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NICOTINIC MODULATION IN TNBS-INDUCED
COLITIS:
FOCUS ON SPLEEN AND T-CELLS

Coordinatore:
Chiar.mo Prof. Marco Mor

Tutor:
Chiar.ma Prof. Elisabetta Barocelli

Dottorando: Dott. Andrea Grandi

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INTRODUCTION

IMMUNE RESPONSE AND INFLAMMATION

Inflammation is a pathophysiological response triggered by the innate immune system upon tissue damage or infection, aimed at eliminating the causing agent and restoring tissue homeostasis. Tissue resident cells (e.g. macrophages, dendritic cells) are specialized in detecting the damaging insult through their *Toll-Like Receptors* (TLRs) (Mantovani et al., 2011) and in producing a wide range of mediators, including cytokines (e.g. IL-1 β , IL-6 and TNF- α), chemokines (e.g. CCL2, CXCL8), eicosanoids (e.g. prostaglandins and leukotriens) and other molecules, such as histamine and bradykinin (Figure 1). These mediators are responsible for the increased vascular permeability, vasodilation and endothelial activation, that allow circulating neutrophils to migrate into the inflamed tissue, where they promote the recruitment of inflammatory monocytes, bacterial clearance and tissue reparation.

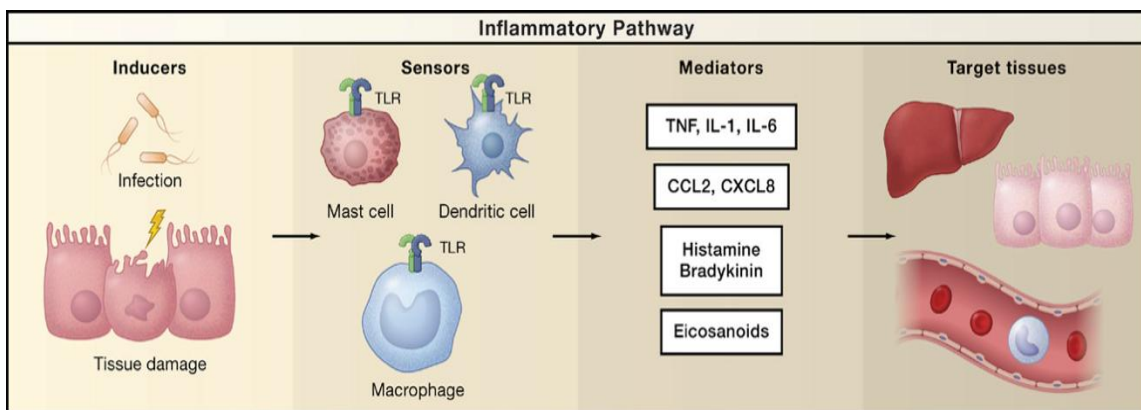


Figure 1: Schematic representation of the inflammatory pathway (Medzhitov et al., 2010).

In physiological conditions the inflammatory response is a finely balanced process that spontaneously resolves, with neutrophils undergoing apoptosis, macrophages ingesting apoptotic

neutrophils and leaving the tissue through lymphatic vessels (Fox et al., 2010; Michlewska et al., 2009). The resolution phase of the inflammatory response is an active and coordinated process, during which different cellular and molecular events lead to the restoration of tissue functionality and integrity (Ortega-Gomez et al., 2013). As shown in figure 2, an impaired resolution phase can widen the actions of pro-inflammatory mediators and pathways, thus leading to a prolonged or chronic inflammatory state (Perretti et al., 2015), and, eventually, to loss of organ function (Serhan and Savill, 2005).

