapten-induced colitis in mice

In a third experiment, healthy or atopic female Balb/c mice were randomly assigned to two different groups: non-atopic or naïve mice (n=24) and atopic animals (n=24). Colitis was induced as previously described (Bailon et al., 2012). On day 0, mice were sensitized by epicutaneous administration of 2,4-dinitrofluorobenzene (DNFB) (0.6% in acetone-olive oil, 4:1) on the shaved abdomen (50 μL) and on paws (50 μL divided between hind legs). On day 1, mice received a boost of DNFB only on the abdomen (50 μL). On day 5, animals from both groups were challenged intrarectally with 0.6% dinitrosulphonic acid (DNS) dissolved in 10% ethanol. The challenge was conducted under light inhalation anesthesia (3% halothane). During the experiment animal body weight and occurrence of diarrhoea were recorded daily. Mice were killed on day 1 (DNFB 24 h, n=8) or day 3 (DNFB 72 h, n=8) after the DNS challenge. All the control groups of the model had been previously evaluated in a extensive manner (Bailon et al., 2012).

2.5. Biochemical and immunological parameters

2.5.1. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al (Krawisz et al., 1984). Colonic specimens where homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0) and MPO activity in supernatant was measured and calculated from absorbance (at 460 nM) changes that resulted from decomposition of H_2O_2 in the presence of O-dianisidine; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol H_2O_2/min at 25ºC.

2.5.2. Determination of immunoglobulin levels

Immunoglobulin (Ig) measurements were performed by ELISA, following the manufacturer's recommendations. Colonic samples were re-suspended (1:5 w/v) in a lysis buffer containing 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.5), 10 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 40 mM β-glycerophosphate, 2,5 mM $MgCl_2$, 1% Igepal®, 1 mM dithiothreitol, 500 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide, 2 mM sodium orthovanadate. The tubes were placed in an orbital rotor (4ºC) for 20 min and centrifuged at 9000 × *g* for 8 min at 4ºC; the supernatants were frozen at -80ºC until assay. Blood was centrifuged (3500 × *g* for 10 min at 4ºC), and plasma aliquots were collected and frozen at −80ºC. 1:10000 dilutions were performed for the determination of total IgG1, IgG2a and IgA in plasma, and 1:2 for IgE. In the colon determinations, dilutions used were 1:1000 (IgG1), 1:500 (IgG2a), 1:5000 (IgA) or no dilution for IgE determination.

2.6. Statistical analysis

Statistical significance was calculated by Student's t-test in the case of parametric parameters such as the ELISA results. All tests were performed with one tail following the 2-sample equal variance model (homoscedastic). For the hypersensitivity score, the Mann-Whitney U-test was used to determine statistical significance. Due to the different age of animals inside the control group (day 19 and day 35), the clinical and macroscopical parameters altered during allergy induction were presented as the mean percentage (%) of variation versus healthy animals sacrificed at the same day (similar body weight, length and age) ± standard error of the mean (SEM) with the exception of score index. The rest of the data are represented as the mean ± SEM, and figures correspond to representative data from one of at least three independent experiments.

3. Results

In order to be able to test our hypothesis regarding the potential increased risk to suffer from colitis in atopic individuals, the first objective of this study consisted in the generation of adulthood atopic mice from a CMPA model developed in just weaning mice. To do that, we used the optimized shorter CMPA protocol previously described with minor modifications (Bailon et al., 2012). This novel CMPA model requires only 2 weeks of oral sensitization to CMP and an IP challenge at day 18 after the first oral sensitization. The optimization of this model with all specific controls was exhaustively evaluated previously (Bailon et al., 2012). In this study, we focus on the analysis of the allergic response to CMP after 24 hours (acute allergic group) and 16 days after the allergy induction (atopic group) in order to obtain adulthood mice with atopic markers.

We intragastrically sensitized the mice (n=24) with CMP plus CT 5 times at three to four days interval. Then, the challenge was made by IP administration. After 30 min of IP challenge, the hypersensitivity symptoms became evident as indicated with a score of 3.65±0.28 (Table 1). Then, the allergic mice were divided in two groups; one group (acute allergic) were killed 24 hours after (day 19) together with a healthy control group (n=8) and the rest of mice were maintained alive 16 days more (atopic mice) and compared with another age-matched healthy group (n=8). At day 35, as expected, any allergic symptom was observed in atopic animals and, obviously, neither in non-sensitized animals.

All the mice corresponding to these active groups did not show any significant alteration in body weight evolution, food consumption or stool consistency during all the experiment compared with untreated mice (data not shown). Once mice were killed, the blood was collected and the small intestine, colon and spleen were obtained to

evaluate the clinical and macroscopic parameters altered during allergy induction. In order to compare the macroscopic parameters between these two groups we presented the values as the mean of % of variation versus healthy animals sacrificed at the same day (similar weight and age) (Table 1). The potential alterations in gut morphology assayed mainly consisted in the evaluation of the weight/length ratio of intestines and the number of colonic patches in the colon (Table 1). Colonic patches are small lymphoid follicles consisting predominantly of B-cell zones (Bailon et al., 2011; Rijnierse et al., 2006) that appear on the mucosal site of the colon. An increase in the number of visible colonic patches is indicative of hypertrophy of these structures and therefore suggests an activation of the intestinal immune system (Bailon et al., 2011; Dohi et al., 1999; Rijnierse et al., 2006).

Table 1: Clinical and macroscopic parameters altered during allergy induction

	Score	Colon weight/length	Small intestine weight/length	Nº colonic patches	Spleen size
Acute allergic[1]	3.65 ± 0.28**	129.92 ± 5.84*	99.69 ± 5.94	193.5 ± 27.5	95.9 ± 4.24
Atopic[2]	ND	73.68 ± 4.15$^{*,\#\#}$	73.75 ± 5.28$^{*,\#}$	140.0 ± 19.6	104.2 ± 3.15

[1]*Acute allergic* animals corresponds to mice sacrificed 24 h after CMP challenge (day 19); [2]*Atopic* animals correspond to mice sacrificed two weeks after CMP challenge (day 35) previously to colitis induction. Values (except score) correspond to the mean % of variation versus healthy animals sacrificed at the same day (similar body weight and age) ± SEM (n=8 per group). * $p<0.05$, ** $p<0.01$ versus healthy animals; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ versus acute allergic animals. ND, not determined.

In this regard, the weight/length ratio of colon, but not small intestine, increased a 29,92% after 24 h of allergy induction, suggesting an acute local inflammatory response produced by infiltrated cells with oedema (Table 1). In contrast, in atopic group (after 16 days) the weight/length ratio of both intestines showed distension

expressed as a reduction of about 27% in weight/length ratio of both intestines in comparison with the respective healthy control group (Table 1). As previously reported, the increase in intestine length in atopic animals has been attributed to the distension of intestine due to a previous inflammatory process (Bailon et al., 2011; Cueto-Sola et al., 2013). In concordance to the alterations in the intestinal inflammatory state, an increase in the number of visible Peyer's patches was seen in the colon of acute allergic animals (93.5% increase), while a weaker increase (40%) was still present in atopic animals (Table 1). None of these values reached statistical significance due to the high variability of the values obtained. Taken together these results suggested that the atopic state, although it could not be considered an active inflammatory process, retains some alterations on the gut physiology and intestinal immune response.

The allergic process was confirmed not only by the intestinal symptoms observed but also by the systemic effects induced. In this case, although it has been previously described an enlargement of spleen in the CMPA protocol used (Bailon et al., 2012), no differences were observed in spleen size after 24 hours nor after 16 days (Table 1) of allergy induction. However, systemic allergic response was further characterized by measuring Ig production in plasma. As expected, the IgE levels increased in both groups confirming the IgE-mediated hypersensibility involved in this model (Table 2).

Table 2: Plasma immunoglobulin levels induced during allergic response

	IgE (ng/ml)	**IgG1 (μg/ml)**	**IgG2a (μg/ml)**	**IgA (μg/ml)**
Healthy[1]	19.84 ± 1.94	301.9 ± 28.5	201.5 ± 30.5	482.1 ± 57.3
Acute allergic[2]	26.22 ± 2.33[*]	368.5 ± 29.6	286.9 ± 49.4	391.0 ± 43.9

Atopic[3]	27.96 ± 3.25*	293.3 ± 44.7	273.4± 78.9	363.3 ± 32.1*

[1]*Healthy* animals correspond to both control groups sacrificed at day 19 and 35; [2]*Acute allergic* animals correspond to mice sacrificed 24 h after CMP challenge (day 19); [2]*Atopic* animals correspond to mice sacrificed two weeks after CMP challenge (day 35) previously to colitis induction. Values correspond to the mean ± SEM (n=8 per group). * p<0.05, ** p<0.01 versus healthy animals; # p<0.05, ## p<0.01 versus acute allergic animals. ND, not determined.

Similarly, IgG2a levels also increased in both groups (Table 2), although they did not reach statistical significance. Regarding other plasma Ig levels, the amounts of IgA decreased in both acute allergy and atopic mice although only reached significance in atopic group (Table 2), while IgG1 levels weakly increased during the acute allergic response but returned to basal levels in atopic mice (Table 1). In concordance with Ig plasma levels, acute allergic and atopic mice showed a similar altered profile of the Ig levels in the colon samples (Table 3). Finally, colonic inflammatory infiltrate was indirectly measured as colonic MPO activity, which although clearly increased in acute allergic animals did not reach significance, and the values returned to basal levels in atopic group (Table 3).

Table 3: Colonic immunoglobulin levels and MPO activity induced during allergic response

	MPO (mU/g)	**IgE (ng/ml)**	**IgG1 (μg/ml)**	**IgG2a (μg/ml)**	**IgA (μg/ml)**
Healthy[1]	39.45 ± 26.7	0.924 ± 0.26	1.528 ± 0.63	1.645 ± 0.32	247.6 ± 32.9
Acute allergic[2]	111.5 ± 55.6	1.281 ± 0.23	2.293 ± 0.52	1.906 ± 0.19	154.5 ± 13.6*
Atopic[3]	25.45± 10.5	1.297 ± 0.29	1.862 ± 0.46	1.878± 0.22	169.1 ± 16.5*

[1]*Healthy* animals correspond to both control groups sacrificed at day 19 and 35; [2]*Acute allergic* animals correspond to mice sacrificed 24 h after CMP challenge (day 19); [2]*Atopic* animals correspond to mice sacrificed two weeks after CMP challenge (day 35) previously to colitis induction. Values correspond to the mean ± SEM (n=8 per group). * p<0.05, ** p<0.01 versus healthy animals; # p<0.05, ## p<0.01 versus acute allergic animals. ND, not determined.

Taken together all of these results, here we report a suitable experimental model of adulthood atopic animals, which consists in the altered immunological state obtained

16 days after the induction of an acute allergy using the well described shorter protocol of CMPA. This model has some intestinal and immunological characteristics common to food-related atopic individuals (Da Silva et al., 2013; Lau 2013; Peters et al., 2013; Wisniewski et al., 2013) such as intestinal distension, probably responsible of an increased permeability, and an increase on plasmatic and colonic IgE levels but a reduced IgA levels in the intestinal mucosa. Moreover, no altered inflammatory markers such as MPO or IgG2a levels are increased in these individuals in a statistical significance manner.

The second goal of the study consisted in explore if the altered intestinal condition observed in atopic animals can modify the severity or the risk of suffering a posterior intestinal inflammatory pathology, such as IBS or IBD. To do that, we initially selected the dextran sodium sulphate (DSS)-induced colitis in mice as colitis model, since is a well-established model to study human IBD (Cavalcanti et al., 2014; Garrido-Mesa et al., 2011; Krause et al., 2014). In this regard, a group of healthy and atopic mice were further divided in three groups; non-treated group (Control) (n=8) and mice treated with DSS at concentrations of 1.5% (w/v) (moderate DSS group) or 3.5% (w/v) (severe DSS group). All DSS-treated animals developed diarrhoea and had weight loss associated with anorexia, which were evident from the first day after DSS administration and progressively increased during the course of the experiment.

As expected, the administration of 1.5% or 3.5% DSS dissolved in the drinking water for 5 days to healthy mice resulted in a progressive increase in DAI values, due to the body weight loss and the excretion of diarrheic/bleeding faeces (Fig. 1A). The disease severity depends on the concentration of DSS since at day 6 the DAI values were 3.0 and 1.5 in severe and moderate DSS groups respectively (Fig. 1A).

Interestingly, in atopic mice, a significant decrease of DAI, mainly associated with an improvement in the weight loss and faecal consistency, was observed in both DSS groups (Fig. 1A).

The reduced inflammatory process of DSS in atopic mice (moderate- and severe-DSS groups) in comparison with DSS in naive mice was also biochemically observed through the analysis of colonic MPO activity (a marker of neutrophils infiltration) (Fig. 1B); suggesting a lower leukocyte infiltration into the inflamed tissue during the DSS-induced colitis in atopic animals. This reduced effect in the DSS-induced inflammation in atopic animals was also macroscopically evidenced by a significant reduction in the colonic weight/length in severe DSS group ratio in comparison with naive mice (Fig. 1C). As expected, the colonic oedema and inflammation was more evident in severe than moderate DSS colitis in both groups of animals (Fig. 1C). However, any significant difference was observed in small intestine weight/length ratio beyond the previously distension described in atopic animals (Fig. 1D).

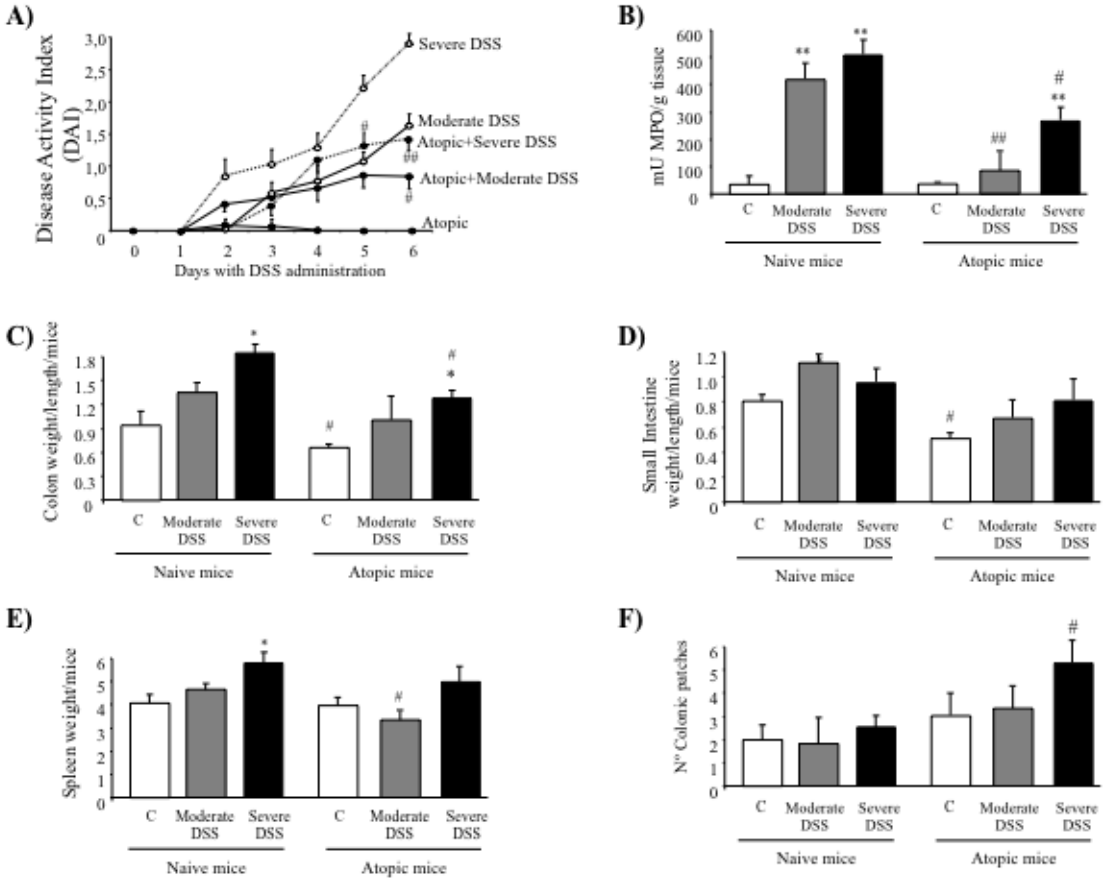

Fig. 1. Clinical amelioration of colitis symptoms induced by DSS was observed in atopic animals A) Disease activity index (DAI) values over the 6-day experimental period, based on the criteria proposed (Bailon et al., 2007; Camuesco et al., 2006). B) Colonic myeloperoxidase (MPO) activity. Weight/length ratio of the colon (C) and small intestine (D), and spleen weight (E) were measured at day 6 after colitis induction as described in material and methods. F) For each colon sample and previous to its sectioning, colon patches were counted. All results are expressed as the mean ± SEM. The Student's t-test was used to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared to healthy animals in each group; # ($p<0.05$), ## ($p<0.01$) compared to naïve mice.

The spleen size increased significantly with the severe DSS treatment in naïve mice in counterpart of the slight downward trend observed in severe DSS atopic animals (Fig. 1E). Interestingly, although the colitis model of DSS did not increase the number of visible colonic Peyer's patches in naïve animals (Fig. 1F), the number of visible patches increased significantly in the severe DSS group in atopic animals (Fig. 1F).

Next, we also evaluated the humoral immune response in plasma and colon samples from the colitic animals in both groups (Fig. 2). The DSS treatment at 3.5%,

but not at 1.5% concentration, induced a significant reduction of IgE, IgG1, IgG2a and IgA levels in plasma of naïve animals (Fig. 2A). Similarly, in colon samples the IgE, IgG1 and IgGA levels were also significantly reduced in severe DSS group, although the IgG2a production was not modified or even significantly increased in moderate DSS group (Fig. 2B).