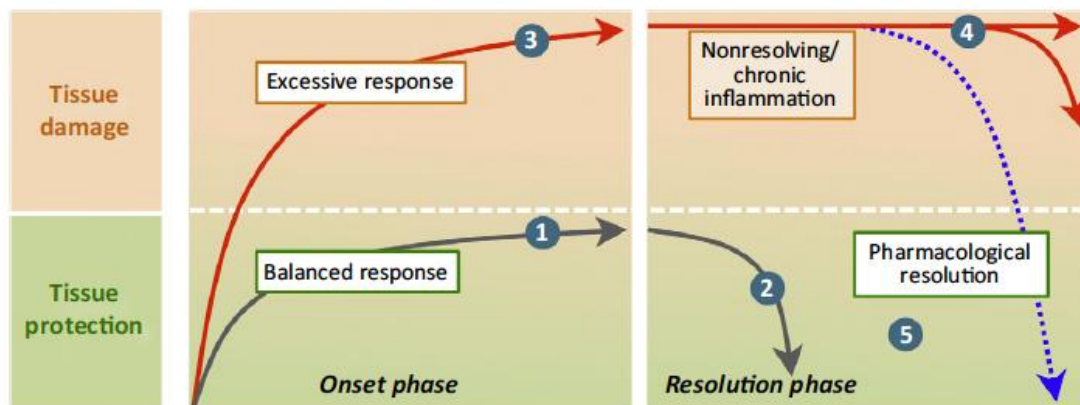


Figure 2: Onset and resolution phases of the inflammatory response (Perretti et al., 2015).

Macrophages are also involved in the adaptive immunity, as they are, together with dendritic cells, professional antigen presenting cells (APC): APC cells process antigens, then migrate to secondary lymphoid organs (i.e. spleen and lymph nodes) to present processed antigens to T-cells. Antigenic peptides are complexed with *Major Histocompatibility Complex* (MHC) on the cell surface of APC cells; naïve T-cells ($CD3^+$, $CD4^+$, $CD8^+$) recognize MHC through their co-receptors (i.e. CD4 or CD8), thus triggering the proliferation of the stimulated T-cells clone. In particular MHC class I is recognized by CD8, stimulating the differentiation of naïve T-cells in cytotoxic T-cells ($CD3^+$ $CD8^+$ $CD4^-$), whereas MHC class II is recognized by CD4, stimulating differentiation in helper T-cells ($CD3^+$ $CD4^+$ $CD8^-$) (Abbas and Lichtman, 2006). $CD8^+$ T-cells kill infected cells through the release of perforins and granzymes, whereas $CD4^+$ T-cells can further differentiate in subsets of effectors T-cells (Th1, Th2, Th17) or regulatory T-cells ($CD4^+$ $CD25^+$ $Foxp3^+$ or Treg), producing a wide range of either pro-inflammatory or anti-inflammatory cytokines to modulate the immune response (Abbas et al., 1996).

Among T-lymphocytes, regulatory CD4⁺ CD25⁺ Foxp3⁺ cells (Tregs) play a crucial role in the resolution phase of the inflammatory response: Tregs modulate inflammation through the production and release of pro-resolving cytokines, such as IL-10 and TGF- β , and play a key role in immune homeostasis (Fahlen et al., 2005). Beneficial roles of Tregs have already been evidenced in many chronic inflammatory disorders, including rheumatoid arthritis (Cao et al., 2003) and atherosclerosis (Ait-Oufella et al., 2006).

THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY (CAP)

The role of the vagus nerve (VN) as a modulator of inflammation has been known since a long time. In fact, the release of pro-inflammatory cytokines (e.g. IL-1 β , IL-6, TNF- α) from intestinal mucosa activate VN afferents terminating in the central nervous system (CNS), where an anti-inflammatory response is triggered through the activation of hypothalamus-pituitary-adrenal (HPA) axis and the production of glucocorticoids (Bonaz et al., 2013; Dantzer et al., 2000). Moreover, in recent years, it has been demonstrated that also VN efferents have anti-inflammatory properties, from which the theory of a Cholinergic Anti-inflammatory Pathway (CAP) has been devised (Borovikova et al., 2000a).

The concept of CAP was introduced in 2000, when Borovikova and colleagues investigated the action of CNI-1493, a p38 MAP kinase inhibitor that prevented the inflammatory response in the rat paw after local injection of carrageenan, but was inefficient in vagotomised rats (Borovikova et al., 2000a). Afterwards, electrical vagus nerve stimulation and intracerebroventricular injection of CNI-1493 were tested in systemic inflammation (Borovikova et al., 2000b; Bernik et al., 2002): in these studies, LPS, derived from *Escherichia Coli*, was i.v. administered to rats at a lethal dose (15 mg/kg), triggering an intense systemic inflammatory response, characterized by a massive production of pro-inflammatory cytokines. The results showed that both electrical vagus nerve stimulation and CNI-1493 administration were efficient in reducing circulating levels of inflammation markers (i.e. TNF- α); cutting the vagi neutralized both protective effects. VN efferents were shown to exert their anti-inflammatory activity through the release of acetylcholine (ACh), subsequently activating its receptors expressed by immune cells. In fact, ACh suppressed the release of pro-inflammatory cytokines from human macrophages upon LPS stimulation (Borovikova et al., 2000b).

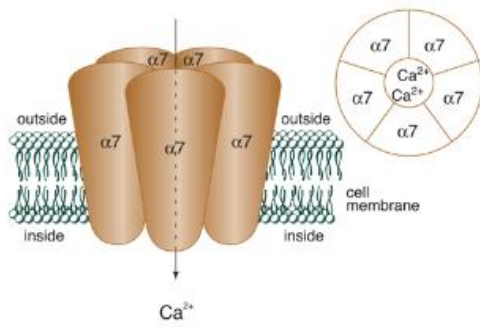
In the following years many studies investigated the CAP in other animal models of inflammatory conditions, such as ischemia-reperfusion injury (Bernik et al., 2006), hemorrhagic shock (Guarini et al., 2003), pancreatitis (van Westerloo et al., 2006) and colitis (Ghia et al., 2006). Tracey and colleagues showed that nicotine was as efficient as ACh in reducing cytokines production from activated macrophages (Tracey et al., 2002), thus indicating that nicotinic acetylcholine receptors (*nAChRs*), rather than muscarinic receptors, are involved in the cholinergic modulation of the inflammatory response. An essential nicotinic link in the CAP was confirmed by the finding that while VN stimulation reduces inflammation in wild-type mice, it does not work in mice lacking the $\alpha 7$ nicotinic receptor subunit (Wang et al., 2003; Vida et al., 2011).

Furthermore, a novel vagus-resolution circuit has been recently identified, indicating that the vagus nerve is a critical regulator in the resolution phase of the inflammatory response (Mirakaj et al., 2014): in fact, the production of pro-resolving mediators in mice has been negatively affected by vagotomy, which shifted the lipid profile from lipoxins to increased pro-inflammatory eicosanoids levels (i.e. leukotriens).

THE NICOTINIC ACETYLCHOLINE RECEPTORS (*nAChRs*)

Nicotinic acetylcholine receptors (*nAChRs*) represent a large and well-characterized family of ligand-gated ion channels broadly expressed throughout the nervous system, either centrally or peripherally, and in non-neuronal cells (Hurst et al., 2013). *nAChRs* modulate cations flow across the cell membrane under the control of an extracellular signalling molecule (i.e. the neurotransmitter ACh). Cations influx through the associated channel pore depolarizes the cell membrane thus increasing cell excitability. *nAChRs* are composed by five subunits (Unwin, 2005) and may exist as homopentamers, formed by five identical subunits (e.g. $\alpha 7$ *nAChRs*), as well as heteropentamers (e.g. $\alpha 4\beta 2$ *nAChRs*), resulting from the combination of different subunits (figure 3). Subunits have been further classified into two subgroups, defined as α and β , whilst three additional subunits (γ , δ and ϵ) have been identified for the muscle receptors (Dani & Bertrand, 2007).