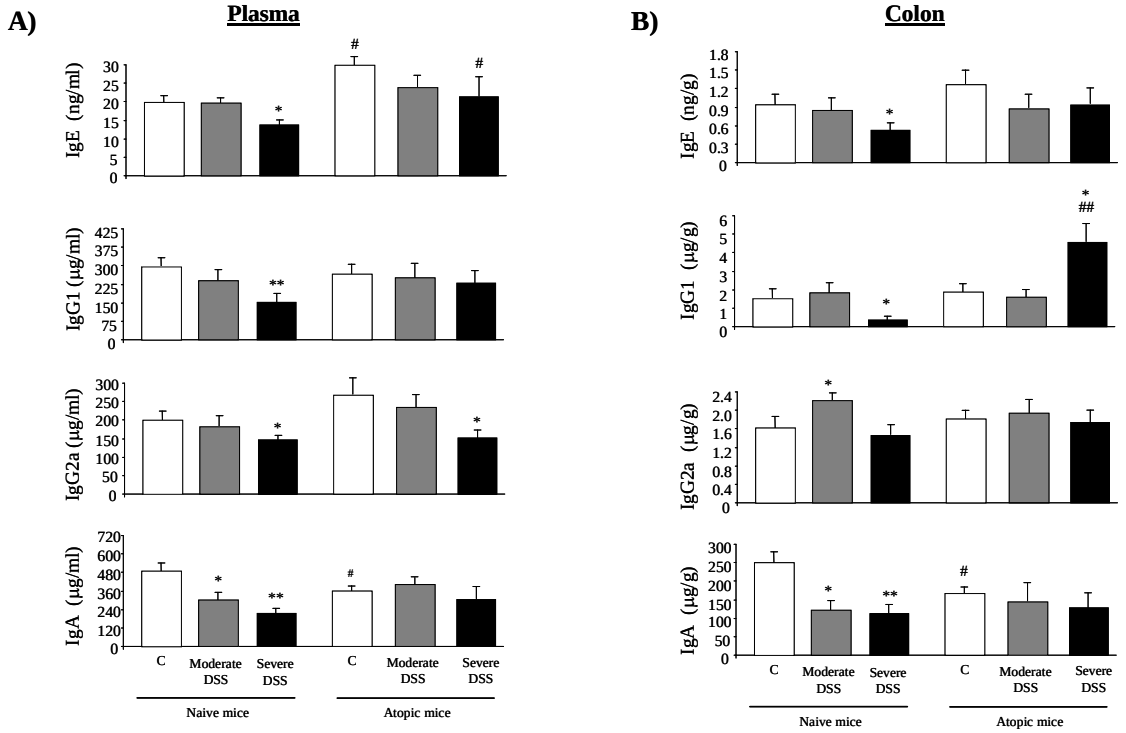

Fig. 2. Analysis of plasma and colon immunoglobulin levels induced by DSS in atopic mice. Plasma (A) and colon (B) from all mice were collected after sacrifice and used to measure total IgE, IgG1, IgG2a and IgA levels by ELISA, as described in material and methods section. Results are expressed as the mean concentration ± SEM. The Student's t-test was used to determine statistical significance. *($p<0.05$), **($p<0.01$) compared to healthy animals in each group; #($p<0.05$), ##($p<0.01$) compared to naïve mice.

In atopic animals although a reduction in IgE and IgG2a production in plasma was observed in severe DSS group in comparison with control group, no differences in IgG1 and IgA were observed compare to naïve mice (Fig. 2A). Moreover in colon, the atopic process induced a significant increase of IgG1 levels in severe DSS group without modifying the other immunoglobulins (Fig. 2B). Therefore, it could be concluded that a trend to reduce the inflammatory process induced by the DSS colitis

was clearly observed in atopic animals, a phenomenon that became more evident in the severe DSS group than in the animals with a moderate inflammation.

However, due to the complexity of the immune response in intestinal mucosa and based on our previous experiences with complex and simultaneous or consecutive experimental procedures, we decided to further confirm our results using a second model of intestinal inflammation and colitis. In this case, we selected the DNFB/DNS model that develops an inflammatory reaction in the colon which is evoked by skin sensitization of mice with DNFB, followed by a local intrarectal challenge with the hapten DNS. This model induces a mild and superficial inflammation of the mucosa, which reverts very fast (Bailon et al., 2011).

In this model, intestinal inflammation measured as colon weight/length was significantly increased 72 h after colitis induction in naive mice (Fig. 3A). Similarly, an increased ratio in small intestine was observed (Fig. 3B). No significant differences in the size or morphology of the spleen were observed although tended to increase (Fig. 3C). The intestinal inflammation was also evidenced by an increase in the number of colonic patches in these animals (Fig. 3D). On the other hand, the evaluation of the same macroscopic inflammatory parameters in the atopic mice suggested a faster onset of the inflammatory process and an increased severity. In this regard, an increased weight/length ration was observed in both intestines at 24 hours after DNFB treatment and also an increase on the number of visible Peyer's patches (Fig. 3). Moreover, a significant increase versus the response observed in naïve animals was observed in most of the parameters assayed after 72 hours of DNFB treatment (Fig. 3), suggesting that the inflammation produced by DNFB/DNS model may be aggravated in atopic animals.

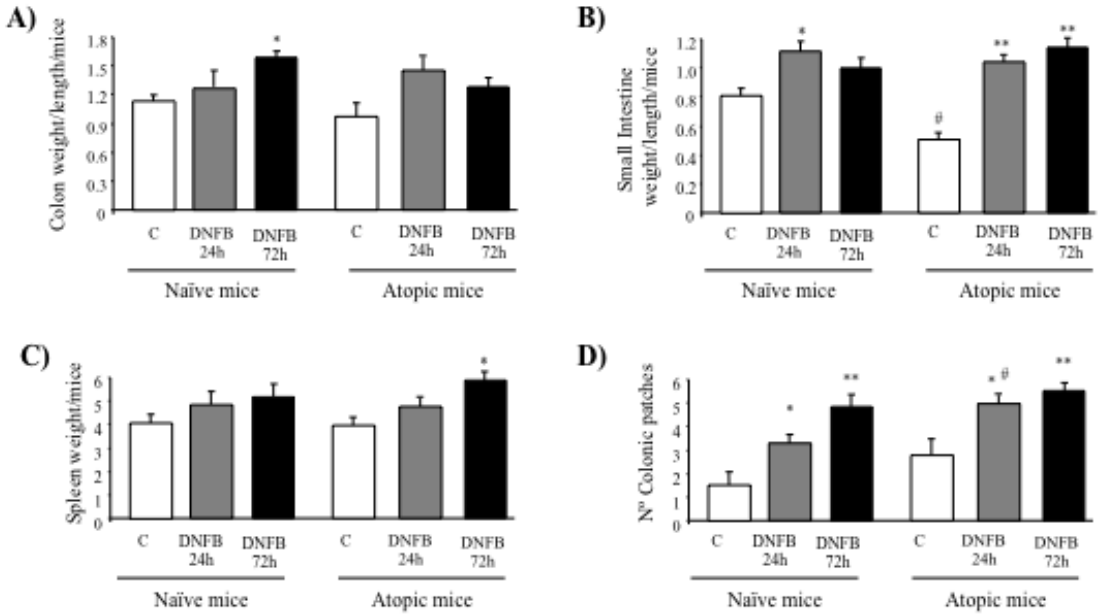

Fig. 3. Clinical exacerbation of macroscopic parameters was observed in atopic animals in the DNFB/DNS model Weight/length ratio of the colon (A) and small intestine (B), and spleen weight (C) were measured at 24 or 72 h after DNS administration in DNFB-sensitized mice as described in material and methods. F) For each colon sample and previous to its sectioning, colon patches were counted. All results are expressed as the mean ± SEM. The Student's t-test was used to determine statistical significance. * ($p<0.05$), ** ($p<0.01$) compared to healthy animals in each group; # ($p<0.05$), ## ($p<0.01$) compared to naïve mice.

Similarly, the colitis induced by DNS in DNFB-sensitized mice significantly increased the IgE levels in plasma at 72 h (Fig. 4), while did not significantly modify other Igs analysed at this time point (Fig. 4A). In contrast, a reduced IgG1 and IgA plasma levels were observed after 24 hours of the colitis induction in naïve animals. A similar response than that observed at the blood compartment could also be found in the inflammatory focus where the expression of IgE increased at 72 h time-point while a reduction of IgA was seen at 24 h (Fig. 4B). An exception was the levels of IgG2a that remained almost constant in the plasma compartment while in the colon initially reduced the levels at 24 hours but highly rose after 72 hours of colitis induction (Fig. 4B). In atopic animals the values obtained in Igs levels reached more significant

increased values than those obtained in naïve mice, especially on the plasma compartment and on colonic IgE (Fig. 4). The increased inflammation observed after DNFB/DNS treatment on atopic animals was also evident by a reduced colonic expression of IgA due to the tissue damage induced on the intestinal mucosa (Fig. 4B).

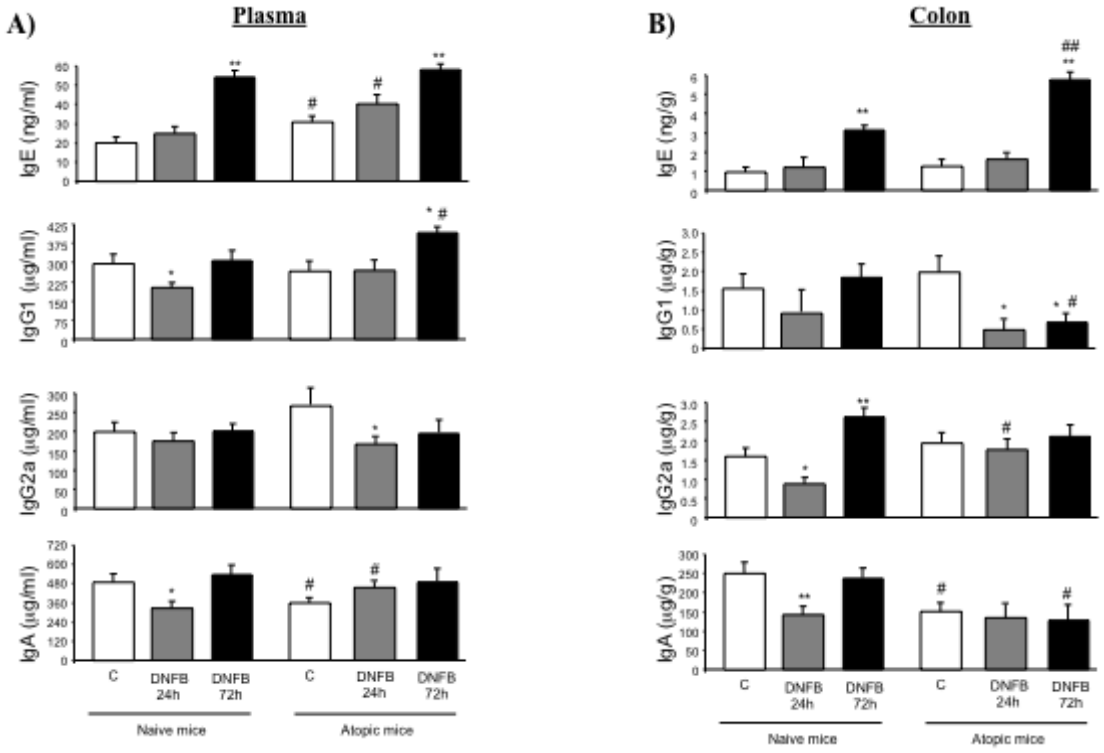

Fig. 4. Analysis of plasma and colon immunoglobulin levels induced by DNFB/DNS model in atopic mice. Plasma (A) and colon (B) from all mice were collected after sacrifice and used to measure total IgE, IgG1, IgG2a and IgA levels by ELISA, as described in material and methods section. Results are expressed as the mean concentration ± SEM. The Student's t-test was used to determine statistical significance. *($p<0.05$), **($p<0.01$) compared to healthy animals in each group; #($p<0.05$), ##($p<0.01$) compared to naïve mice.

DISCUSSION

Food allergy, especially to CMP, is probably one of the most prevalent pathologies during infancy (Hill, 1996). Despite CMPA in infants could have severe consequences and even death in a reduced number of cases, the majority of them could be easily managed using exclusion diets or even without any treatment, allergic signs disappear around two years of age (Hill, 1996). However, most of the allergic infants develop an altered immune state known as atopy (Da Silva et al., 2013; Lau 2013; Peters et al., 2013; Wisniewski et al., 2013), which has been related to an increased risk to be sensitized to other allergens and to a skin prone to develop topical irritations and eczemas. Moreover, some epidemiologic studies relate an increased risk to suffer other pathologies during adult life on atopic individuals. These pathologies include asthma, diabetes, multiple sclerosis and intestinal inflammatory processes such as IBD or IBS (Broberg et al., 2000; Oehlling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007). This association must not be surprising, since perinatal defects in the induction of mucosal tolerance are associated with the later development of allergies, autoimmune diseases (such as rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus) and chronic inflammation of the gut and respiratory mucosa (Broberg et al., 2000; Oehlling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007).

In this regard, abnormal immunologic reactions against endogenous or exogenous antigens, infectious agents, intestinal microbiota or environmental factors contribute to the pathogenesis of several intestinal conditions, including IBD or IBS (Bouma and Strober, 2003; Kaser et al., 2010; Renz et al., 2012; Scharl and Rogler, 2012). Since 1961, when Truelove (Truelove, 1961) proposed that ulcerative colitis (UC) was due to an abnormal response towards food allergens such as milk, the

hypothesis that allergic reactions to diet antigens are involved in the etiology and pathogenesis of IBD has remained controversial (Farah et al., 1985; Jenkins et al., 1984; Knoflach et al., 1987; Pearson et al., 1993; Rosekrans et al., 1980; Zwetchkenbaum and Burakoff, 1988). In fact, practices based on the elimination of a particular food or the use of elemental diets is still common among many IBD and IBS patients (Borrelli et al., 2006; Farah et al., 1985; Pearson et al., 1993; Rajendran and Kumar, 2010).

The objective of this work was to demonstrate the veracity of this hypothesis using animal models. Research into human pathology has been widely supported by laboratory data thanks to the use of diverse experimental animal models. Experimental animal models are commonly used to simplify and study complex human pathologies such as allergy or colitis (Bailon et al., 2011; 2012; Dearman and Kimber, 2009; Diesner et al., 2008; Galvez, 2009; Ganeshan et al., 2009; Gonipeta et al., 2010; Hsieh et al., 2003; Lara-Villoslada et al., 2004; 2005; Mizoguchi and Mizoguchi, 2010; Strid and Strobel, 2005; Takeda and Gelfand, 2009; Thang et al., 2011; Untersmayr et al., 2010; Valeur et al., 2009), and they allow the discovery of interactions and common elements of particular signaling events and molecular pathways. Mouse and rat models have been used satisfactorily in studies of various allergic diseases and intestinal inflammatory pathologies. Although several models of food allergy have been described, they differ in the route of sensitization and challenge, the strain of mouse used, the use of adjuvant or co-administered drugs, the type of antigen or the doses required and the duration of the sensitization period (Diesner et al., 2008; Ganeshan et al., 2009; Gonipeta et al., 2010; Hsieh et al., 2003; Strid and Strobel, 2005; Thang et al., 2011; Untersmayr et al., 2010; Valeur et al., 2009). Similarly, current experimental animal models for intestinal inflammatory conditions do not perfectly match the patho-

physiological features observed in humans, and a myriad of them have been described both in mice and rats to expand the amount of human symptoms and pathological conditions available (Galvez, 2009). As a result, studies use distinct animal models, thereby hindering the comparison of results. Any experimental model should be characterized by the robustness and reproducibility of the symptoms, the selectivity and specificity of the response, and the easiness of the protocols, both regarding time and resources. With this aim we have optimized several allergy, IBD and IBS models in the last years (Bailon et al., 2011; 2012; Garrido-Mesa et al., 2011).

In this work we have used some of these optimized experimental models, such as the CMP allergy model (Bailon et al., 2012) or the DNFB/DNS model resembling human IBS (Bailon et al., 2011). However, to demonstrate our hypothesis we required a new experimental model not described so far to our knowledge; an atopy experimental animal model. In this regard, data presented in this work support and validates the atopy model described since most of the alterations showed could be translationally correlated with the human condition. This is the case of the increased levels of IgE observed both in plasma and colon samples, the intestinal distension and the reduced IgA colonic levels, while no other signs of acute inflammation were seen, namely MPO colonic activity or alterations on the IgG2a levels (Da Silva et al., 2013; Lau 2013; Peters et al., 2013; Wisniewski et al., 2013).

IgA decrease could reflect an alteration of the correct intestinal homeostasis (Dong-Yan et al., 2011; Shimada et al., 1999) while intestinal distension has been associated with increased permeability (Ait-Belgnaoui et al., 2005; Gecse et al., 2007). Since recent evidence suggests a role for increased colonic permeability on the ethiology of IBS and other colitic process, and the presence of an allergic/atopic

background correlates with a more severe colitic diseases and diarrhoea predominance, possibly by enhancing mucosal mast cell activation and paracellular permeability in those pathologies (Vivinus-Nebot et al., 2012), it is rational to suspect that an increased risk to suffer subsequent colitic processes will be easily observable in atopic individuals.

The use of two different colitic models in this work points out the difficulties to demonstrate sometimes an easy hypothesis using consecutive experimental animal models. In this sense, we have observed opposite effects using two colitis models, namely DSS, a well-known model of IBD (Cavalcanti et al., 2014; Garrido-Mesa et al., 2011; Krause et al., 2014) and DNFB/DNS model, more resembling the human IBS condition (Bailon et al., 2011). In our model, atopic animals present an exacerbated severity to subsequent DNFB/DNS colitis while remain protected to DSS colitis. Abnormal immunologic reactions against endogenous or exogenous antigens, intestinal microbiota or environmental factors contribute to the pathogenesis of both intestinal conditions (Broberg et al., 2000; Oehlling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007). However, while recent studies have indicated that the colonic mucosa in IBS shows an increase in inflammatory cells, this inflammation is quantitatively less than in IBD and of a different nature with a predominance of mast cells (Spiller, 2009). Since mast cell activation is also a hallmark of allergic responses (Fried and Akin, 2013; Kawakami et al., 2014; Siracusa et al., 2013), the reactivation of these mast cells during the DNFB/DNS model could further increase gut permeability, and may allow an increased activation of the systemic immune system and the severity of the colitis observed. Moreover, the expression of IL-5 and IL-13 was enhanced in IBS while IL-12 and IL-10 were reduced (Kindt et al., 2009), showing a biased Th2 response in this

pathology that also correlates with the cytokine profile observed during allergy/atopy processes (Bailon et al., 2012; Lara-Villoslada et al., 2004; 2005).

In contrast, DSS model, although also involve humoral and Th2 cytokine components (Bailon et al., 2009), presents a clear inflammatory and Th1 immune response, especially in the mice models (Bailon et al., 2008; Mikami et al., 2014; Ustyogova et al., 2012). Moreover, since intestinal permeability is totally disrupted in this model a weak influential effect could be attributed to this condition. Based on that, it could be possible that the biased Th2 response observed in atopic individuals could diminish the Th1 cytokine response required to initiate the inflammatory state observed in the DSS model.

In conclusion, data presented in this work confirm that the intestinal and immune alterations observed in atopic individuals could modify the course of subsequent mucosal inflammatory conditions, but this modification could be in both directions depending on the main ethiological characteristics of each inflammatory condition. Due to that, the selection of adequate experimental models could facilitate or mislead the conclusions drawn.

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REFERENCES

Ait-Belgnaoui A, Bradesi S, Fioramonti J, Theodorou V, Bueno L. Acute stress-induced hypersensitivity to colonic distension depends upon increase in paracellular permeability: role of myosin light chain kinase. Pain. 2005;113:141-7.

Bailon E, Camuesco D, Nieto A, et al. The intestinal anti-inflammatory effects of the novel agent UR-1505 in the TNBS model of rat colitis are mediated by T-lymphocyte inhibition. Biochem Pharmacol. 2007; 74: 1496-1506.

Bailon E, Comalada M, Román J, Michelena P, Ramis I, Merlos M, Nieto A, Concha A, Zarzuelo A, Gálvez J. UR-1505, a salicylate able to selectively block T-cell activation, shows intestinal anti-inflammatory activity in the chronic phase of the DSS model of rat colitis. Inflamm Bowel Dis. 2008; 14: 888-97.