Five $\alpha 7$ subunits form an $\alpha 7$ homo-oligomeric nAChR



Two $\alpha 4$ and three $\beta 2$ subunits form an $\alpha 4\beta 2$ heteromeric nAChR

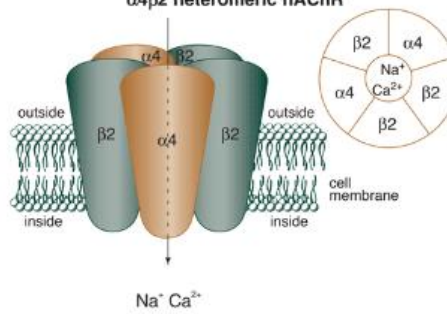


Figure 3: Schematic representation of the two most common subtypes of nAChRs. In both nAChRs, the subunits are arranged around a central pore that opens when ligands (i.e. ACh or nicotine) bind to the ligand-binding site. The $\alpha 7$ nAChR principally allows passage of Ca^{2+} , whereas the $\alpha 4\beta 2$ nAChR allows passage of both Ca^{2+} and Na^+ (Davis & de Fiebre, 2006).

Binding of either endogenous ligand (i.e. ACh) or exogenous agonists to the ligand binding domain (LBD) modifies the transition rates between three distinct functional states of the receptor (figure 4): the resting, open and desensitized states. The rate constants between the functional states are highly dependent on the specific combination of subunits and the chemical nature of the agonist that is bound at the LBD. Importantly, channel conformational states differentially influence the activity of the target cell through the electrogenic action of Na^+ , K^+ and Ca^{2+} that can pass through the open channel or through the activation of signalling cascades that are modulated by Ca^{2+} (Hurst et al., 2013).

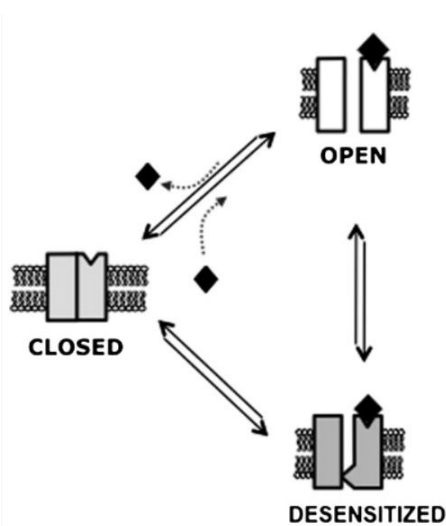


Figure 4: Functional states of ligand-gated ion channels (Hurst et al., 2013).

Homopentameric receptors, like $\alpha 7$ *nAChRs*, are widely expressed in the central and peripheral nervous systems and their ability to form a functional receptor implies that both principal and complementary binding sites are on the same subunit (Hurst et al., 2013), which confers some unique features to this subtypes of *nAChRs*, such as fast desensitization and high permeability to Ca^{++} (Yu & Role, 1998).

Recently PCR (*Polymerase Chain-Reaction*) analysis showed that genes encoding for *nAChRs* are expressed in various extra-neuronal cells such as leukocytes, lungs, kidneys, skin and adipose tissue (Gault et al., 1998). In particular the gene encoding for the $\alpha 7$ subunit (*CHRNA7*) has been identified in T-cells, macrophages and dendritic cells.

The VN has been demonstrated to exert its anti-inflammatory activity by reducing cytokines production by immune cells (e.g. macrophages), through the stimulation of $\alpha 7$ *nAChRs* (Wang et al., 2003). Recently, many studies advanced our understanding on the intracellular signalling pathways involved in the anti-inflammatory potential of ACh: stimulation of the $\alpha 7$ *nAChR* elicits an increase in intracellular Ca^{2+} levels that triggers activation of PI3K/Akt and Jak2/STAT3 phosphorylation (Arredondo et al., 2006; De Jonge & Ulloa, 2007). Phosphorylation of STAT3 leads to the inhibition of NF- κ B transcriptional activity (figure 5), thus reducing the production of pro-inflammatory mediators, such as IL-6 and iNOs (Wang et al., 2004).