Bailon E, Román J, Ramis I, Michelena P, Balsa D, Merlos M, Zarzuelo A, Gálvez J, Comalada M. The new salicylate derivative UR-1505 modulates the Th2/humoral response in a dextran sodium sulphate model of colitis that resembles ulcerative colitis. J Pharmacol Sci. 2009; 109: 315-8.

Bailon E, Cueto-Sola M, Utrilla P, et al. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis. 2011; 17: 2087-2101.

Bailon E, Cueto-Sola M, Utrilla P, et al. A shorter and more specific oral sensitization-based experimental model of food allergy in mice. J Immunol Methods. 2012; 381: 41-49.

Bouma G1, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003; 3: 521-33.

Borrelli O, Cordischi L, Cirulli M, et al. Polymeric diet alone versus corticosteroids in the treatment of active paediatric Crohn's disease: a randomized controlled open-label trial. Clin Gastroenterol Hepatol. 2006; 4: 744-753.

Broberg A, Svensson A, Borres MP, Berg R. Atopic dermatitis in 5-6-year-old Swedish children: cumulative incidence, point prevalence, and severity scoring. Allergy. 2000; 55:1025-9.

Camuesco D, Comalada M, Concha A, et al. Intestinal anti-inflammatory activity of combined quercitrin and dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, in rats with DSS-induced colitis. Clin Nutr. 2006; 25: 466-476.

Cavalcanti E, Vadrucci E, Delvecchio FR, Addabbo F, Bettini S, Liou R, Monsurrò V, Huang AY, Pizarro TT, Santino A, Chieppa M. Administration of reconstituted polyphenol oil bodies efficiently suppresses dendritic cell inflammatory pathways and acute intestinal inflammation.

Cueto-Sola M1, Bailon E, Utrilla P, Rodríguez-Ruiz J, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. Active colitis exacerbates immune response to internalized food antigens in mice. Int Arch Allergy Immunol. 2013; 162:214-24.

Custovic A, Lazic N, Simpson A. Pediatric asthma and development of atopy. Curr Opin Allergy Clin Immunol. 2013;13:173-80.

Da Silva L, Kweku Sagoe Amoah S, Da Silva J. Relationship between atopy, allergic diseases and total serum IgE levels among HIV-infected children. Eur Ann Allergy Clin Immunol. 2013; 45:155-9.

D'Arienzo A, Manguso F, Astarita C, et al. Allergy and mucosal eosinophil infiltrate in ulcerative colitis. Scan J Gastroenterol. 2000; 6: 624-631.

Dearman RJ, Kimber I. Animal models of protein allerginicity: potential benefits, pitfalls and challenges. Clin Exp Allergy. 2009; 39: 458-468.

Diesner SC, Knittelfelder R, Krishnamurthy D, Pali-Schöll I, Gajdzik L, Jensen-Jarolim E, Untersmayr E. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. Immunol Lett. 2008; 121:45-51.

Dohi T1, Fujihashi K, Rennert PD, Iwatani K, Kiyono H, McGhee JR. Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. J Exp Med. 1999; 189: 1169-80.

Dong-Yan L, Weiguo J, Pei L. Reduction of the amount of intestinal secretory IgA in fulminant hepatic failure. Braz J Med Biol Res. 2011; 44:477-82.

El-Salhy M, Ostgaard H, Gundersen D, Hatlebakk JG, Hausken T. The role of diet in the pathogenesis and management of irritable bowel syndrome. Int J Mol Med. 2012; 29: 723-31.

Eswaran S, Tack J, Chey WD. Food: the forgotten factor in the irritable bowel syndrome. Gastroenterol Clin North Am. 2011; 40:141-62.

Farah DA, Calder I, Benson L, Mackenzie JF. Specific food intolerance: Its place as a cause of gastrointestinal symptoms. Gut. 1985; 26: 164-168.

Fried AJ, Akin C. Primary mast cell disorders in children. Curr Allergy Asthma Rep. 2013; 13:693-701.

Galssman MS, Newman LJ, Berezin S, Gryboski JD. Cow's milk protein sensitivity during infancy in patients with inflammatory bowel disease. Am J Gastroenterol. 1990; 85: 838-840.

Gálvez J. Experimental models of inflammatory bowel disease in rodents. In: Peppelenbosch MP, Comalada M, ed. Preclinical Research into Crohn's disease: a practical guide. Kerala, India: Transworld Research Network; 2009: 1-27.

Ganeshan K, Neilsen CV, Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. J Allergy Clin Immunol. 2009; 123: 231-238.

Garrido-Mesa N, Utrilla P, Comalada M, Zorrilla P, Garrido-Mesa J, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. The association of minocycline and the probiotic Escherichia coli Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice. Biochem Pharmacol. 2011; 82: 1891-900.

Gecse K, Róka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztóczy A, Izbéki F, Fioramonti J, Wittmann T, Bueno L. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. Gut. 2008; 57: 591-9.

Gonipeta B, Parvataneni S, Tempelman RJ, Gangur V. An adjuvant-free mouse model to evaluate the allergenicity of milk whey protein. J Dairy Sci. 2009; 92: 4738-44.

Hill DJ, Hosking CS. Emerging disease profiles in infants and young children with food allergy. Pediatr Allergy Immunol. 1997; 10: 21-28.

Hsieh KY, Tsai CC, Wu CH, Lin RH. Epicutaneous exposure to protein antigen and food allergy. Clin Exp Allergy. 2003; 33: 1067-75.

Jenkins HR, Pincott JR, Soothill JF, Milla PJ, Harries JT. Food allergy: The major cause of infantile colitis. Arch Dis Child. 1984; 59: 326-329.

Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol. 2010; 28:573-621.

Kawakami T, Kashiwakura J, Kawakami Y. Histamine-releasing factor and immunoglobulins in asthma and allergy. Allergy Asthma Immunol Res. 2014; 6:6-12.

Kindt S, Van Oudenhove L, Broekaert D, Kasran A, Ceuppens JL, Bossuyt X, Fischler B, Tack J. Immune dysfunction in patients with functional gastrointestinal disorders. Neurogastroenterol Motil. 2009; 21: 389-98.

Knoflach P, Park BH, Cunningham R, Weisser MM, Albini B. Serum antibodies to cow's milk proteins in ulcerative colitis and Crohn's disease. Gastroenterol. 1987; 92: 479-485.

Krause P, Zahner SP, Kim G, Shaikh RB, Steinberg MW, Kronenberg M. The Tumor Necrosis Factor Family Member TNFSF14 (LIGHT) is Required for Resolution of Intestinal Inflammation in Mice. Gastroenterology. 2014: S0016-5085(14)00221-2. doi: 10.1053/j.gastro.2014.02.010.

Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. Gastroenterology. 1984; 87: 1344-50

Lara-Villoslada F, Olivares M, Jimenez J, Boza J, Xaus J. Goat milk is less immunogenic than cow milk in a murine model of atopy. J Pediatr Gastroenterol Nutr. 2004; 39: 354-360.

Lara-Villoslada F, Olivares M, Xaus J. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J Dairy Sci. 2005; 88: 1654-1660.

Lau S. What is new in the prevention of atopy and asthma? Curr Opin Allergy Clin Immunol. 2013; 13:181-6.

Mikami Y, Mizuno S, Nakamoto N, Hayashi A, Sujino T, Sato T, Kamada N, Matsuoka K, Hisamatsu T, Ebinuma H, Hibi T, Yoshimura A, Kanai T. Macrophages and dendritic cells emerge in the liver during intestinal inflammation and predispose the liver to inflammation. PLoS One. 2014; 9: e84619.

Mizoguchi A, Mizoguchi E. Animal models of IBD: linkage to human disease. Curr Opin Pharmacol. 2010; 10: 578-587.

Morcos A, Dinan T, Quigley EM. Irritable bowel syndrome: role of food in pathogenesis and management. J Dig Dis. 2009; 10:237-46.

Oehling A, Resano A, Sanz ML, Fernández Benítez M. Importance of food allergy in atopic dermatitis. Allergy. 1998; 53:139-42.

Ozol D, Mete E. Asthma and food allergy. Curr Opin Pulm Med. 2008; 14: 9-12.

Pallone F, Monteleone G. Interleukin 12 and Th1 responses in inflammatory bowel disease. Gut. 1998; 48: 735-736.

Pearson M, Teahon K, Levi AJ, Bjarnason I. Food intolerance and Crohn's diasease. Gut. 1993; 34: 783-787.

Peters JL, Boynton-Jarrett R, Sandel M. Prenatal environmental factors influencing IgE levels, atopy and early asthma. Curr Opin Allergy Clin Immunol. 2013; 13:187-92.

Rajendran N, Kumar D. Role of diet in the management of inflammatory bowel disease. World J Gastroenterol. 2010; 16: 1442-1448.

Renz H, Brandzaeg P, Hornef M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol. 2012; 12: 9-23.

Rijnierse A, Koster AS, Nijkamp FP, Kraneveld AD. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. J Immunol. 2006; 176: 4375-84.

Rosekrans PCM, Meijer CJLM, van der Wal AM, Lindeman J. Allergic proctitis, a clinical and immunological entity. Gut. 1980; 21: 1017-1023.

Scharl M, Rogler G. Inflammatory bowel disease pathogenesis: what is new? Curr Opin Gastroenterol. 2012; 28: 301-309.

Shimada S, Kawaguchi-Miyashita M, Kushiro A, Sato T, Nanno M, Sako T, Matsuoka Y, Sudo K, Tagawa Y, Iwakura Y, Ohwaki M. Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. J Immunol. 1999; 163: 5367-73.

Siracusa MC, Kim BS, Spergel JM, Artis D. Basophils and allergic inflammation. J Allergy Clin Immunol. 2013; 132: 789-801

Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol. 2007; 120:1172-7.

Spiller RC. Overlap between irritable bowel syndrome and inflammatory bowel disease. Dig Dis. 2009; 27:48-54.

Strid J, Strobel S. Skin barrier dysfunction and systemic sensitization to allergens through the skin. Curr Drug Targets Inflamm Allergy. 2005; 4: 531-41.

Takeda G, Gelfand EW. Mouse models of allergy diseases. Curr Opin Immunol. 2009; 21: 660-665.

Thang CL, Baurhoo B, Boye JI, Simpson BK, Zhao X. Effects of Lactobacillus rhamnosus GG supplementation on cow's milk allergy in a mouse model. Allergy Asthma Clin Immunol. 2011; 7:20.

Truelove SC. Ulcerative colitis provoked by milk. Br Med J. 1961; 1061: 154-160.

Untersmayr E, Diesner SC, Oostingh GJ, Selzle K, Pfaller T, Schultz C, Zhang Y, Krishnamurthy D, Starkl P, Knittelfelder R, Förster-Waldl E, Pollak A, Scheiner O, Pöschl U, Jensen-Jarolim E, Duschl A. Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. PLoS One. 2010; 5: e14210.

Ustyugova IV, Zhi L, Wu MX. Reciprocal regulation of the survival and apoptosis of Th17 and Th1 cells in the colon. Inflamm Bowel Dis. 2012; 18: 333-43.

Valeur J, Lappalainen J, Rita H, Lin AH, Kovanen PT, Berstad A, Eklund KK, Vaali K. Food allergy alters jejunal circular muscle contractility and induces local inflammatory cytokine expression in a mouse model. BMC Gastroenterol. 2009;9 :33.

Vivinus-Nebot M, Dainese R, Anty R, Saint-Paul MC, Nano JL, Gonthier N, Marjoux S, Frin-Mathy G, Bernard G, Hébuterne X, Tran A, Theodorou V, Piche T.

Combination of allergic factors can worsen diarrheic irritable bowel syndrome: role of barrier defects and mast cells. Am J Gastroenterol. 2012; 107: 75-81

Wisniewski J, Agrawal R, Woodfolk JA. Mechanisms of tolerance induction in allergic disease: integrating current and emerging concepts. Clin Exp Allergy. 2013; 43:164-76.

Zwetchkenbaum JF, Burakoff R. Food allergy and the irritable bowel syndrome. Am J Gastroenterol. 1988; 83: 901-904.

Chapter 7

Discussion

CHAPTER 7: DISCUSSION

Since long time ago it is known that occidental style life is related with a higher incidence of several pathologies, mainly with inflammatory cause as IBD, allergy, IBS, and also cancer, autoimmune diseases, etc. The contribution of environment is critical not only in the beginning but also in the evolution of these pathologies. People of industrialized countries who live in a big city suffer more from these pathologies that those who live in a little village or in the countryside. The explanation of this fact is the presence of pollution, processed foods, conservatives in the foods and contact with others chemical substances with uncertain safety. However, multifactorial stimuli are necessary to develop an allergy or IBD including genes, microenvironment, age and concomitant triggering events (Guarner et al., 2006; Molodecky et al., 2012).

Tolerance is the key to maintain a good relationship between external and internal environment without developing an excessive response against external or internal substances. However, excessive tolerance may lead to invasion by microorganisms or parasites, or to the development of cancer. Clearly, regulation of tolerance is essential for life. The precise balance between the frequency of Th2, Th1 and Treg responses define a healthy or ill status. The observed increment of the incidence of allergic diseases is due to the loss of peripheral T-cell tolerance to allergens (Chehade & Mayer, 2005).

Allergic immune response is elicited by APCs which process and present allergenic epitopes to T-helper lymphocytes. In presence of IL-4 the response is switch to Th2, and if IL-13 is present, B cells produce IgE specific antibodies which activate mast cells and basophils releasing inflammatory mediators and cytokines, perpetuating the proinflammatory response. In delayed phases, IL-5 is responsible for the activation and tissue recruitment of eosinophils which perpetuate the allergic disease. Treg cells keep an unresponsiveness state producing IL-10 and TGFβ which suppress the Th2 response and IgE production in healthy people. The presence of Th1 lymphocytes has been described in some situations (Montero Vega, 2006). Others allergy participants have been described such different type of antibodies. For example, it has been noticed that an increase in production of allergen-specific-IgG1 often takes place in allergic situations. Regarding other immunoglublins, increased levels of IgG2a (more indicative of a Th1 response) or decreased IgA concentration have been observed in some circumstances (Berin, 2012; Liu et al., 2012).

Similarly, inflammatory response is initiated also with the interaction of APCs with naïve T lymphocytes, which evolve to Th1 cells in presence of IL-12 and IL-18. Th1 activated cells produce IFNγ leading to the activation of macrophages and inhibition of plasma B cells. Accumulation of macrophages and its cytokines TNFα and IL-1, produce a chronic inflammation with the participation of B cells and antigen-specific antibodies. Regarding the role of antibodies, it has been found that about 60-80% of patients with an inflammatory disease, such as IBD, have specific-IgG antibodies against various antigens (for example anti-neutrophil cytoplasmic antibodies), suggesting an increased humoral response in these diseases (van Schaik et al., 2013). An overproduction of

IgG1 and IgG2 has been observed in UC and CD, respectively (Furrie, Macfarlane, Cummings, & Macfarlane, 2004). Furthermore it is known that human IgA immunodeficiency may be related to UC (Brandtzaeg, Carlsen, & Halstensen, 2006). Although any Th2 contribution is generally accepted in the inflammatory response, in some complex pathologies as IBD a participation of Th2 subset has been demonstrated with a Th1/Th2 balance controlled by Treg cells (Allez & Mayer, 2004). Same cells, but in a different cytokine scene, are the picture of allergy and inflammation.

In absence of experimental proofs, a lot of evidences in patient with IBD related the food allergy to CD, UC or IBS. Some studies have found that a big number of immunological cells like eosinophils, mast cells and IgE-positive cells are infiltrated in small bowel of patients with atopic eczema, rhinitis or asthma. Moreover, authors suggest that patients with atopic diseases have a gut barrier dysfunction as it is indicated by an increased intestinal permeability. In fact, it has been established the expression “atopic bowel” to name the patients with intestinal symptoms associated to an atopic disease as rhinitis or asthma. Their intestinal symptoms worsen when allergic signs are increased (Lillestol et al., 2010).

The relationship between allergy and IBD has not been demonstrated in experimental conditions yet (see figure 4), although several retrospective analyses into paediatric populations have shown a strong connection between allergy suffered in childhood and IBD in adulthood. Virta et al. published some data from the Finnish health system that point out a preceding diagnosis of asthma in 12% of the paediatric patients with CD and this relation is stronger when asthma is diagnosed beyond 3 years of age. Moreover, cow’s milk allergy (CMA) is related with CD as well as UC. Interestingly, the strongest association to contract CD was seen when CMA has predominantly abdominal symptoms and not skin symptoms (Virta, Ashorn, & Kolho, 2013).

Moreover, the experimental conditions of two different pathologies as allergy and IBD in the same animal have not being published by now. Reasons for this fact are numerous; first, the election of the animal model is a critical point. In both cases, pathophysiological conditions are complex and a big number of components (cytokines, immune cells, antibodies) play a role in the evolutions of symptoms. Secondly, interpretation of the results is more difficult if the experimental model is not good established.

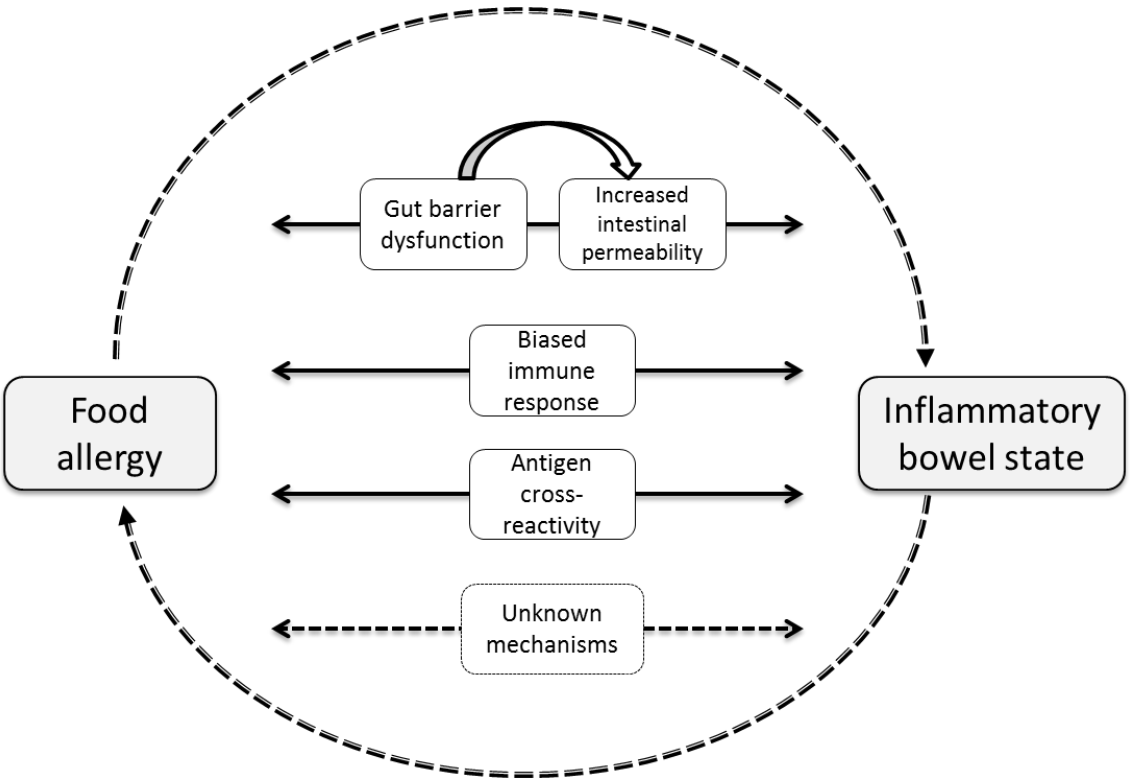

Figure 4.- General mechanism of allergy development. The allergy development occurs in various stages. Firstly the allergen is internalized,

We have attempted to find the best animal model of food allergy and colitis in mice to do a combined experimental assay to observe the repercussion of each other, and with that objective we studied separately both experimental conditions and after that we suggested two mixed allergy-colitis models.