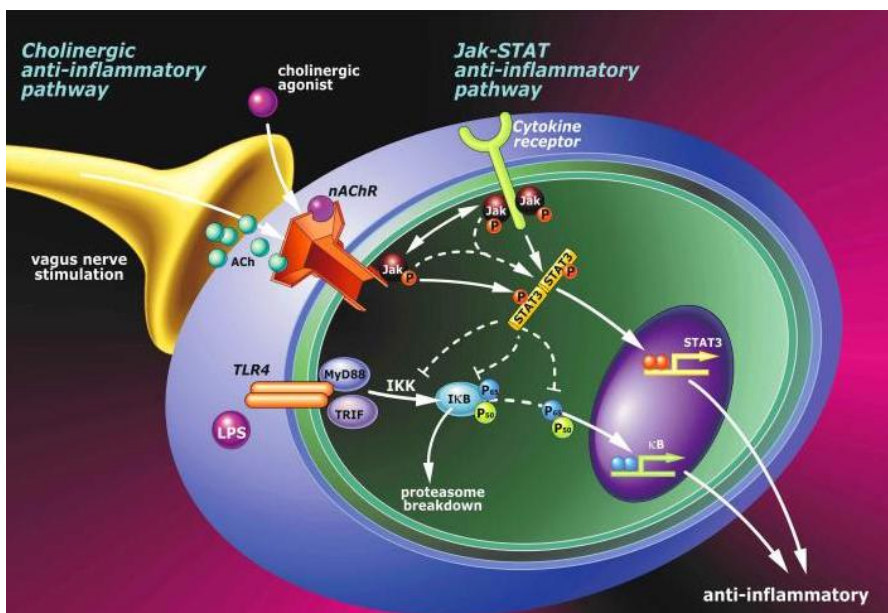


Figure 5: The intracellular ‘nicotinic anti-inflammatory pathway’ (De Jonge & Ulloa, 2007).

Since a wide range of diseases are related to an overproduction of pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-6), the role played by the VN and *nAChRs* in modulating their biosynthesis may be crucial in restoring physiological conditions. Hence, developing new drugs targeting $\alpha 7$ *nAChRs* might represent an innovative approach, paving the way to new therapeutic strategies for the treatment of inflammatory conditions, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), osteoarthritis, asthma, obesity, type 2 diabetes and sepsis (Bencherif et al., 2013).

Pharmacological agents able to stimulate *nAChRs* showing anti-inflammatory activity are represented in figure 6: among these, AR-R17779, GTS-21, TC-7020, PNU-282987, CAP55, DMAB, PHA568487 show high selectivity for the $\alpha 7$ subunit.

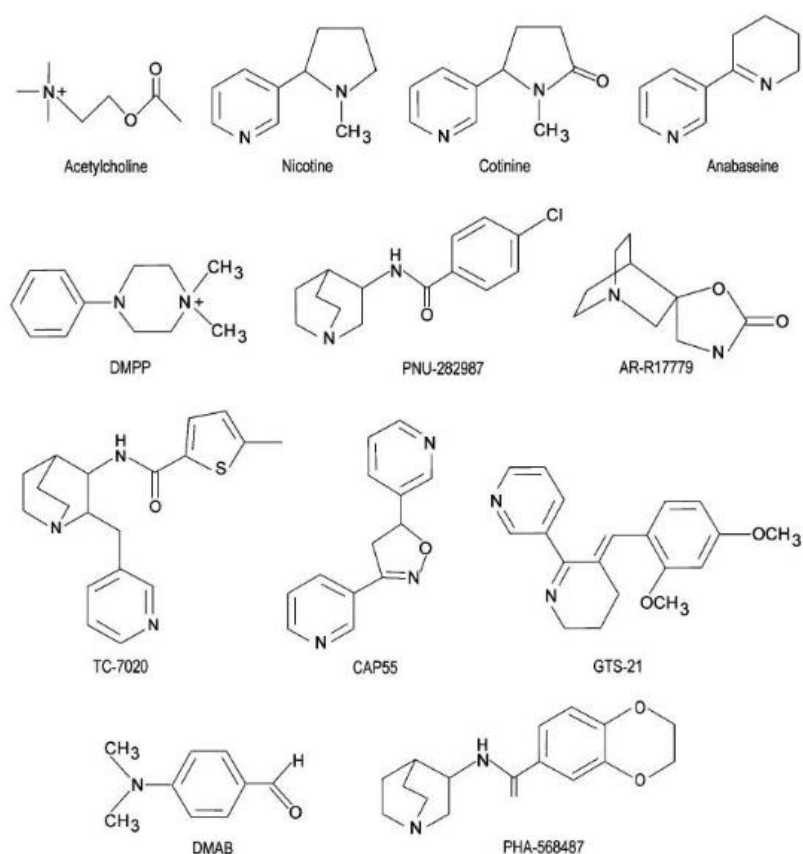


Figure 6: Structures of $\alpha 7$ *nAChRs* agonists that demonstrated anti-inflammatory activity (Bencherif et al., 2013).