Animal models of IBD try to mimic the human disease but they have some deficiencies because the complex scene that occurs in the pathophysiology of UC and CD. While CD is a Th1 biased pathology, UC is quite more complex with participation of lymphocytes Th1, Th2 but without any significant modification in IL-4 levels. For this reason, there are several animal models that match CD, but we have only found two hapten-induced experimental protocols for UC: the oxazolone one and the DNFB model, the last one with scarce information about biochemical characteristics.

We attempted to find an animal model of IBD which permits us to evaluate the relationship between allergy and IBD. To reach this objective we analyzed the immunological profile of the DNFB/DNS protocol proposed by Brkic et al in 1992 (Brkic et al., 1992) and redefined by Rijnierse et al in 2006 (Rijnierse, Koster, Nijkamp, & Kraneveld, 2006) in order to use a perfectly characterized protocol and find the best animal model.

An experimental protocol, based on the one proposed by Brkic, was designed with different groups of animals which were sacrificed at different times for monitoring the evolution of biochemical changes. In addition, the proposed model

included another group of animals with an additional challenge to observe the behaviour of pathology and confirm the observations that were seen with the first challenge. Moreover, we compared the DNFB/DNS model with the TNBS-induced colitis model, a very well-known protocol of CD (Bailon et al., 2011).

Although those mice had mild diarrhoea just after 24 hours of DNS colonic instillation any significant loss of weight was observed, and this effect gradually increase during the first 72 hours but disappeared at day 5. Inflammation associated with diarrhoea was also of medium intensity as we observed with the macroscopic examination of patches number. Microscopically, our results indicated that a big infiltration of mast cells was present but only a modest increasing of histamine levels was found in plasma samples, what indicated a moderate activation and degranulation of mast cells. Similar situation was observed in relation to lymphocytes; there was a big infiltration but a little cytokine expression. But it is important to emphasise the rise in IL-2 and IL-5, with a minimal participation of TNF-α which is according with the limited number of macrophages infiltrated. IL-5 can be produced by lymphocytes because few mast cells were found in the microscopic analysis of colonic samples, and its function consists, at least partially, in the regulation of B cells and antibodies production. Relative to humoral response to this inflammation, an increase of IgE and IgG1 was observed in both colon and plasma samples while IgA was increased in colon and reduced in plasma. This allowed us to think about a mainly atopic response mediated by B cells in this experimental model.

On the other hand, a second challenge in those mice did not exacerbate or accelerate the process occurred after first challenge. The absence of significant differences between groups of mice in the production of cytokines, except the higher expression of IL-10 and TNF-α, complicates the interpretation of the results obtained after the second challenge. Curiously, the Ig profile after the second challenge was very different from the one after the first challenge and there was not any clear biased immune response that let us to categorise the process as an atopic reaction.

Participation of IL-5 in this model could remember us an experimental model of IBD similar to Oxazolone-induced colitis and human UC, but when all results are observed together it is impossible to arrive at this conclusion, mainly because if there is not a disruption of mucosal integrity, there will not be any significant antigen recruitment and therefore, any inflammatory cells infiltration, a key point in the beginning and the evolution of every IBD illness. However, if this protocol is not useful as an experimental approximation to human IBD, it may be a good option for studying other diseases as allergic intestinal responses due to the Igs profile. We chose this model to carry out our objectives because its mild inflammatory reaction and its simple cytokines response.

Animal models of allergy partially reflect the symptoms of human disease but they are closer to biochemical changes. Among the allergens used to mimic food allergy, OVA triggers a strong, reproducible and well characterized allergic reaction, while Cow's milk protein allergy (CMPA) requires just-weaned mice and it unleashes a slow reaction with a peak at 6 weeks; moreover the results are not reproducible in a

high rate. In fact, a useful method in the study of the more and more frequent CMPA was published by Li et al. in 1999 and re-evaluated in BalbC mice by Lara-Villoslada et al in 2004 (Lara-Villoslada, Olivares, Jimenez, Boza, & Xaus, 2004; Li, Schofield, Huang, Kleiner, & Sampson, 1999). Three-week-old C3H/HeJ mice were sensitized by intragastric administration of cow's milk (CM) plus cholera toxin and boosted 5 times at weekly intervals. This method has a first problem which is the long time required to obtain the allergic response (6 weeks after the initial feeding). Although a Th2 biased response is evident and systemic symptoms are similar to human allergy with increased plasma histamine and increased intestinal permeability to casein, the results do not appear to be similar in different situations.

In a second step of this thesis, we improved the method proposed by Lara-Villoslada et al (Lara-Villoslada et al., 2004) with a significant reduction in the time of induction of the allergic response together with an exacerbated immune response (Bailon et al., 2012). In the original protocol, a systemic sensitization and oral challenge with milk protein and cholera toxin as adjuvant was applied to obtain the allergic response. One innovation in our method was the use of intraperitoneal administration in the challenge moment which increase the immune response with a significant reduction in the time needed (2 weeks versus 6 weeks).

Adjuvants in allergy animal methods are necessary for the innate tendency to develop oral tolerance in mice. We also analysed the effect of cholera toxin as adjuvant in the immune reaction in both long and short protocols, and we observed that cholera toxin led to a significant increase in several allergy response markers, such as diarrhoea, colon oedema, spleen enlargement, and colonic IgE and IgG2a production among others in animals without challenge. These results can be explained by the fact that this adjuvant possibly promotes an unspecific response against other food proteins different from milk casein and another possible option is the cross-reaction between caseins and soy-derived proteins present in the diet.

Unquestionably, in our experiment, a good allergic response is found in the group of the original protocol, with significant differences in various parameters such as spleen size, plasma histamine and plasma and colon antibodies (IgE, IgG1, IgA, CMP-specific IgG1) versus control group. However, the novel proposed protocol reduces the experimental time by two-thirds and triggers a more allergen-specific response since an increase in plasma and colon levels of CMP-specific IgG1 is observed.

Short protocol can be questionable in some aspects as the utilization of i.p. route for the challenge. While the sensitization is made usually by systemic administration, challenge is made by oral route in all experimental method of food allergies. Although this via of administration is not physiological for food allergy in human, it is better to give little concentration of allergen and guarantee the internalization of minimal amounts of intact protein and therefore to achieve a better specific response. Another matter in the proposed method is the behaviour of antibodies IgE and IgA. Regarding with the original protocol, the new short method does not increase levels of the hallmark of allergy, IgE, neither in plasma nor in colon. This result can be explained by the fact that IgE production is highly dependent on the

allergen type and casein from cow's milk protein is not always effective inducing IgE. On the other hand, absence of IgA in our short protocol may be due to its short duration. This fact can be considered an advantage since the main action of IgA is opposite to others Igs.

In our intention to study a double pathological condition, allergy-bowel inflammation, it is important to achieve a good correlated temporal evolution and a clearly established immune response with as minimal interferences as possible. Moreover, the experimental model should be reproducible and the data obtained not confused.

In the combined protocol that we developed, OVA allergic response was evident 30 minutes after challenge, because animals felt an intense scratching and other allergic symptoms as piloerection and general reduced activity. After sacrifice, colonic macroscopic alterations did not show any difference between colitic groups, DNS and OVA+DNS, indicating a similar diarrheic status in both groups, but the results of immunological conditions demonstrated a robust allergic response to OVA and a bigger inflammatory response in the OVA+DNS group. This last group suffered a bigger spleen enlargement with an increased amount in splenocytes number. When these cells were stimulated with OVA "in vitro", its proliferation was more intense that splenocytes proliferation in the others groups. Moreover in this experiment, any proliferation was observed using LPS or Concanavalin A (ConA) as activators what indicates a specific response to OVA and not against other antigens. Analysis of humoral response also evidence an exacerbated response since IgG1 plasma levels are increased in OVA group versus healthy group and in OVA+DNS group versus all other groups. Similar response was found in splenocytes stimulated with OVA "in vitro" showing that humoral response was specific against the allergen used. Finally, cytokine production observed in treated animals indicates a Th2 biased response with bigger intensity in allergic+colitic OVA stimulated splenocytes with an increased secretion of IL-2, IL-4 and IL-5.

Altogether, results explain an important specific allergic response to OVA in two allergic groups and which is bigger in concomitant allergic and colitic animals, indicating a good design of the experimental model and no interference in the interpretation of results. Similarly to IBD patients, inflammation increases the allergic symptoms and this fact may be relevant to prevent the risk of anaphylaxis in allergic or IBD patients.

In the last part of this thesis, we have studied the influence of atopy in the symptoms of two experimental protocols of IBD, DSS colitis, which mimics human IBD, and DNFB/DNS colitis which is similar to food allergy and also IBS. Innovations of this work are not only the establishment of a model of atopic adult mice, but also the combination with an experimental model of colitis, and interrelate both results to study how important is the atopic situation which is initiated in childhood, in the posterior colitis process developed in adulthood.

"Atopic career" is the most usual way of earlier allergic children to develop other inflammatory diseases subsequently in their life. In this situation we

hypothesized that abnormalities in the intestinal mucosa produced by allergy, frequently subclinical, are responsible of a higher response to diverse inflammatory situation as IBD or IBS.

The animal model of atopic adult mice was based in the short CMPA model which was established and discussed previously in this chapter (Bailon et al., 2012). This model allowed us to reproduce an atopic status of animals 35 days after the first oral challenge with CMP plus cholera toxin as adjuvant and boosted 5 times. Atopic status was evident in internal organs as colon and small intestine with a diminished weight/length ratio and also bigger spleen size, whereas external aspect (weight, food consumption or stool consistency) was similar to healthy group. In addition, IgE plasma levels were elevated in atopic mice at day 35 but biochemical colonic determination of MPO and Igs showed normal levels after an elevation in acute allergic status. These results showed a classical humoral allergic response associated with an intestinal distention which is probably related with a subclinical increased intestinal permeability. Most of the alterations observed are similar to human atopic condition.

Once atopic adult mice group was obtained, we explored the possibility of combining this situation with an experimental model of colitis and observe the response of atopic individuals to this new aggression. We utilized two different hapten-induced colitis models: DSS and DNFB/DNS.

In the DSS induced colitis model, two doses were used (1.5% and 3.5%) with the aim to observe the different response to a weak or intense colitis. Differences in the seriousness of colitis between low and high doses were evident in DAI (disease activity index) at day 6 of administration of DSS in drink water and a significant decrease in this value was observed in atopic mice in both doses which was associated with an improvement in the weight loss and faecal consistency. Similarly, lower MPO values indicating less leukocytes infiltration was observed in atopic colitic mice versus naïve colitic mice, as well as colon weight/length ratio showed a recovery in the disease. When the humoral response in colitic animals is analysed, a complex response is observed, with a significant reduction of IgE, IgG1, IgG2a and IgA plasma levels in severe naïve and atopic colitic group, whereas in colonic samples the results are not correlated. Nevertheless, a clear reduction in inflammatory response to both doses of DSS is evident in atopic mice even though conclusion cannot be extracted about humoral behaviour in this combined experiment.

Finally, another colitis experimental model was analysed in atopic mice with the aim to clarify, as far as possible, the humoral response in this combined experimental protocol. Thus, we used DNFB/DNS, an hapten that induces a mild and superficial inflammation in the mucosa which reverts very fast and which may be useful to imitate several human conditions as food allergy or IBS as we have previously discussed (Bailon et al., 2011). Unlike DSS induced colitis, a more intense colitis developed in atopic mice in response to DNFB/DNS with intestinal inflammation in accordance with macroscopic features as colonic patches, weight/length ratio and spleen weight at 24 hours after DNS administration. In the humoral response, quite more complex results were also observed in this experiment with an increment of IgE in both plasma and

colon significant at 72 hours after DNS administration. Moreover, a decrease in IgA colonic levels could add evidences to the bigger inflammation observed in atopic mice.

The new experimental models developed have important features such as robustness, reproducibility and simplicity in the protocols and closeness to human illness. In this regard a new animal model of atopy to the allergen cow´s milk protein (CMP) has been characterized. The distension observed in both small and large intestine may indicate an increase in intestinal permeability which allows the activation of immune cells. Also, lower levels of IgA in colon samples may contribute to moderate inflammation status which remains subclinically. DSS induced colitis is characterized by a Th1/Th17 biased response in Balb/c mice and for that, an allergic reaction characterized by a Th2 biased response could be the origin of a lower colitic response due to competition between Th1 and Th2 cells which inhibit each other by the cytokines released by them. Unlike that, DNFB/DNS colitis model induce a hypersensitivity reaction in the colon with lymphocytic infiltration and IL-5 production typical of Th2 immune reaction. Mast cells activation in this colitic model and the increase in intestinal permeability caused by atopy could be added and they could also explain the bigger colitic response observed in this combined experimental method.

Therefore, as a conclusion of our work we must emphasize the importance of the selection of the animal model when conducting a project. To achieve this objective it is vitally important the model optimization considering the mice strain, the parameters to be analysed, etc., because a lack of rigor at this point can lead to hasty conclusions as occurred with the model of DNFB/DNS which was considered as a model of IBD instead of an IBS model.

In this thesis we have extended the analysis and optimization of two animals models, a food allergy one (CMPA) and an intestinal inflammation model (DNFB/DNS). We have also made clear the importance of model selection for the demonstration of one hypothesis since in function of the chosen model it is possible to have an answer or another. That was confirmed when we studied how IBD affects allergy, because DSS improve the allergy condition whereas DNFB worsens it. Also, it is important to highlight that this was not an easy task for various reasons such as having to comply with the ethical standards of animal studies because we used several protocols simultaneously.

And finally, for the first time we have demonstrated, through the simultaneous use of different animal models, that it is possible to answer the kind of questions that we made to us at the beginning of this thesis.

REFERENCES

Allez, M., & Mayer, L. Regulatory T cells: peace keepers in the gut. Inflamm Bowel Dis. 2004;10(5):666-676.

Bailon, E., Cueto-Sola, M., Utrilla, P., Nieto, A., Garrido-Mesa, N., Celada, A., . . . Comalada, M. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis. 2011;17(10):2087-2101.

Bailon, E., Cueto-Sola, M., Utrilla, P., Rodriguez-Ruiz, J., Garrido-Mesa, N., Zarzuelo, A., . . . Comalada, M. A shorter and more specific oral sensitization-based experimental model of food allergy in mice. J Immunol Methods. 2012;381(1-2):41-49.

Berin, M. C. Mucosal antibodies in the regulation of tolerance and allergy to foods. Semin Immunopathol. 2012;34(5):633-642.

Brandtzaeg, P., Carlsen, H. S., & Halstensen, T. S. The B-cell system in inflammatory bowel disease. Adv Exp Med Biol. 2006;579:149-167.

Brkic, T., Banic, M., Anic, B., Grabarevic, Z., Rotkvic, I., Artukovic, B., . . . Hernandez, D. E. A model of inflammatory bowel disease induced by 2,4-dinitrofluorobenzene in previously sensitized BALB-c mice. Scand J Gastroenterol. 1992;27(3):184-188.

Chehade, M., & Mayer, L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol. 2005;115(1):3-12; quiz 13.

Furrie, E., Macfarlane, S., Cummings, J. H., & Macfarlane, G. T. Systemic antibodies towards mucosal bacteria in ulcerative colitis and Crohn's disease differentially activate the innate immune response. Gut. 2004;53(1):91-98.

Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H. S., McGuirk, P., van Eden, W., . . . Rook, G. A. Mechanisms of disease: the hygiene hypothesis revisited. Nat Clin Pract Gastroenterol Hepatol. 2006;3(5):275-284.

Lara-Villoslada, F., Olivares, M., Jimenez, J., Boza, J., & Xaus, J. Goat milk is less immunogenic than cow milk in a murine model of atopy. J Pediatr Gastroenterol Nutr. 2004;39(4):354-360.

Li, X. M., Schofield, B. H., Huang, C. K., Kleiner, G. I., & Sampson, H. A. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol. 1999;103(2 Pt 1):206-214.

Lillestol, K., Helgeland, L., Arslan Lied, G., Florvaag, E., Valeur, J., Lind, R., & Berstad, A. Indications of 'atopic bowel' in patients with self-reported food hypersensitivity. Aliment Pharmacol Ther. 2010;31(10):1112-1122.

Liu, L. L., Yao, H., Zhang, X. L., Zhang, H. L., Chao, P. L., Tong, M. L., . . . Yang, T. C. Characteristics of patients suffering from cow milk allergy. Int Immunopharmacol. 2012;14(1):94-98.

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., . . . Kaplan, G. G. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;142(1):46-54 e42; quiz e30.

Montero Vega, M. T. New aspects on inflammation in allergic diseases. Allergol Immunopathol (Madr). 2006;34(4):156-170.

Rijnierse, A., Koster, A. S., Nijkamp, F. P., & Kraneveld, A. D. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. J Immunol. 2006;176(7):4375-4384.

van Schaik, F. D., Oldenburg, B., Hart, A. R., Siersema, P. D., Lindgren, S., Grip, O., . . . Bueno-de-Mesquita, H. B. Serological markers predict inflammatory bowel disease years before the diagnosis. Gut. 2013;62(5):683-688.

Virta, L. J., Ashorn, M., & Kolho, K. L. Cow's milk allergy, asthma, and pediatric IBD. J Pediatr Gastroenterol Nutr. 2013;56(6):649-651.

Chapter 8

Conclusions

CHAPTER 8: CONCLUSIONS

The main objective of this thesis has consisted in answer the question of whether alterations in the immune system in early life, for example as a result of a food allergy, may pose a greater risk of experiment other diseases of immunological character in adulthood as inflammatory bowel disease. Also we wanted to figure out if an allergic process is modified in individuals which also suffer from bowel inflammation. Using various animal models we have tried to answer these questions. Thus, the conclusions we have drawn from this dissertation are:

CONCLUSION I: Optimization of various experimental models:

A) It has been developed a shorter CMPA mouse model based on oral sensitization to cow´s milk protein which the following advantages:
   - It triggers a more intense, specific and targeted immune response to the antigen, favouring the consistency of the results.
   - It allows reducing the number of animals in each experimental group, and this together with the reduction of the sensitization period required (from 6 weeks to 2 weeks) also means a decrease in the cost of the experiment.
   - With the reduction of the experimental time, the exposure to the adjuvant (cholera toxin, CT) has also decreased, therefore, CT control groups do not interfere with the data interpretation.

B) It has been optimized the DNFB/DNS mouse model, originally described as a model of intestinal bowel disease (IBD). In this model the main conclusions we have reached are:
   - It should not been consider as an IBD model because it features resemble more the immune response detected during irritable bowel syndrome, so it could be a good model for this pathology where the experimental models are scarce.
   - T and B cell responses are involved and the humoral response also plays a key role, in contrast with the modest participation of macrophages.
   - It is an acute inflammation model where the humoral response does not experiment a significant increase after a second exposure to the antigen. The fast return to basal levels let to use a posterior model in the same animals without interferences.

CONCLUSION II: The possible influence of intestinal inflammation on allergic individuals has been analysed through the DNFB/DNS and the OVA model respectively. We have demonstrated that:
   - There is an exacerbated antigen-specific allergic response in animals with colitis, mainly due to the immune modulation resulting during the DNFB/DNS model.

CONCLUSION III: The modulatory effect of CMPA on others intestinal pathologies with a immune component has been evaluated. Using the CMPA, DSS and DNFB/DNS models we have proved that:

- Atopic process of the CMPA model attenuates the response observed in DSS model.
- Atopic process of the CMPA model increases the response observed in DNFB/DNS model.

Resumen de la tesis doctoral en español

(Spanish summary of the doctoral thesis)

RESUMEN DE LA TESIS DOCTORAL EN ESPAÑOL

INTRODUCCIÓN

1. Antedecentes

La mucosa intestinal es uno de los principales órganos de defensa del organismo debido a su constante contacto con el exterior a través de los alimentos y de los alérgenos, microorganismos y carcinógenos que los acompañan. Dicha función defensora se lleva a cabo de una forma inespecífica con su especial estructura anatómica (capa mucosa, células epiteliales, lámina propia y capa submucosa) y, de una forma específica, con la ayuda de múltiples componentes externos e internos especializados, donde la microbiota y el sistema inmune juegan un papel clave.

La homeostasis intestinal se alcanza no solo a través de la regulación de la función de barrera de la mucosa intestinal, sino también reduciendo la respuesta inmune frente a bacterias y antígenos presentes en los alimentos sobre todo gracias a células como las células epiteliales intestinales. Uno de los principales dilemas del sistema inmune es la identificación de las sustancias extrañas al organismo, reconociendo claramente los componentes propios y diferenciando las sustancias peligrosas de aquéllas que no lo son. En condiciones normales, esta tarea se realiza eficazmente en el proceso denominado tolerancia con una participación importante del aparato digestivo (tolerancia oral) donde la vía oral es la principal ruta de entrada de sustancias ajenas y potencialmente peligrosas como alérgenos y carcinógenos. Se puede definir la tolerancia oral como el estado de inhibición activa de la respuesta inmune frente a un antígeno, por medio de la exposición previa a ese antígeno por vía oral, que confiere una tolerancia al antígeno tanto local como sistémica.

Durante los primeros años de vida el sistema inmune va entrando en contacto con antígenos ajenos y por tanto, se va desarrollando la tolerancia oral a ellos. Un sistema inmune inmaduro puede llevar a que se altere la tolerancia oral u homeostasis intestinal de forma que no se diferencie de forma adecuada lo propio de lo ajeno. Esto conducirá a la aparición de enfermedades inflamatorias como por ejemplo la IBD o alérgicas como la alergia alimentaria, la celiaquía o el asma. Cuando se rompe la tolerancia u homeostasis intestinal se produce la inflamación. La inflamación es una respuesta fisiológica de defensa frente a agentes extraños y se puede clasificar en aguda y crónica. En la inflamación aguda se produce una activación de células del sistema inmune como macrófagos, mastocitos y células dendríticas (DC), las cuales son activadas por los antígenos liberando mediadores proinflamatorios como TNFα, IL-8, IL-6, IL-1, Prostaglandina E_2 (PGE2) y Óxido nítrico (NO). Cuando este sistema (inmunidad innata) no es capaz de eliminar el agente patógeno, se pone en marcha la respuesta de células especializadas como los linfocitos B productores de anticuerpos, linfocitos T colaboradores (helper) $CD4^+$ y linfocitos T citotóxicos $CD8^+$. En función de

cómo se activen las células T CD4+ generarán distintos tipos de respuesta y secreción de citocinas. Así los linfocitos T activados por el antígeno y otras señales co-estimuladores como la presencia de IL-12 generarán linfocitos Th1 que secretaran IFNγ, la co-estimulación con IL-4 (producida por células NK, mastocitos, basófilos y linfocitos $CD4^+$maduros) generará un tipo de linfocito Th2 secretor de IL-4, IL-10 e IL-13 y, por último, la presencia de IL-23 (producida por macrófagos y células dendríticas) favorecerá un fenotipo Th17 que secretará IL-17 principalmente.

Cuando no sea posible finalizar la inflamación aguda o bien se vayan produciendo procesos inflamatorios recurrentes aparecerá la inflamación crónica. En ella se detecta una infiltración continua en el foco dañado de células como macrófagos, linfocitos y células plasmáticas que conducen a la formación de tejido fibroso característico de la cronicidad del proceso. Esta inflamación crónica conduce a distintas patologías como por ejemplo la IBD.

La IBD, engloba la colitis ulcerosa (Ulcerative colitis, UC) y la enfermedad de Crohn (Crohn's Disease, CD), caracterizadas por su comportamiento cíclico con periodos de remisión y periodos de agudización y con diarrea, dolor abdominal, pérdida de peso, hemorragia intestinal, fiebre, fatiga y malestar general como signos más importantes. A veces aparecen síntomas extra-intestinales como artralgias. Aunque existen similitudes entre ambas enfermedades, las diferencias son notables en cuanto a la distribución de la inflamación, la afectación de la mucosa intestinal y la consistencia de las heces. Al igual que las alergias, son enfermedades relacionadas directamente con el estilo de vida occidental, con una incidencia creciente en todos los países industrializados.

Aunque la causa de la enfermedad inflamatoria intestinal no se conoce, se supone una ruptura de la tolerancia oral como consecuencia de factores genéticos y medioambientales. En los pacientes con la enfermedad activa o en fase de remisión, se produce un incremento de la permeabilidad intestinal que permite la llegada de sustancias hasta el sistema inmune de la lámina propia, desencadenando la respuesta inflamatoria. Hasta hace poco se pensaba que la enfermedad de Crohn se caracterizaba por una reacción de tipo Th1 asociada a un exceso de IFNγ, TNFα e IL-12, mientras que la colitis ulcerosa era una respuesta Th1/Th2 atípica con una producción de TNFα, IL1β e IL-6. Pero en la actualidad se dibuja un escenario más complejo con la participación de linfocitos Th17 e IL-17 en ambas patologías.

El tratamiento de estas patologías es sintomático y se basa en la disminución de la inflamación y los síntomas con la utilización de glucocorticoides, aminosalicilatos, inmunosupresores, anticuerpos monoclonales, antibióticos y probióticos.

Por otro lado, la alergia es una respuesta inflamatoria de tipo Th2 en la que individuos susceptibles responden a antígenos inocuos. Brevemente, la alergia se inicia con la participación de linfocitos Th2 que producen IL4 e IL-13, responsables del cambio en los linfocitos B para la producción de Inmunoglobulina E (IgE). Ésta activa a mastocitos y basófilos liberadores, entre otras sustancias, de aminas biógenas (histamina, serotonina) responsables de los síntomas de la alergia. También se liberan

mediadores como TNFα, IL-3, IL-5 y GM-CSF responsables de las fases temprana y tardía de la alergia y de la activación de linfocitos Th2. La participación de los eosinófilos es activada por los linfocitos Th2 y resulta crucial para el desarrollo de las reacciones alérgicas como el asma alérgica, gastroenteropatías eosinofílicas e incluso en las alergias IgE independientes.

La participación de los linfocitos T reguladores (Treg) en la alergia es importante ya que pueden disminuir la producción de IgE por parte de las células plasmáticas, pero no son capaces de anular una respuesta alérgica ya establecida.

Una alergia alimentaria se puede definir como "un efecto adverso para la salud derivado de una respuesta inmune específica que ocurre de forma repetida tras la exposición a un determinado alimento". El creciente número de alergias en los países desarrollados se puede explicar por la "hipótesis de la higiene" que justifica los fallos en la respuesta inmunológica en base a un déficit en el contacto con sustancias extrañas durante los primeros años de la vida. En la actualidad se sospecha que cualquier tipo de respuesta inmune (Th1 y/o Th2) durante la infancia ayuda a la maduración del sistema inmune y el desarrollo de la tolerancia.

El incremento de la permeabilidad intestinal es una constante en los pacientes que sufren de alergia alimentaria, pero no se puede afirmar con certeza si se trata de la causa o la consecuencia de la misma. Además de los síntomas intestinales como diarrea y dolor abdominal, la alergia alimentaria puede afectar a otros órganos como la cavidad oral, la piel o el tracto respiratorio. Aunque la alergia alimentaria se caracteriza por la presencia de anticuerpos IgE, también existe una alergia independiente de esta inmunoglobulina, con una respuesta intestinal retardada debido a la producción de TNFα.

Los alimentos que más frecuentemente provocan alergia son la leche de vaca, los frutos secos y las frutas, siendo la alergia a las proteínas de la leche de vaca la que afecta a un mayor número de niños (2-3 % de la población infantil). En el caso de identificar el agente alergógeno, el principal tratamiento de la alergia alimentaria es la supresión total del consumo del alimento. En caso de no haber identificado al agente causal, la principal estrategia farmacológica consiste en el empleo de glucocorticoides y antihistamínicos administrados de forma sistémica. Algunos tratamientos específicos del alérgeno consisten en la exposición al mismo por vía oral, sublingual o cutánea para conseguir la desensibilización del individuo. Otras terapias incluyen la utilización de probióticos, proteínas modificadas, terapia anti-IgE, y otras técnicas experimentales que obligan a una investigación constante en la búsqueda de soluciones a una de las más frecuentes enfermedades de hoy en día.

La alergia a la leche de vaca durante la infancia parece estar relacionada con la lactancia artificial, ya que la mayor incidencia se encuentra entre niños que no han sido alimentados mediante lactancia materna. Un tema importante es la relación entre la alergia infantil y el desarrollo posterior de otras enfermedades en la edad adulta; la denominada "carrera atópica" que parece justificar el dato de que entre 30-80% de los niños que han padecido alergia a la leche de vaca en la infancia y/o dermatitis atópica, desarrollan asma u otras alergias respiratorias en la edad adulta.

2. MODELOS ANIMALES

La utilización de modelos experimentales en animales persigue la observación de los cambios bioquímicos básicos característicos de las patologías humanas. Debido a las diferencias anatómicas, fisiológicas e inmunológicas entre los animales y el hombre, la interpretación y extrapolación de los resultados debe hacerse con precaución.

2.1 Modelos animales de alergia

La mayor parte de la investigación en alergia se hace utilizando ratones como animal de experimentación debido a la disponibilidad de reactivos inmunológicos y de biología molecular, incluyendo animales modificados genéticamente. Otros animales como cobayas, ratas, perros, etc., son poco útiles por la dificultad de manejo.

Es importante que el modelo animal sea lo más parecido posible a lo que ocurre en el humano en cuanto a vía de administración del alérgeno, respuesta inmune, reactividad de proteínas (carácter alérgico), ausencia de adyuvantes y propiedades reproducibles.

La cepa de ratones Balb/c se usa frecuentemente por su capacidad para desarrollar una respuesta de tipo Th2 frente a numerosos antígenos como ovoalbúmina (OVA), aspergillus, huevo o el polvo doméstico. También son capaces de desarrollar síntomas como diarrea y signos de anafilaxis, además de las respuestas típicas mediadas por mastocitos y eosinófilos, así como la producción de IgE e IgG1.

Los protocolos incluyen una fase de sensibilización seguida de otra de re-sensibilización para establecer definitivamente la respuesta alérgica. En las primeras 24 horas tras el segundo contacto, los ratones producen anticuerpos específicos tipo IgE e IgG1 característicos de la respuesta Th2. Se ha investigado la influencia de la vía de administración del antígeno alimentario en la aparición de anticuerpos específicos, encontrándose que la administración intraperitoneal (i.p.) produce una respuesta más intensa que la vía oral, sin embargo, esta vía requiere menor cantidad de alérgeno con un contacto intermitente. Por otra parte, la gran capacidad para desarrollar tolerancia oral en los roedores puede interferir en la respuesta obtenida. Para solventar el problema de la tolerancia oral se suelen utilizar coadyuvantes como el hidróxido de aluminio, la toxina colérica o el adyuvante de Freund, todos ellos destinados a mejorar la respuesta inmunológica de los animales frente al alérgeno administrado. Se observan síntomas generales como la bajada de temperatura, pérdida de peso o diarrea, así como una respuesta Th2 caracterizada por la aparición de IgE e IL-4. También se aprecia la participación de linfocitos Treg en su papel de control de la tolerancia oral y de otras citocinas supresoras de la respuesta inmune como IFNγ, IL-10 e IgA.

La mayor limitación de los modelos animales de alergia alimentaria viene de la incapacidad para detectar alérgenos en los alimentos, predecir el poder de nuevas

proteínas de alimentos para producir alergia o imitar la sensibilización y respuestas alérgicas en humanos.

Dentro de los modelos de alergia alimentaria existen dos muy interesantes como son la alergia inducida por la Ovoalbúmina (OVA) y la alergia a las proteínas de la leche de vaca (APLV). En el caso del modelo de la OVA, se utiliza la proteína del huevo, la OVA, que es uno de los alérgenos mejor conocidos en experimentación animal. Se suele administrar i.p. con un coadyuvante para obtener una buena respuesta alérgica ya que la vía oral da lugar a una reacción menos intensa. El adyuvante suele ser el hidróxido de aluminio ($AlOH_3$) que facilita la respuesta de tipo Th2. Se puede apreciar claramente una elevación de IL-4, IL-5, IL-13 e IL-10 así como la producción de IgE y eosinofilía. Además la elevación de IL-17 demuestra la participación de linfocitos Th17 y Th1 al igual que ocurre en pacientes con alergia.

En el caso de la APLV, se administra leche de vaca a ratones con toxina colérica como coadyuvante que estimula la respuesta Th2, incrementa la permeabilidad intestinal y la producción de IgG1. Los ratones recién destetados (Balb/c o C3H/HeJ) se sensibilizan con leche de vaca administrada vía oral semanalmente. A partir de la 3ª semana desarrollan la alergia y a la 6ª semana se obtiene la máxima respuesta. Tras una dosis de carga, este modelo reproduce síntomas generales como el picor, diarrea, pilo-erección, dificultad respiratoria, cianosis, temblor, convulsiones y muerte.

2.2 MODELOS ANIMALES DE INFLAMACIÓN INTESTINAL

La mayoría de los modelos animales reproducen una respuesta Th1, por lo que permiten un adecuado estudio de la enfermedad de Crohn. Sin embargo, los modelos con respuesta Th2 para el estudio de la colitis ulcerosa son limitados.

Uno de los modelos de IBD más utilizados en ratón es el de la colitis inducida por sulfato de dextrano sódico (DSS). El DSS (2-5%) se administra en el agua de bebida durante 4-9 días produciendo inflamación en el colon que puede ser aguda o bien crónica dependiendo del tipo de tratamiento del DSS y de la cepa de ratón. Los síntomas son similares a la enfermedad humana con presencia de sangre en heces, pérdida de peso, acortamiento del intestino, úlceras en la mucosa e infiltración de neutrófilos. El perfil inmunológico de la inflamación intestinal obtenida es típico de la respuesta Th1/Th17 correspondiente a una inflamación aguda. En el caso de la colitis crónica se puede apreciar una respuesta de tipo Th2.

Existe otro modelo de tipo Th1, el modelo de colitis inducida por ácido trinitrobencenosulfónico (TNBS) que ha sido ampliamente utilizado en ratas durante años. Este modelo consiste en la administración intracolónica de TNBS disuelto en alcohol que origina una inflamación aguda en el intestino grueso de los animales con una gran infiltración de linfocitos T y macrófagos, debido a una respuesta típica Th1. Este modelo posee una gran similitud con la enfermedad de Crohn humana como así lo refleja la participación parcial de los linfocitos Th2 observada en ratones $IL12^{-/-}$. Así mismo, los linfocitos Th17 juegan un importante papel en este modelo experimental.

La administración crónica de TNBS puede originar una colitis crónica con presencia de fibrosis intestinal.

Por último, en el año 2006, Rijnierse y colaboradores describieron un modelo de colitis con dinitrofluorobenceno/ácido dinitrosulfónico (dnfb/dns) en ratones que presentaba un perfil Th1 especial. El modelo consiste en la sensibilización cutánea de los ratones Balb/c con DNFB, seguida de una dosis intrarrectal de DNS. La respuesta encontrada en los animales entre las 6-72 horas tras el segundo contacto se caracteriza por la activación de mastocitos, acompañada de una producción de TNFα en el colon producido por estas células y de la participación de la población de linfocitos T reguladores $CD8^{+}CD28^{-}$. El problema de este modelo es que no ha sido del todo caracterizado y por tanto no se sabe si reproduce la IBD en humanos ni si puede ser de utilidad.

OBJETIVOS

Para intentar contestar a los interrogantes que se nos plantearon en un inicio se tuvieron que optimizar modelos animales de distintas patologías y sobretodo optimizar distintos parámetros que nos interesaban para contestar el objetivo general del proyecto.

El grupo de Investigación ya disponía de experiencia en varios modelos experimentales en ratón como el del DSS o TNBS de colitis experimental o bien el de la OVA o APLV como modelos de alergia alimentaria. No obstante, se introdujeron adaptaciones en el modelo de la APLV ya descrito y se optimizó un nuevo modelo de colitis experimental.

El proyecto se dividió en los siguientes objetivos concretos:

OBJETIVO I: Selección de los modelos experimentales a estudiar:

A) Establecimiento del modelo experimental de la APLV en ratón con un protocolo más corto.
B) Optimización y análisis del modelo de DNFB/DNS en ratón como modelo de inflamación intestinal.

OBJETIVO II: Análisis del posible efecto de una inflamación intestinal sobre individuos alérgicos. Así, se analizará el efecto del modelo de DNFB/DNS sobre la incidencia y gravedad de la alergia a la OVA.

OBJETIVO III: Evaluación del posible efecto modulador de la APLV sobre segundas patologías. Así, se analizará el efecto de la APLV sobre la incidencia y gravedad de enfermedades como la enfermedad inflamatoria intestinal.

RESULTADOS

Los resultados de la presente tesis doctoral se han organizado en cuatro apartados, siendo cada uno de estos un artículo original que han sido publicados en diversas revistas científicas o como en el caso del último artículo, se encuentra enviado pero aún no se ha publicado.

1. LA COLITIS INDUCIDA EN RATONES POR DNFB-DNS NO DEBERÍA SER CONSIDERADA UN MODELO DE ENFERMEDAD INFLAMATORIA INTESTINAL.