In mice, AR-R17779 treatment potently prevented postoperative ileus (POI) (The et al, 2007) and reduced TNF- α levels in both plasma and synovial tissue in experimental models of arthritis, whilst

mice lacking the $\alpha 7$ subunit ($\alpha 7 nAChRs^{-/-}$) showed a significant increase in arthritis incidence, severity and in synovial inflammation (van Maanen et al., 2010).

GTS-21 has been shown to reduce in vitro TNF- α production by murine alveolar macrophages upon LPS stimulation and, in vivo, significantly reduced lung TNF- α concentration in an animal model of sepsis (Giebelen et al., 2007).

DMAB, PNU-282987 and PHA 558487 reduced cytokines production from macrophages and inhibited neutrophils trans-alveolar migration in experimental models of asthma (Su et al., 2010).

These results evidenced the key role played by $\alpha 7 nAChRs$ stimulation in attenuating inflammatory and immune responses.

Recent studies analyzed the critical role played by another nicotinic receptor, the $\alpha 4\beta 2$ subtype. $\alpha 4\beta 2 nAChRs$ show lower permeability to Ca^{++} and slower desensitization with respect to $\alpha 7$ (Van der Zanden et al., 2009), and, despite the paucity of studies investigating the anti-inflammatory activity of this receptor, some preliminary evidence about its involvement in the CAP has been documented (Van der Zanden et al., 2009; Vishnu et al., 2011). In particular $\alpha 4\beta 2$ stimulation triggers an intracellular cascade that leads to the inhibition of NF- κB (Vishnu et al., 2011) and influence the phagocytic activity of isolated murine macrophages (Van der Zanden et al., 2009).

INVOLVEMENT OF THE SPLEEN IN THE CAP

The central role played by the spleen in the inhibition of the inflammatory response by VN stimulation has been addressed by Huston and colleagues who demonstrated that electrical VN stimulation failed to suppress inflammation in splenectomised animals (Huston et al., 2006). Indeed, the spleen is responsible for most of the production of pro-inflammatory cytokines upon inflammatory stimuli (i.e. LPS) and it is the site where the biosynthesis of such mediators is suppressed by the CAP (Martelli et al., 2014).

However, in rodents, the spleen receives no vagal cholinergic fibers, but only noradrenergic innervation from splenic nerve terminals (Bellinger et al., 1993; Nance & Sanders, 2007). Hence, the model of CAP postulated by Tracey (Tracey et al., 2002), implicating a direct action by VN efferents on splenic immune cells (e.g. macrophages) (Fig. 7A), had to be modified. Huston and colleagues conceived a new formulation of the CAP, defined as “disynaptic model”, proposing that, in the celiac ganglion, preganglionic vagal fibers synapsed with postganglionic noradrenergic

splenic neurons (Huston et al., 2008). In this model, shown in figure 7B, *nAChRs* are proposed to be located in the postganglionic splenic neuron (Vida et al., 2011). Further support to this formulation was provided by Rosas-Ballina and colleagues (Rosas-Ballina et al., 2008), by showing that the integrity of the sympathetic splenic nerve is essential for VN stimulation to dampen inflammation. Nevertheless, later studies showed that there was no synaptic contact between VN efferent terminals and splenic-projecting sympathetic neurons; furthermore, most of those noradrenergic neurons were not located in the celiac ganglia (Bratton et al., 2012). In fact, electrical stimulation of the peripheral end of the vagus did not drive action potentials in the splenic nerve (Bratton et al., 2012), thus discrediting the “disynaptic model” (Fig. 7B, 7C).