(Bailón E, Cueto-Sola M, Utrilla P, Nieto A, Garrido-Mesa N, Celada A, Zarzuelo A, Xaus J, Gálvez J, Comalada M. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis. 2011; 17:2087-101.)

Ante la ausencia de modelos aceptables para el estudio preclínico de la UC, se investigó el perfil de respuesta inmunológica en el modelo experimental de colitis que emplea DNFB y DNS, propuesto recientemente como modificación de otro ya existente en el que se utilizaba exclusivamente el DNFB como hapteno productor de colitis.

Rijnierse y col. describieron en el año 2006 el modelo del DNFB/DNS como un modelo de colitis colónica que estaba asociado con la activación de mastocitos sin participación de macrófagos y donde se demostraba que la expresión de TNFα derivada de mastocitos cumplía un papel clave. En nuestro caso nos interesaba encontrar un modelo Th2 para el desarrollo de la presente tesis doctoral con una mayor similitud a la UC, puesto que el modelo del TNBS en ratón era un modelo Th1 y el DSS era un modelo Th1-atípico. Por tanto, decidimos profundizar y optimizar más este modelo.

Aunque el modelo experimental original de colitis inducida por DNFB permite el estudio de algunos aspectos de la UC humana, el principal problema que entraña es la dificultad de encontrar una respuesta homogénea entre experimentos. En la mayor parte de los casos se observa una inflamación moderada de la mucosa intestinal, mientras que en otras ocasiones aparece una rápida implantación de la inflamación con intensa hemorragia intestinal y la muerte de muchos animales. El modelo del DNFB/DNS parece permitir una evolución más lenta de la inflamación intestinal y la obtención de resultados más consistentes. Sin embargo, en los animales tratados aparece una alta respuesta de mastocitos sin la participación de macrófagos con un papel predominante de TNFα. Debido a la escasez de datos aportados por Rijnierse y col. que profundizaran en dicho modelo experimental, llevamos a cabo una exploración del perfil inmunológico de esta propuesta con el fin de afianzar su utilidad en el estudio de la UC.

Para llevar a cabo el estudio se desarrollaron distintos experimentos con ratones machos Balb/c. En el primer experimento se valoró la colitis inducida tras la sensibilización de los animales en el día 0 por la administración de forma tópica de DNFB en el abdomen rasurado y en las patas, un segundo contacto con DNFB el día 1 en el abdomen y el día 5 se realizó la re-sensibilización vía rectal con DNS. Los grupos experimentales fueron: i) sano, ii) DNFB/DNS, iii) DNFB, iv) DNS. Los ratones se sacrificaron los días 1, 2, 3, 5 y 7 tras la re-sensibilización.

En un segundo experimento se estudió la colitis que se desarrollaba al administrar una segunda re-sensibilización rectal con DNS el día 15. Los animales se sacrificaron tras 24-72 horas de esta segunda administración intrarrectal. Finalmente,

se realizó un tercer experimento en el que se provocaba la colitis con la administración rectal de TNBS, modelo típico de respuesta Th1 y que lo utilizamos como control para comentar ambos modelos.

Los cambios observados por el modelo del DNFB/DNS en ratones Balb/c en el tejido del colon se corresponden con una inflamación moderada del intestino si lo comparamos con la inflamación provocada por el TNBS, y que se resuelve espontáneamente en menos de 5 días. Además, de forma similar a lo publicado para el modelo de DNFB, el inicio de la colitis es poco homogéneo, siendo inexistente en el 10% de los animales y letal para el 30-40%. El análisis microscópico confirma una escasa infiltración de macrófagos, sugiriendo una mayor participación de los linfocitos T y B en el proceso inflamatorio, como lo indica el mayor número de lesiones observadas en el intestino grueso en comparación con el protocolo del TNBS y confirmado por la inmunohistoquímica. La contribución de los mastocitos en este modelo es muy modesta, a pesar de lo publicado con anterioridad. Estos resultados son acordes con la mínima presencia de histamina en el plasma durante la fase inflamatoria. Al contrario de lo que ocurre en otras especies animales (hombre, rata, etc.), en las que los mastocitos juegan un importante papel durante la inflamación, en el ratón se ha descrito una mínima contribución en distintos modelos animales a menos que exista una infección por nematodos. La presencia de TNFα es insignificante después de la re-sensibilización con DNS, mientras que IL-2 e IL-5 están muy aumentadas, un escenario muy diferente al expuesto por los autores que desarrollaron el modelo experimental, algo de difícil explicación. La escasa presencia de macrófagos puede justificar la ausencia de TNFα, aunque los niveles de esta citocina no se correlacionan con el grado de la enfermedad, ni siquiera en humanos. Los altos niveles de IL-5 pueden tener un origen linfocitario y, aunque su papel no está completamente dilucidado en la inflamación intestinal, parece que cumple funciones reguladoras de linfocitos B y la producción de anticuerpos, aunque la ausencia de esta citocina no altera la evolución de la enfermedad. La evaluación de la respuesta humoral del modelo tampoco coincide con la expuesta por los autores avalistas, ya que fue expuesto como IgE independiente, mientras que se han encontrado niveles elevados de IgE tanto en plasma como en colon, y una expresión retardada de IgA.

Debido a todo lo anterior, este modelo experimental no debe ser considerado válido para el estudio de la enfermedad inflamatoria intestinal, teniendo en cuenta que la inflamación moderada que produce la presencia del hapteno no es suficiente para provocar la pérdida de la integridad de la mucosa intestinal que permita la infiltración de antígenos luminales y ponga en marcha la respuesta de la inmunidad adquirida. Además, la inflamación se resuelve espontáneamente en los primeros 5 días sin capacidad para agudizarse tras una segunda re-sensibilización con el antígeno. A pesar de ello, este modelo aporta una respuesta inflamatoria similar a la hipersensibilidad retardada de contacto, con la participación de linfocitos T y B y la respuesta humoral en un papel predominante, como se ha demostrado en modelos de dermatitis de contacto provocada por DNFB. Debido a la complejidad de la respuesta encontrada en este modelo experimental, se puede deducir que se asemeja más a situaciones de alergia alimentaria con respuestas IgE-dependientes e independientes, así como a la respuesta del síndrome de intestino irritable (IBS), una patología que

también requiere de nuevos modelos experimentales para su estudio. La optimización que hemos llevado a cabo de este modelo nos ha servido de base para posteriores estudios de la presente tesis doctoral. Es el caso de la evaluación de cómo la colitis influye en la alergia alimentaria (ver artículo 3) o del papel que tiene la alergia alimentaria a la leche de vaca en ratones sanos sobre la inflamación intestinal (ver artículo 4).

2. MODELO EXPERIMENTAL BASADO EN LA SENSIBILIZACIÓN ORAL, MÁS CORTO Y ESPECÍFICO, DE ALERGIA ALIMENTARIA EN RATONES.

(Bailón E, Cueto-Sola M, Utrilla P, Rodríguez-Ruiz J, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. A shorter and more specific oral sensitization-based experimental model of food allergy in mice J Immunol Methods. 2012; 381:41-9.)

Los modelos animales de alergia en ratas o ratones nos permiten estudiar nuevos abordajes terapéuticos y los mecanismos moleculares involucrados en la enfermedad. Sin embargo, en los modelos establecidos hasta hoy día, la sensibilización oral de alimentos en ratones requiere varias semanas y están asociados habitualmente a respuestas inmunológicas inespecíficas, por lo que ningún modelo animal en ratones resulta aceptable completamente, ya sea para la alergia a la leche de vaca u otras alergias alimentarias. Los modelos descritos utilizan varias vías de administración, diferentes coadyuvantes, tipos de antígenos, dosis y duración del periodo de sensibilización. El principal obstáculo en todos ellos es la capacidad innata de los ratones para desarrollar tolerancia oral a antígenos digestivos y por tanto se necesitan largos periodos de sensibilización.

Por ello, el principal objetivo que nos planteamos fue optimizar un protocolo de alergia alimentaria a la leche de vaca (CMPA) que fuera más corto que el descrito previamente en la literatura pero que presentara características similares. Para ello se emplearon ratones hembra Balb/c y comparamos 2 protocolos, uno que llamamos "protocolo largo" que consiste en un modelo convencional de alergia a la leche ya descrito previamente y que consta de 6 semanas de sensibilización oral y de una re-sensibilización también por vía oral. Y un segundo esquema, propuesto por nosotros, que llamamos "protocolo corto" donde el periodo de sensibilización constaba sólo de 2 semanas aunque la re-sensibilización debía hacerse por vía i.p.

El estudio se ha llevado a cabo con varios grupos experimentales: i) sano, ii) sensibilizado 6 semanas + re-sensibilización, iii) toxina colérica 6 semanas, iv) no sensibilizado 6 semanas + re-sensibilización, v) sensibilizado 2 semanas + re-sensibilización, vi) toxina colérica 2 semanas, vii) no sensibilizado 2 semanas + re-sensibilización. En el protocolo original largo se realiza una sensibilización oral con proteína de leche de vaca más toxina colérica como coadyuvante durante 6 semanas (una vez por semana) y una re-sensibilización oral el día 48, mientras que en el protocolo corto se hace una sensibilización vía oral cada 3 días durante 2 semanas y una re-sensibilización i.p. el día 18. Los grupos no sensibilizados pero que reciben la re-sensibilización así como los que reciben solo toxina colérica sirven de control.

Según nuestros resultados, la administración de toxina colérica a los animales de experimentación, en ausencia de proteína de leche de vaca, provoca una respuesta inmune no selectiva caracterizada por diarrea, edema de colon, agrandamiento del bazo, aumento de histamina plasmática, producción de IL-4 y aparición de IgG1, IgE o IgG2a. El mecanismo de esta inducción inmunológica de tipo Th2 todavía no está completamente dilucidado y así, se han encontrado resultados contradictorios, con una supresión de la respuesta inmune en lugar de inducción cuando se promueve la secreción de IgA o una respuesta de IgG2a asociada a Th1.

La observación de una respuesta inmune reducida en los animales tratados exclusivamente con toxina colérica podría deberse, no a un efecto directo sino a la elevación de una respuesta alérgica a otros antígenos alimentarios presentes en la dieta, un proceso que probablemente necesitará más de 2 semanas para llevarse a cabo. En este aspecto, la proteína de la leche de vaca sola, necesita de al menos 5 semanas para inducir una reacción alérgica, según otros autores. También se ha publicado una reactividad cruzada entre las proteínas de soja y la de la leche de vaca en animales de experimentación, lo cual puede justificar algunos de los resultados encontrados en este experimento.

Los datos encontrados demuestran que el nuevo protocolo presenta algunas ventajas respecto a los otros métodos publicados. En resumen, se reduce el tiempo de experimentación en dos tercios, el grupo control con toxina colérica sola no interfiere en la interpretación de los resultados, permitiendo alcanzar significación en parámetros cruciales de la alergia como el score clínico o los niveles de IL-4. Por último, la respuesta obtenida es más específica de antígeno que la encontrada en el protocolo original ya que no se incrementa la cantidad de inmunoglobulinas totales en plasma o colon, mientras que se produce un incremento claro en la cantidad de IgG1 específica de la proteína de la leche de vaca, un hecho característico de la respuesta alérgica.

Un posible argumento en contra de este nuevo método sería la utilización de una re-sensibilización i.p. para conseguir una respuesta óptima. Aunque esta vía de administración no es la más fisiológica para el estudio de la alergia a la leche de vaca, es la mejor opción para poner mínimas cantidades de antígeno en los animales, consiguiendo el mantenimiento de una respuesta específica de antígeno. Si se utilizara la vía oral para la re-sensibilización se necesitarían grandes cantidades de proteína para asegurar la absorción de una cantidad suficiente de proteína intacta. Algunos autores proponen la utilización de inhibidores de la secreción gástrica para conseguir el mismo objetivo, sin embargo se puede perder la selectividad del antígeno.

También se observa una ausencia en las diferencias de IgE producida tanto en plasma como en colon, siendo esta inmunoglobulina de vital importancia en la alergia. Sin embargo ya se ha comunicado una pérdida de la respuesta dependiente de IgE en la alergia a la leche de vaca y otros antígenos alimentarios, puesto que la aparición de IgE depende del tipo de antígeno utilizado. Por ello, el resultado encontrado en el protocolo convencional puede deberse a la presencia de otros antígenos en la alimentación. Por último, la ausencia de IgA en el protocolo corto puede explicarse por la brevedad del mismo, sin embargo este hecho puede resultar beneficioso ya que esta inmunoglobulina podría bloquear el efecto de las otras.

En conclusión, el nuevo modelo desarrollado para el estudio de la alergia a la proteína de la leche de vaca que requiere solo 2 semanas de sensibilización, produce una respuesta inmune más intensa, específica y selectiva frente al antígeno utilizado. Por tanto favorece la consistencia de los resultados y permite la reducción del número de animales utilizados. Este modelo fue empleado para evaluar la respuesta inflamatoria en animales que presentaban atopia a la CMP (ver artículo 4).

3. LA COLITIS ACTIVA INCREMENTA LA RESPUESTA INMUNE A ANTÍGENOS ALIMENTARIOS EN RATONES.

(Cueto-Sola M, Bailon E, Utrilla P, Rodríguez-Ruiz J, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. Active colitis exacerbates immune response to internalized food antigen in mice. Int Arch Allergy Immunol. 2013; 162:214-24.)

Debido a las condiciones particulares que genera la enfermedad inflamatoria intestinal (IBD) en el aparato digestivo, se sospecha que se puede ver agravada una respuesta alérgica a alimentos. Sin embargo, la diferente especialización de la respuesta inmune en ambas patologías, Th1 en IBD y Th2 en alergia, pone en entredicho la posibilidad de que exista una interrelación entre ambas. Hasta el momento no se conoce cómo la sensibilización a antígenos alimentarios puede estar relacionada con la inflamación intestinal, sin embargo, en casi un tercio de los pacientes con IBD aparecen síntomas de la enfermedad como consecuencia del consumo de algún alimento. Así, la eliminación de la dieta de algunos alimentos como leche, huevos o pescado resulta en un beneficio evidente para el paciente.

Por tanto, el objetivo fundamental que nos propusimos fue analizar la posible relación existente entre la respuesta alérgica alimentaria y la inflamación intestinal usando métodos experimentales sencillos. Para ello, utilizamos un modelo de colitis (DNFB/DNS) y un modelo de alergia basado en la sensibilización y re-sensibilización con OVA i.p. y los combinamos (ver Figura 1). En ambos modelos la respuesta inmunológica es simple y reproducible, además, la administración i.p. del alérgeno permite conocer exactamente la cantidad que llega al interior del animal.

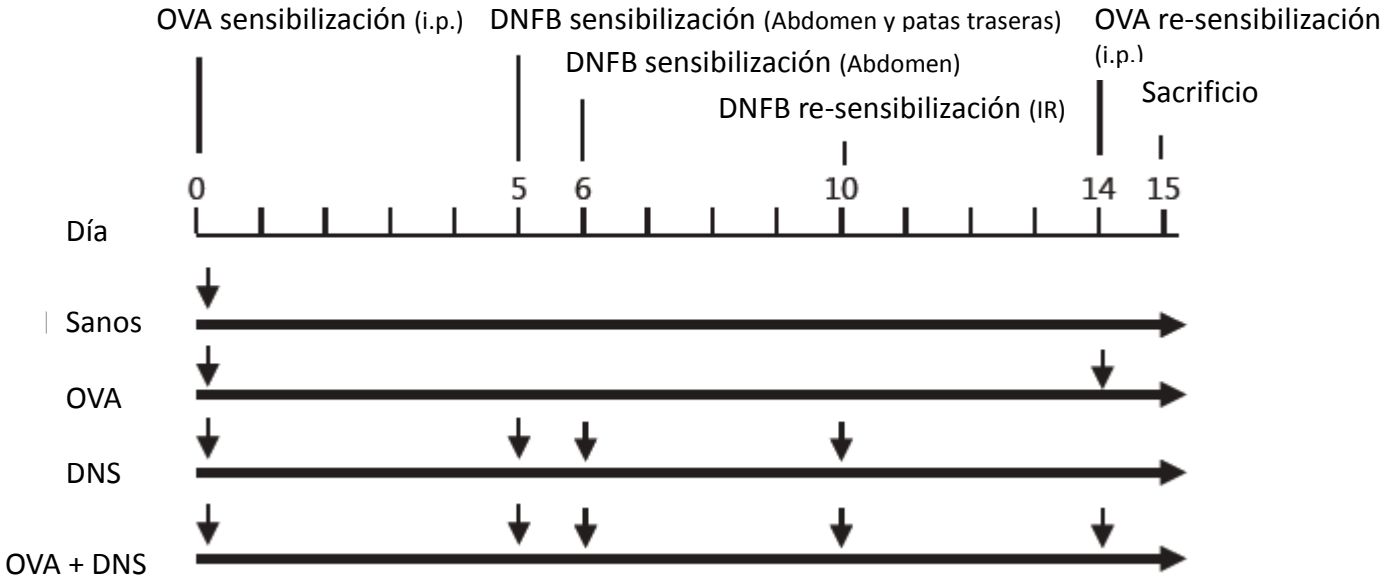

Figura 1. Esquema del protocolo empleado. (i.p.: intraperitoneal; IR: intrarrectal)

A modo de resumen (ver Figura 1) se planteó un estudio con diversos grupos experimentales: i) sano, ii) OVA, iii) DNS y iv) OVA + DNS. Se emplearon ratones

hembra Balb/c. Para la inducción de la alergia se utilizó OVA administrada vía i.p. con hidróxido de aluminio como coadyuvante. Cinco días después de la sensibilización con OVA se realizó la sensibilización con DNFB por vía tópica en el abdomen rasurado y las patas; el día 6 se administró sólo DNFB en el abdomen y el día 10 se realizó la re-sensibilización intrarrectal (IR) con DNS. Finalmente, el día 14 se procedió a la re-sensibilización con OVA administrada i.p.

La relación entre ambas patologías puede explicarse por tres factores principalmente. El primero son las alteraciones de la mucosa intestinal y la mayor permeabilidad que ocurre durante la inflamación intestinal que facilita la entrada de antígenos y el desencadenamiento de los síntomas, fundamentalmente en sujetos sensibilizados por factores ambientales. Sin embargo, esta hipótesis no solo no ha sido confirmada en ningún estudio, sino que se ha refutado en otros. Además, la variabilidad en la ruptura de la barrera intestinal de los distintos modelos experimentales hace que la interpretación de los resultados sea difícil. Por ello, se usó el modelo del DNFB/DNS en el cual se provoca una inflamación moderada que no altera la integridad de la mucosa.

En segundo lugar, se ha postulado como punto de relación entre las patologías, una posible reactividad cruzada entre los anticuerpos específicos de antígeno de la alergia y los anticuerpos generados durante la inflamación intestinal. Dicha relación se ha encontrado, por ejemplo, en la diabetes tipo I. La probabilidad de que se origine dicha interacción en nuestros modelos experimentales es mínima debido a la naturaleza de los agentes causantes de la enfermedad DNFB y OVA. Además, observando los resultados obtenidos del estudio “in vitro”, la respuesta que desencadenan los linfocitos tras la estimulación con OVA denota que es específica para este antígeno y no como consecuencia de una reacción cruzada con el DNFB/DNS.

Por último, se ha sugerido que la especialización de la respuesta inmune de la alergia y la inflamación intestinal pueden ser responsables de la interrelación. Las citocinas y factores de crecimiento originados como IL-3, IL-5 y GM-CSF de eosinófilos e IL-4 de mastocitos que se generan durante la UC contribuyen de forma crucial en los síntomas de la alergia ya que son claves para el desarrollo y el reclutamiento de las células efectoras de la alergia así como de su funcionalidad y la liberación de mediadores. Así, los pacientes con UC sufren con más frecuencia alergia alimentaria que los enfermos de CD debido a su comportamiento mayoritariamente Th2. A este respecto, el modelo de colitis empleado tiene una respuesta Th2 atípica y genera también mecanismos Th1 reflejando la complejidad de la enfermedad humana.

Sin embargo, el modelo empleado no es completamente satisfactorio. Así, la inyección i.p. de OVA provoca una sintomatología alérgica tan intensa que no se observan diferencias clínicas entre los grupos colíticos y no colíticos. Además, se aprecia una distensión en el intestino delgado, en ambos grupos tratados, de etiología desconocida y por último, hay una pobre respuesta sistémica no relacionada con la producción de IgE, algo que, por otra parte, ha sido descrito en algunos modelos de alergia alimentaria.

A pesar de las pequeñas sombras de los modelos experimentales usados, los resultados obtenidos demuestran que la especialización en la respuesta inmune es el principal punto de conexión entre la alergia y la inflamación intestinal. El proceso colítico y la particular respuesta originada durante la sensibilización con DNFB/DNS producen factores que estimulan y refuerzan el mantenimiento y prolongación de las células generadas durante la inducción de la alergia a OVA. El incremento de la respuesta linfocitaria observado “in vitro” tras la estimulación por OVA en los grupos OVA + DNS en comparación con los otros grupos experimentales apoya esta hipótesis. Además, la participación de la inmunidad adquirida en la respuesta exagerada se puso en evidencia también por el agrandamiento del bazo y la mayor producción de IL-2.

En conclusión, los resultados encontrados sugieren que se produce una mayor respuesta alérgica específica de antígeno en los animales con colitis. Esta observación es importante para reducir el riesgo de anafilaxis en los pacientes co-diagnosticados de IBD y alergia alimentaria en los cuales aparece alta reactividad inmunológica y permeabilidad intestinal elevada.

4. LOS RATONES ATÓPICOS MUESTRAN UNA RESPUESTA INMUNE AUMENTADA O REDUCIDA DURANTE LA COLITIS, DEPENDIENDO DEL MODELO ANIMAL USADO.

(Cueto-Sola M, Utrilla P, Bailon E, Garrido-Mesa N, Rodriguez-Ruiz J, Vidald JM, Zarzuelo A, Xaus J, Galvez J, Comalada M. Atopic mice show an increased or reduced immune response during colitis depending on the animal model used. Pendiente de publicación)

La alergia alimentaria originada en la infancia, sobre todo la alergia a la proteína de la leche de vaca, se relaciona frecuentemente con el riesgo de sufrir otras enfermedades alérgicas en la edad adulta como asma, rinitis o dermatitis atópica. Esta evolución se ha denominado "carrera atópica". El incremento en la permeabilidad intestinal que se origina en la alergia alimentaria y que perduraría durante toda la vida, podría considerarse relacionada con el origen de patologías como la IBD o el IBS. De hecho, la coexistencia de ambas enfermedades es cada vez más frecuente en los últimos años.

La relación entre IBD y alergia en humanos ha sido ya demostrada, pero respecto al IBS, los enfermos relatan una cierta intolerancia a algunos alimentos aunque no se ha podido constatar dicha relación. A pesar de los numerosos estudios realizados, todavía no se ha puesto en evidencia experimentalmente una mayor tendencia a sufrir colitis en individuos atópicos.

En estudios anteriores, utilizamos un modelo combinado de alergia alimentaria y colitis para demostrar que los síntomas de la alergia eran más notables cuando se sufría una colitis activa (ver artículo 3). En este trabajo los objetivos eran, en primer lugar establecer un modelo animal que se asemejara a la atopia en adultos y posteriormente observar como esta condición puede alterar la respuesta a la inflamación intestinal provocada por haptenos como DSS y DNFB/DNS.

Se utilizaron ratones hembra Balb/c para establecer el modelo de atopia en ratones. Para ello se inició la alergia a la proteína de la leche de vaca a las tres semanas de edad con ratones recién destetados siguiendo el "protocolo corto" que se puso a punto en nuestro laboratorio y que se ha discutido anteriormente (ver artículo 2) aunque con algunas pequeñas modificaciones. La re-sensibilización fue realizada el día 18 y 24 horas más tarde un grupo de animales (grupo de "alergia aguda") fue sacrificado para constatar el estado de alergia deseado. Otro grupo de animales (grupo de ratones "atópicos") se sacrificaron el día 34 con el fin de demostrar que el estado de alergia se mantenía, asemejándose a la atopia en humanos. Tras esto, con ratones atópicos se inició el experimento de colitis con dos modelos experimentales: colitis inducida por DNFB/DNS, modelo caracterizado por una diarrea con escasa influencia en la permeabilidad intestinal que podría relacionarse con IBS en humanos, y colitis inducida por DSS, modelo experimental para IBD. En este último caso se provocó una colitis moderada utilizando DSS al 1,5% y una colitis severa con la utilización de DSS al 3,0%, en ambos casos incorporado al agua de bebida durante 5 días.

Los resultados obtenidos en el caso del modelo de atopia adulta en ratones han permitido constatar la presencia de IgE plasmática que se acompaña de una ligera alteración intestinal como se demuestra con la disminución de la relación peso/longitud tanto del intestino delgado como del colon, a pesar de la ausencia de síntomas de alergia y otras alteraciones macroscópicas como el peso de los animales, consumo de comida o consistencia de las heces. Todo ello refleja que la alergia originada en la infancia produce un daño permanente en el intestino que se prolonga hasta la edad adulta.

En el caso de la colitis inducida a los animales atópicos, la respuesta encontrada en los dos modelos experimentales utilizados es diferente. En la colitis por DSS, los animales atópicos han demostrado una sintomatología mucho menos aguda que los no atópicos, tanto en la colitis moderada como en la severa. Sin embargo, el modelo de colitis inducida por DNFB/DNS provoca una mayor respuesta diarreica en los animales atópicos que en los sanos.

La diferente respuesta encontrada puede deberse al distinto mecanismo inmune que acompaña a los haptenos empleados en su proceso inflamatorio. Así, el DSS es capaz de provocar una respuesta preferentemente Th1, por tanto la respuesta Th2 observada en el proceso atópico podría atenuar la expresión de citocinas de tipo Th1 requeridas para el inicio del estado inflamatorio en este modelo DSS, debido a la competencia existente entre ambas derivaciones de la respuesta inmune. Mientras, la colitis inducida por DNFB/DNS se caracteriza por una inflamación mediada por linfocitos Th2 que podría sumarse a la respuesta alérgica que sigue el mismo modelo de respuesta.

Como conclusión, puede afirmarse que las alteraciones intestinales e inmunes observadas en el modelo de atopia desarrollado, pueden modificar la respuesta de inflamación intestinal, en un sentido u otro, según el modelo experimental utilizado, por ello, es muy importante la elección del modelo animal y se debe ser cauto a la hora de extraer conclusiones y de extrapolar los resultados a la especie humana.

DISCUSIÓN

Desde hace tiempo se viene observando que en los países más industrializados cada vez hay una mayor prevalencia de enfermedades sobre todo de tipo inflamatorio y aunque la causa no está clara, parece que hay diversos factores implicados. Uno de los aspectos que podrían participar en el desarrollo de estas enfermedades es la pérdida de la tolerancia. La tolerancia es clave para mantener una relación adecuada entre el exterior (o lo ajeno) y el interior (o lo propio) sin que se desencadene una respuesta inmune excesiva, por falta de tolerancia, ni haya una infección, por exceso de tolerancia.

Tanto en la respuesta inmune como en la alergia están implicadas la respuesta celular y la humoral, pudiendo diferenciarse dentro de esta última la presencia de anticuerpos específicos de antígeno. Aunque aún no se ha podido demostrar de manera experimental, hay muchas evidencias y estudios retrospectivos donde se ha visto una relación entre pacientes que han sufrido una alergia en la edad temprana y que luego en la etapa adulta han desarrollado IBD. Sin embargo en el caso de la CD y UC no está tan clara esta relación como en el caso de la dermatitis atópica y la alergia.

Por otra parte, no se ha publicado hasta ahora la recreación de las condiciones de dos patologías diferentes como la alergia y la IBD en un mismo animal de experimentación. Esto puede deberse a varias complicaciones como la elección del modelo animal y la compleja interpretación de resultados. Con este fin en mente, llevamos a cabo la optimización de un modelo de alergia alimentaria y un modelo de inflamación intestinal por separado para luego estudiar estas condiciones de forma conjunta en dos modelos mixtos.

En primer lugar, pretendíamos encontrar un modelo animal de IBD que nos permitiera evaluar la relación entre la alergia y la IBD. Para alcanzar este objetivo, analizamos el perfil inmunológico del modelo del DNFB/DNS descrito por Rijnierse en 2006. Además comparamos este modelo con el modelo del TNBS que es un protocolo ampliamente estudiado de CD.

En el modelo del DNFB/DNS observamos que tanto la diarrea como la inflamación asociada a esta eran de intensidad media y prácticamente desaparecían en el día 5. Por otro lado la activación y desgranulación de mastocitos y la expresión de citocinas fue moderada. Sin embargo sí que se vio un aumento en IL-2 e IL-5 con una mínima participación de TNF-α. En cuanto a la respuesta humoral, observamos un aumento tanto en colon como en plasma de IgE e IgG1 mientras que IgA estaba aumentada en colon y disminuida en plasma. Esto nos hace pensar que la respuesta inmune en este modelo está mediada principalmente por células B. La participación de IL-5 podría hacernos pensar que se trata de un modelo que se asemeja a la UC humana pero observando todos los resultados en conjunto la conclusión es diferente. Aunque no sea un modelo adecuado para el estudio de la IBD sí podría serlo para otras enfermedades con respuestas intestinales alérgicas. Elegimos este modelo para llevar

a cabo alguno de nuestros objetivos por su reacción inflamatoria moderada y su respuesta simple de citocinas.

Por otro lado buscábamos un modelo adecuado de alergia alimentaria. Hay modelos con diferentes alergenos, entre los que destaca la ovoalbúmina (OVA) que desencadena una respuesta fuerte, reproducible y bien caracterizada; y la proteína de leche de vaca (CMP) que requiere ratones recién destetados, los resultados no tienen una tasa muy alta de reproducibilidad y desarrolla una respuesta lenta con un pico a las 6 semanas. Sin embargo este modelo que había sido reevaluado por Lara-Villoslada y col. en 2004 tenía como ventajas que los síntomas sistémicos eran similares a la alergia humana. Así que nos propusimos mejorar este modelo con una reducción significativa en el tiempo necesario (2 semanas versus 6 semanas) para que se obtenga la respuesta alérgica junto con una respuesta incrementada. Una de las innovaciones fue la administración intraperitoneal de CMP en el momento de la re-sensibilización que aunque podría considerarse que no es una opción muy fisiológica sí que permite la administración de cantidades mínimas de la proteína intacta consiguiendo una respuesta más específica. También comprobamos el papel que tenía la toxina colérica tanto en el modelo de 6 como en el de 2 semanas, viendo que incrementa significativamente algunos de los marcadores de respuesta alérgica. En cuanto a la respuesta humoral, vimos que en nuestro protocolo no se veían aumentados los niveles de IgE (CMP no siempre es efectiva aumentando IgE) y tampoco los de IgA (podría ser una ventaja ya que la acción de IgA es contraria a la del resto de Igs).

Una vez llegados a este punto, nuestro objetivo era estudiar de forma combinada ambas patologías y para conseguir esto había que conseguir una buena correlación temporal y una respuesta inmune claramente establecida con las mínimas interferencias posibles.

En el modelo combinado de OVA y DNFB/DNS, se vio un aumento de la respuesta humoral en los grupos que combinaban alergia alimentaria y modelo de inflamación intestinal. Esto también ocurrió con la respuesta celular, principalmente Th2, donde se vio el aumento de IL-2, IL-4 e IL-5.

En la última parte de la tesis, estudiamos la influencia que podría tener la atopia en los síntomas de dos modelos: DSS (modelo que se asemeja a IBD humana) y DNFB/DNS (similar a IBS). La importancia de esto no solo fue el establecimiento de un modelo animal atópico adulto sino también la combinación con un modelo experimental de colitis y la interrelación de ambos resultados para estudiar la importancia de la situación atópica desarrollada en la infancia con el posterior desarrollo de procesos colíticos en el adulto.

El modelo de ratón atópico adulto empleado fue el protocolo corto de CMPA comentado anteriormente que nos permitía reproducir un estado atópico 35 días después de la primera sensibilización con CMP junto con toxina colérica. Los resultados obtenidos indicaban una condición muy parecida a la situación atópica en humanos. Una vez adoptado este modelo, para ver la respuesta de individuos atópicos a una

nueva agresión de tipo inflamatorio utilizamos en estos mismos ratones dos modelos diferentes de colitis inducida por haptenos (DSS y DNFB/DNS).

En el modelo del DSS se observó una clara reducción de la respuesta inmune en ratones atópicos (CMPA) tanto en la concentración de DSS al 1,5% como al 3,5% aunque en cuanto al comportamiento humoral no se pudo extraer ninguna conclusión clara en este modelo combinado. Para clarificar esta respuesta inmune se diseñó otro experimento combinado con CMPA y DNFB/DNS. En este caso, al contrario de lo que ocurría con el DSS, se vio una potenciación de la colitis en ratones atópicos. En la respuesta humoral se vio un aumento de IgE en colon y en plasma y una disminución de IgA en colon.

La posible explicación para estos comportamientos opuestos es que el modelo del DSS se caracteriza por una respuesta Th1/Th17 que podría competir con la respuesta Th2 característica de la atopia. Sin embargo el modelo DNFB/DNS induce una reacción de hipersensibilidad en el colon con un patrón de citocinas típico de una respuesta Th2 que se potenciaría con la respuesta de la atopia.

Por tanto y como conclusión, en la presente tesis doctoral hemos ampliado el análisis y la optimización de dos modelos animales, uno de alergia alimentaria (CMPA) y otro de inflamación intestinal (DNFB/DNS). Además hemos dejado patente la importancia de la selección del modelo para la demostración de la hipótesis de estudio puesto que en función del modelo elegido se puede tener una respuesta u otra. Es importante destacar que no ha sido una tarea fácil por cuestiones diversas como tener que cumplir con las normas éticas de experimentación animal al utilizar varios protocolos simultáneamente. Y finalmente, por primera vez hemos demostrado, mediante la utilización simultanea de distintos modelos animales, que se pueden contestar preguntas como las que nos hacíamos inicialmente en esta tesis.

CONCLUSIONES

Las conclusiones que hemos podido extraer de este trabajo de tesis doctoral son:

CONCLUSIÓN I: Optimización de varios modelos de experimentación animal:

C) Se ha desarrollado un modelo más corto de CMPA con las siguientes ventajas:

- Se consigue una respuesta inmune más intensa, más específica y más dirigida al antígeno, lo que favorece la consistencia de los resultados.
- Permite conseguir una disminución del coste del experimento al reducir el tiempo y el número de animales necesarios.
- Al reducirse el tiempo de sensibilización también disminuye la exposición al adyuvante (toxina colérica) con lo que la respuesta de los grupos control no interfiere en la interpretación de los datos.

D) Se ha optimizado el modelo del DNFB/DNS, que originalmente se había descrito como un modelo de enfermedad inflamatoria intestinal. Las principales conclusiones son:

- Este modelo no debería ser considerado como un modelo de IBD, sino más bien como un modelo del síndrome del intestino irritable, donde los modelos animales escasean.
- Las respuestas T y B y la humoral tienen un papel clave en este modelo.
- Que sea un modelo de inflamación aguda que vuelve rápidamente a valores basales permite usar otro modelo a continuación en el mismo animal sin que haya interferencias.

CONCLUSIÓN II: Mediante el estudio de la posible influencia de la inflamación intestinal (modelo DNFB/DNS) en individuos alérgicos (modelo OVA) hemos demostrado que:

- Hay una respuesta específica de antígeno incrementada en animales con colitis.

CONCLUSIÓN III: Se ha evaluado el efecto modulador de la CMPA en otras patologías intestinales con un componente inmune, utilizando los modelos CMPA, DSS y DNFB/DNS probándose que:

- El proceso atópico de CMPA atenúa la respuesta observada en el modelo del DSS.
- El proceso atópico de CMPA aumenta la respuesta observada en el modelo del DNFB/DNS.

List of abbreviations

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
APCs	Antigen presenting cells
CD	Crohn´s disease
CMP	Cow's milk protein
CMPA	Cow's milk protein allergy
ConA	Concanavalin A
CT	Cholera toxin
CTGF	Connective tissue growth factor
DCs	Dendritic cells
DNFB	Dinitrofluorobenzene
DSS	Dextran Sulfate Sodium
Foxp3	Forkhead box protein 3
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICAM-1	Intercellular adhesion molecule class I
IELs	Intraepithelial lymphocytes
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate lymphoid cells
ILFs	Isolated lymphoid follicles
i.p.	Intraperitoneal
IR	Intrarectal
kDa	Kilo Dalton
KO	Knockout
LFA-1	Leukocyte function antigen-1
LPLs	Lamina propria lymphocytes
LPS	Lipopolysaccharide
MCP-1	Mast cell protease-1
M cells	Microfold cells
MHC	Major histocompatibility complex
NK cells	Natural killler cells
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain containing 2
OVA	Ovalbumin
PAMPs	pathogen associated molecular patterns
PDGF	Platelet derived growth factor

PGE2	Prostaglandin E2
PRR	Pattern recognition receptors
sIgA	Secretory Immunoglobulin A
SCF	Stem cell factor
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumor necrosis factor alpha
Treg cells	Regulatory T cells
UC	Ulcerative colitis
VCAM-1	Vascular adhesion molecule class I

Annex: CURRICULUM VITAE

Annex: curriculum vitae

El trabajo desarrollado en el grupo de investigación "Farmacología de Productos Naturales" del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Granada, me ha permitido desarrollar esta Tesis Doctoral. En paralelo, he podido colaborar en otros proyectos de investigación, cuyos resultados han sido también publicados en prestigiosas revistas científicas, citadas a continuación. A su vez, he podido asistir a diversos congresos y cursos.

Publicaciones científicas:

Artículos:

1. **Cueto-Sola M**, Bailon E, Utrilla P, Rodríguez-Ruiz J, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. Active colitis exacerbates immune response to internalized food antigens in mice. Int Arch Allergy Immunol. **2013**; 162(3):214-24.

2. Bailón E, **Cueto-Sola M**, Utrilla P, Rodríguez-Ruiz J, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. A shorter and more specific oral sensitization-based experimental model of food allergy in mice. J Immunol Methods. **2012**; 381(1-2):41-9.