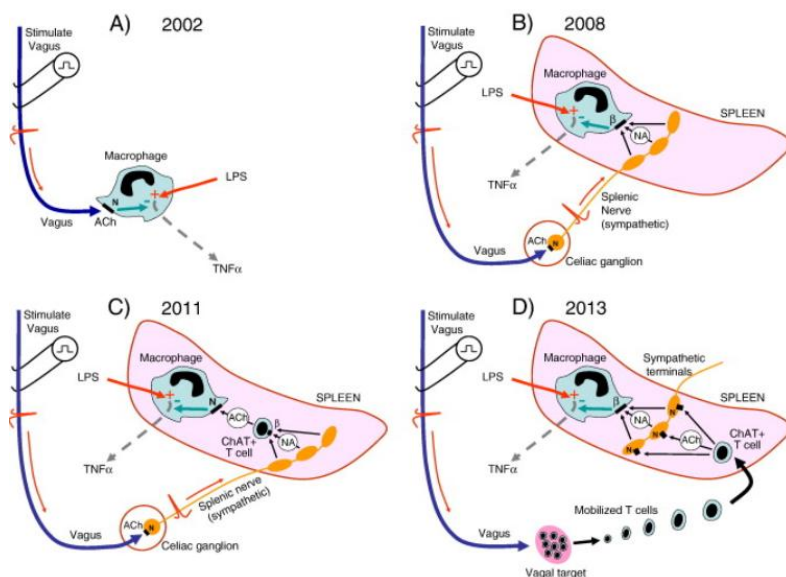


Figure 7: The evolution of the *Cholinergic anti-inflammatory pathway* (Martelli et al., 2014).

Recent findings indicate that the ACh necessary for the anti-inflammatory activity of the vagus is not neural in origin (Rosas-Ballina et al., 2011). Interestingly a subset of T-cells able to synthesize ACh has been identified: these cells express the Choline Acetyl Transferase (ChAT) enzyme and have been shown to be present within murine spleens (Rosas-Ballina et al., 2011; Gautron et al., 2013). In mice lacking functional T-cells (i.e. nude mice) VN stimulation failed to suppress inflammation, but the adoptive transfer of ChAT⁺ T-cells in these mice restored the anti-inflammatory activity of the vagus (Rosas-Ballina et al., 2011). These evidences suggest that the link between the VN and the spleen might be non-neural and, despite the mechanism is currently unclear, the hypothesis of cellular migration is increasingly being corroborated.

If, on one hand, the vagus does not directly innervate the spleen, on the other hand, VN efferents widely innervate the gastrointestinal (GI) tract, where a large quantity of lymphoid cells are located (Berthoud et al., 1991). Moreover, enteric neurons are strongly associated with immune cells in the lymphoid tissue of the GI tract (Gautron et al., 2013). In the most recent formulation of the CAP (Figure 7D) it has been hypothesized that, upon inflammatory stimuli (e.g. cytokines), VN stimulation can drive T-cells, including ChAT⁺ T-cells, from the GI tract to the spleen (Martelli et al., 2014), where they release ACh to suppress pro-inflammatory cytokines production, through the activation of $\alpha 7$ nAChRs. Whether the $\alpha 7$ nAChRs mediate their anti-inflammatory action directly or indirectly remains to be elucidated. Some authors suggest that activation of $\alpha 7$ nAChRs expressed by splenic immune cells triggers an increase in intracellular Ca⁺⁺ levels and the activation of Jak2/STAT3 pathway, leading to the inhibition of NF-kB (Wang et al., 2003), whereas some others propose that nAChRs are located in splenic nerve terminals, where they stimulate the release of norepinephrine that inhibits NF-kB through the activation of β -adrenergic receptors expressed on splenic macrophages (Rosas-Ballina et al., 2008, Bonaz et al., 2016). A protective effect independent of the spleen and mediated by the stimulation of $\alpha 7$ nAChRs expressed on intestinal resident macrophages by ACh released by enteric neurons reached by vagal efferents has been finally speculated (Goverse et al., 2016).