3. Camuesco D, Rodríguez-Cabezas ME, Garrido-Mesa N, **Cueto-Sola M**, Bailón E, Comalada M, Arribas B, Merlos M, Balsa D, Zarzuelo A, Janer G, Xaus J, Román J, Gálvez J. The intestinal anti-inflammatory effect of dersalazine sodium is related to a down-regulation in IL-17 production in experimental models of rodent colitis. Br J Pharmacol. **2012**; 165(3):729-40.

4. Bailón E, Cueto-Sola M, Utrilla P, Nieto A, Garrido-Mesa N, Celada A, Zarzuelo A, Xaus J, Gálvez J, Comalada M. DNFB-DNS hapten-induced colitis in mice should not be considered a model of inflammatory bowel disease. Inflamm Bowel Dis. **2011**; 17(10):2087-101.

5. Garrido-Mesa N, Camuesco D, Arribas B, Comalada M, Bailón E, **Cueto-Sola M**, Utrilla P; Nieto A, Zarzuelo A, Rodriguez-Cabezas ME, Gálvez J. The intestinal anti-inflammatory effect of minocycline in experimental colitis involves both its immunomodulatory and antimicrobial properties. Pharmacological Research. **2011**; 63(4):308-19

6. Rodríguez-Cabezas ME, Camuesco D, Arribas B, Garrido-Mesa N, Comalada M, Bailón E, **Cueto-Sola M**, Utrilla P, Guerra-Hernández E, Pérez-Roca C, Gálvez J, Zarzuelo A. The combination of fructooligosaccharides and resistant starch shows prebiotic additive effects in rats. Clin Nutr. **2010**; 29(6):832-9

7. Bailón E, **Cueto-Sola M**, Utrilla P, Rodríguez-Cabezas ME, Garrido-Mesa N, Zarzuelo A, Xaus J, Gálvez J, Comalada M. Butyrate in vitro immune-modulatory effects might be mediated through a proliferation-related induction of apoptosis. Immunobiol. **2010**; 215(11):863-73

Capítulos de libros:

1. Aula de la Farmacia. Revista Profesional de Formación Continuada. Actualizaciones Farmacológicas. Artículo: Interacciones hipolipemiantes. Autor/es: Antonio Zarzuelo Zurita, **Margarita Cueto Sola**. Nº revista: 57. Fecha de publicación: 01 Marzo 2009.

2. Aula de la Farmacia. Revista Profesional de Formación Continuada. Actualizaciones Farmacológicas. Artículo: Interacciones hipolipemiantes. Generalidades de las dislipemias y aspectos farmacológicos de los hipolipemiantes. Autor/es: Antonio Zarzuelo Zurita, **Margarita Cueto Sola**. Nº revista: 56. Fecha de publicación: 01 Febrero 2009

Congresos:

Comunicaciones orales:

1. **Cueto-Sola M**, Bailón E, Garrido-Mesa N, Celada A, Zarzuelo A, Xaus J, Galvez J, Comalada M. Active colitis exacerbates immune response to food antigens. 18th United European Gastroenterology Week (UEGW), Oct 23-27, 2010, Barcelona (Spain).

2. **M. Cueto**, E Bailón, N. Garrido, B Arribas, M Comalada, ME Rodríguez-Cabezas, J.C. Quintela, E. de la Fuente, M. Olivares, J Gálvez. Intestinal anti-inflammatory effects of an extract from the olive tree, Aquolive®, in the TNBS model of rat colitis. I Reunión de Jóvenes Farmacólogos de Andalucía. Granada (Spain). 2009.

Póster:

1. Comalada M, Bailón E, **Cueto-Sola M**, Garrido-Mesa N, Utrilla P, Rodríguez-Cabezas ME, Zarzuelo A, Xaus J, Gálvez J. The specificity and magnitude of the immune response raised against food allergens depends on the murine

experimental model used. XXXIII Congreso Nacional de la Sociedad Española de Farmacología, Málaga (Spain), 3-5 oct, 2011.

2. Utrilla MP, **Cueto-Sola M**, Garrido-Mesa N, Bailón E, Rodríguez-Cabezas ME, Zarzuelo A, Xaus J, Gálvez J, Comalada M. The immune response in atopic animals modifies the risk to acquire other immune-related diseases. XXXIII Congreso Nacional de la Sociedad Española de Farmacología, Málaga (Spain), 3-5 oct, 2011.
3. **Cueto-Sola M**, Bailón E, Utrilla P, Garrido-Mesa N, Zarzuelo A, Xaus J, Galvez J, comalada M. DNFB/DNS, an unconventional intestinal inflammatory model for what?. XXXII Congreso de la Sociedad Española de Farmacología, Leon (Spain), 15-17 sept, 2010.
4. Garrido-Mesa N, Camuesco D, Arribas B, Comalada M, Bailón E, **Cueto-Sola M**, Utrilla P, Zarzuelo A, Nieto A, Rodriguez-Cabezas ME, Galvez J. Orally administered minocycline promotes the recovery of the colonia damage in the chronic phase of the DSS model of mouse colitis. XXXII Congreso de la Sociedad Española de Farmacología, Leon (Spain), 15-17, sept, 2010.
5. Garrido-Mesa N, Camuesco D, Arribas B, Comalada M, Bailón E, **Cueto-Sola M**, Utrilla P, Zarzuelo A, Nieto A, Rodriguez-Cabezas ME, Galvez J. Oral administration of minocycline promotes colonic recovery in the DSS model of Mouse colitis. Role of its immunomodulatory properties. 18th United European Gastroenterology Week (UEGW), Oct 23-27, 2010, Barcelona (Spain).
6. Comalada M, Arribas B, Garrido-Mesa N, **Cueto M**, Bailón E, Rodríguez-Cabezas ME, Suárez-Pereira E, Camuesco D, Ortiz-Mellet C, García-Fernández J, Zarzuelo A, Gálvez J. Intestinal anti-inflammatory effects of a new prebiotic, difructose dianhydride-enriched caramel, in the TNBS model of rat colitis. Gastro 2009 UEGW/WCOG. Publicación: Gut 2009, 58 (Suppl II) A453. London (United Kingdom). 2009.
7. Rodríguez-Cabezas ME, Arribas B, Bailón E, Garrido-Mesa N, **Cueto M**, Utrilla P, Comalada M, Quintela JC, Olivares M, de la Fuente E, Gálvez J. Intestinal anti-inflammatory effects of an extract from the olive tree, Aquolive®, in the TNBS model of rat colitis. Gastro 2009 UEGW/WCOG. Publicación: Gut 2009, 58 (Suppl II) A155. London (United Kingdom). 2009.
8. Garrido-Mesa N, Camuesco D, Arribas B, Comalada M, Bailón E, **Cueto M**, Zarzuelo A, Nieto A, Rodríguez-Cabezas ME, Gálvez J. The immunodulatory properties of minocycline contribuye to its intestinal anti-inflammatory effects in the trinitrobencenesulphonic acid model of rat colitis. Gastro 2009 UEGW/WCOG. Publicación: Gut 2009, 58 (Suppl II) A454. London (United Kingdom). 2009.
9. **Cueto M**, Rodríguez-Cabezas ME, Arribas B, Bailón E, Comalada M, Garrido-Mesa N, Nieto A, Román J, Balsa D, Merlos M, Zarzuelo A, Gálvez J. The intestinal anti-inflammatory effect of Dersalazine sodium in the DSS modelo f

colitis in mice is associated with a downregulated in Th1 and Th17 cytokine production. Gastro 2009 UEGW/WCOG. Publicación: Gut 2009, 58 (Suppl II) A453. London (United Kingdom). 2009.

10. Utrilla P, Arribas B, Bailón E, **Cueto M**, Garrido N, Comalada M, Rodríguez-Cabezas ME, Zarzuelo A, Gálvez J. Probiotic pretreatment diminishes T cell cytokine production in an experimental model of rheumatoid artritis. XXXI Congreso de la Sociedad Española de Farmacología (SEF). Publicación: Methods and Findings in Experimental and Clinical Pharmacology; 31(A): 106. Sevilla (Spain). 2009.
11. Comalada M, Garrido N, Camuesco D, Arribas B, Bailón E, **Cueto M**, Utrilla P, Zarzuelo A, Nieto A, Rodríguez-Cabezas ME, Gálvez J. Intestinal anti-inflammatory effects of tetracyclines in the TNBS model of rat colitis. XXXI Congreso de la Sociedad Española de Farmacología (SEF). Publicación: Methods and Findings in Experimental and Clinical Pharmacology; 31(A): 116. Sevilla (Spain). 2009.
12. Rodríguez-Cabezas ME, Camuesco D, Arribas B, Bailón E, Comalada M, Garrido N, **Cueto M**, Román J, Balsa D, Merlos M, Zarzuelo A, Gálvez J. Dersalazine sodium downregulates inflammatory bowel disease-associated gene expression. Falk Symposium 170. IBD and IBS: Novel mechanisms and future practice. Glasgow (Great Britain). 2009.
13. Garrido N, Camuesco D, Arribas B, Comalada M, Bailón E, **Cueto M**, Zarzuelo A, Nieto A, Rodríguez-Cabezas ME, Gálvez J. Intestinal anti-inflammatory effects of tetracycline and minocycline in the TNBS model of rat colitis. Falk Symposium 170. IBD and IBS: Novel mechanisms and future practice. Glasgow (Great Britain). 2009.
14. Arribas B, Garrido N, Comalada M, Bailón E, **Cueto M**, Zarzuelo A, Rodríguez-Cabezas ME, Adalid JM, Tabuenca D, Navarro A, Gálvez J. Evaluation of the sinergism of oligossacharides (BENEOTM-p95) and resistan dextrin (FIBERSOL-2TM) in the prebiotic effect and in the intestinal anti-inflammatory activity in the trinitrobenzene sulfonic acid model of rat colitis. Falk Symposium 170. IBD and IBS: Novel mechanisms and future practice. Glasgow (Great Britain). 2009.
15. E Bailón, **M Cueto**, B Arribas, N Garrido, M Comalada, A Zarzuelo, ME Rodríguez-Cabezas, JM Adalid, D Tabuenca, A Navarro, J Gálvez. Evaluation of the sinergism of oligossacharides (BENEOTM-p95) and resistan dextrin (FIBERSOL-2TM) in the prebiotic effect and in the intestinal anti-inflammatory activity in the trinitrobenzene sulfonic acid model of rat colitis. I Reunión de Jóvenes Farmacólogos de Andalucía. Granada (Spain). 2009.
16. N. Garrido, D Camuesco, B Arribas, M Comalada, E Bailón, **M Cueto**, A Zarzuelo, A. Nieto, ME Rodríguez-Cabezas, J Gálvez. Intestinal anti-inflammatory effects of tetracycline and minocycline in the TNBS model of rat colitis. I Reunión de Jóvenes Farmacólogos de Andalucía. Granada (Spain). 2009.

17. B. Arribas, E. Bailón, **M Cueto**, N Garrido, M Comalada, ME Rodríguez-Cabezas, A Zarzuelo, J Gálvez, P Utrilla. Probiotic Pretreatment Diminishes T Cell Cytokine Production In An Experimental Model Of Rheumatoid Arthritis. I Reunión de Jóvenes Farmacólogos de Andalucía. Granada (Spain). 2009.
18. Bailón E, Rodríguez-Cabezas ME, Salcedo I, **Cueto M**, Zarzuelo A, Xaus J, Gálvez J, Comalada M. El efecto antiinflamatorio intestinal del butirato in vitro está mediado por una inducción de la apoptosis dependiente del estado proliferativo de las células inflamatorias. LXVII Congreso Anual de la Sociedad Española de Patología digestiva. Publicación: Revista Española de Enfermedades Digestivas; 100(1): 47-48. Sitges, Barcelona (Spain). 2008. Ganador de la mejor comunicación en forma de póster en dicho Congreso.

Cursos:

1. "Formación a distancia en Protección y experimentación animal para experimentadores en ciencias biomédicas. Categoría C. V edición". (Curso acreditado por la Dirección General de la Producción Agrícola y Ganadera. Consejería de Agricultura y Pesca. Junta de Andalucía, en virtud del RD 1201/2005).
   Organizado por: Fundación Empresa Universidad de Granada y el Centro de Enseñanzas Virtuales de la Universidad de Granada.
   Duración: 80 horas teórico-prácticas (60 horas virtuales y 20 horas presenciales).
   Fechas: 18 de mayo 2009 – 14 de julio 2009.

2. "Young Investigator Workshop".
   Organizado por: ASNEMGE (Association des Sociétés Nacionales Européennes et Méditerranéennes de Gastroenterology)
   Lugar de realización: Viena (Austria)
   Fechas: 23 al 25 de enero de 2009

Por otra parte, desde mayo de 2011 realizo la residencia de Farmacia Hospitalaria en el Hospital Universitario y Politécnico La Fe de Valencia como farmacéutica interna residente. En este contexto, también he tenido la opción de acudir a varios congresos y cursos.

Publicaciones científicas:

Artículos:

1. Cueto Sola M, Belda Furió M, Borrell García C, Escobar Cava P, López Briz E, Poveda Andrés JL. Incompatibility of undiluted busulfan with a needle-free valve. Am J Health Syst Pharm. 2014. Pendiente de publicación.
2. Ruiz Ramos J, Lorente Fernández L, Gil Gómez I, Cueto Sola M, Monte Boquet E, Poveda Andrés, JL. Análisis de las causas de suspensión de tratamiento con triple terapia antiviral en pacientes con hepatitis C. Farm Hosp. 2014. Vol. 38, núm. nº3. Pendiente de publicación.

Congresos:

Comunicaciones orales:

1. Gil Gómez I, Lorente Fernández L, Monte Boquet E, **Cueto Sola M**, Ruiz Ramos J, Poveda Andrés JL. Estimación de la carga de trabajo de una unidad de pacientes externos ante los nuevos procedimientos de autorización de la triple terapia para el tratamiento de la Hepatitis C crónica. 58° Congreso Nacional de la Sociedad Española de Farmacia Hospitalaria y Encuentro Iberoamericano de Farmacéuticos de Hospital, Málaga (Spain), 23-25 oct, 2013.

Póster:

1. García Robles A, Bosó Ribelles V, **Cueto Sola M**, Ruiz Ramos J, Company Albir MJ, Herrero Cervera MJ, Aliño Pellicer SF, Poveda Andrés JL. Adverse events associated with Single Nucleotide Polymorphisms in breast cancer patients treated with docetaxel-based chemotherapy. EAHP 19th Annual Congress (Congreso Europeo de Farmacia Hospitalaria, Barcelona (Spain), 26-28 mar, 2014.
2. **Cueto Sola M**, Monte Boquet E, Gil Gomez I, Ruiz Ramos J, Roman Ivorra JA, Poveda Andrés JL. Impacto presupuestario del ajuste en el día de administración de adalimumab, certolizumab y golimumab en pacientes reumatológicos. 58° Congreso Nacional de la Sociedad Española de Farmacia Hospitalaria y Encuentro Iberoamericano de Farmacéuticos de Hospital, Málaga (Spain), 23-25 oct, 2013.
3. Lorente Fernández L, Arocas Gomariz L, **Cueto Sola M**, Monte Boquet E, Ruiz Ramos J, Poveda Andrés JL. Análisis de la adherencia al tratamiento con adalimumab en pacientes con enfermedad de Crohn. 58° Congreso Nacional de

la Sociedad Española de Farmacia Hospitalaria y Encuentro Iberoamericano de Farmacéuticos de Hospital, Málaga (Spain), 23-25 oct, 2013.

4. Gil Gómez I, Ruiz Ramos J, Lorente Fernández L, Monte Boquet E, **Cueto Sola M**, Poveda Andrés JL. Estudio de utilización de aztreonam inhalado en un hospital terciario". 58° Congreso Nacional de la Sociedad Española de Farmacia Hospitalaria y Encuentro Iberoamericano de Farmacéuticos de Hospital, Málaga (Spain), 23-25 oct, 2013.
5. Ruiz Ramos J, García Robles A, Pérez Huertas P, **Cueto Sola M**, Marqués Miñana MR, Poveda Andrés JL. Blood levels of immunosuppressant drugs in patients with cystic fibrosis after lung transplantation. EAHP 18th Annual Congress (Congreso Europeo de Farmacia Hospitalaria, París (France), 13-15 mar, 2013.
6. **Cueto Sola M**, Marrero Álvarez P, Ruiz Ramos J, Martínez Cercos L, García Pellicer J, Poveda Andrés JL. Medicamentos extranjeros: evolución del gasto en un hospital de tercer nivel. 57 Congreso Español de Farmacia Hospitalaria, Bilbao (Spain), 2-5 oct, 2012.
7. Reig Aguado J, Ruiz Ramos J, Megías Vericat JE, **Cueto Sola M**, Tordera Baviera M, Poveda Andrés JL. Impacto económico de los ensayos de Mieloma Múltiple en un hospital terciario. 57 Congreso Español de Farmacia Hospitalaria, Bilbao (Spain), 2-5 oct, 2012.

Cursos:

- "VI curso sobre calidad y seguridad del medicamento". H.U.P. La Fe, Valencia, 4-15 de noviembre de 2013.
- "Xª Jornadas de Patología Infecciosa Multidisciplinar para Facultativos Internos Residentes". Calpe (Alicante), 8-9 de noviembre de 2013.
- "Jornadas de Medicamentos Huérfanos y Enfermedades Raras: Sumando esfuerzos, multiplicando resultados en salud". Valencia, 11-13 de abril de 2013.
- "Iniciación a la investigación en Farmacia Hospitalaria" (1ª Edición). Curso pre-congreso. 57° Congreso Nacional de la Sociedad Española de Farmacia Hospitalaria y Encuentro Iberoamericano de Farmacéuticos de Hospital. Bilbao, 2 de Octubre de 2012.
- Jornada: "Formación en los aspectos más importantes de la Buena Práctica Clínica (BPC)". H.U.P. La Fe. Valencia, 22 de octubre de 2012.
- "IV Curso de Metodología en Gestión Farmacoterapéutica".Valencia-Denia, 22-25 de Mayo de 2012.
- "Curso de Protección Radiológica para dirigir instalaciones de Rayos X con fines de diagnóstico médico (IRD)". Valencia, 2011.

Participación como ponente:

1. "XI curso de atención farmacéutica al paciente trasplantado". Valencia, 4-7 de marzo de 2014. Sesión impartida: "Seminario práctico: casos clínicos" con una

duración de 180 min. Actividad acreditada por la Comisión de Formación Continuada del Sistema Nacional de Salud con 9,3 créditos, por la sociedad española de farmacia hospitalaria y por la sociedad valencia de farmacia hospitalaria.

2. “X curso de atención farmacéutica al paciente trasplantado”. Valencia, 4-8 de marzo de 2013. Sesión impartida: “Seminario práctico: casos clínicos” con una duración de 180 min. Actividad acreditada por la Comisión de Formación Continuada del Sistema Nacional de Salud con 9,3 créditos, por la sociedad española de farmacia hospitalaria y por la sociedad valencia de farmacia hospitalaria.