VAGUS NERVE AND INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder of the GI tract, characterized by an aberrant immune response against antigens of the luminal flora in genetically susceptible individuals in response to some environmental factors, such as cigarette smoking and diet (Abraham et al., 2009). Crohn's Disease (CD) and Ulcerative Colitis (UC) are the two principal types of IBD: the incidence and the prevalence of these diseases have strongly increased in the last 50 years, especially in northern Europe and North America (Cosnes et al., 2012). In patients affected by IBD, chronic intestinal inflammation alters the integrity of the intestinal epithelial barrier and modifies intestinal motility and secretions, leading to diarrhoea and abdominal pain. Moreover, extra-intestinal symptoms, including fever, erythemas and arthritis are frequently observed (Abraham et al., 2009). Both UC and CD show a relapsing and remitting course and there is a significant reduction in quality of life during the exacerbations of the disease (Casellas et al., 2001).

Evidences arising from clinical studies showed that IBD is associated with structural and functional alterations of the autonomic nervous system: in fact up to the 35% of patients affected by IBD show decreased efferent vagus nerve activity (Lindgren et al., 1993), resulting in parasympathetic dysfunction and sympathetic dominance.

Studies in animal models confirmed that autonomic imbalance could lead to the development of intestinal inflammation, as chemical sympathectomy exerted a protective effect in TNBS-colitis (McCafferty et al., 2007), whereas, after vagotomy, an exacerbation of colitis, associated to increased NF- κ B and pro-inflammatory cytokines levels, occurs (Ghia et al., 2006; Ghia et al., 2008; Munyaka et al., 2014). Moreover, the vagus nerve has been shown to play a counter-inflammatory role in acute DSS- and DNBS-colitis through the nicotinic α 7 subunit receptor, since α 7nAChR^{-/-} vagotomised mice developed a more severe form of colitis than wild type animals (Ghia et al., 2008). Recently it has been reported that activation of the CAP can be centrally mediated, as central cholinergic activation stimulates a vagus nerve-to-spleen circuit that ameliorates experimental colitis in mice, presumably via α 7nAChRs (Ji et al., 2014). On the other hand, contradictory results were reported by studies-indicating that treatment with selective α 7 agonists fails to improve clinical parameters of chemically induced colitis (Snoek et al., 2010; Galitovskiy et al., 2011) and that the increased susceptibility to develop intestinal inflammation after vagotomy in mice is α 7nAChRs -independent (Di Giovangiulio et al., 2016).

THE IMMUNE SYSTEM IN IBD PATHOGENESIS

Although the pathogenesis of IBD remains unknown the inflammatory response that exacerbates the disease apparently results from an aberrant immune response against luminal antigens (Abraham et al., 2009): hence, the local mucosal immune system (i.e. *Mucosal-Associated Lymphoid Tissue*, MALT) plays a key role in the development of either UC or CD.

At the gut level, complex interactions between the different immune cell types take place, affecting the interactions with the body immune system (Ilan, 2016). The first defence against pathogens is represented by the intestinal epithelium, which is a physical (protein-protein intercellular network tightly sealing the paracellular space) and functional barrier to luminal antigens, containing lymphocytes, macrophages, as well as other cell types specialized in the production of mucus (i.e. Goblet cells) or antimicrobial peptides (i.e. Paneth cells). The intestinal epithelium is in contact with a wide range of microbial species and must discriminate between harmful and inoffensive

components (Galvez, 2014): intraepithelial lymphocytes recognize specific *Pathogens-Associated Molecular Patterns* (PAMPs) through *Toll-Like Receptors* (TLRs) and *Nucleotide Oligomerization Domains* (NOD) expressed on their cell surface, whilst dendritic cells (DCs) are involved in controlling immunity against pathogens and tolerance towards commensals. Because of their unique TLRs and NOD pattern DCs are able to discriminate between commensals and pathogens and to either stimulate or suppress T-cell response (Iwasaki et al., 2004). In healthy individuals DCs promote tolerance against commensal bacteria by stimulating the differentiation of naïve T-cells in regulatory T-cells through cytokine-mediated signalling (Banchereau et al., 1998), regulatory T-cells which contribute to maintain intestinal homeostasis through IL-10 and TGF- β -dependent mechanisms (figure 8).

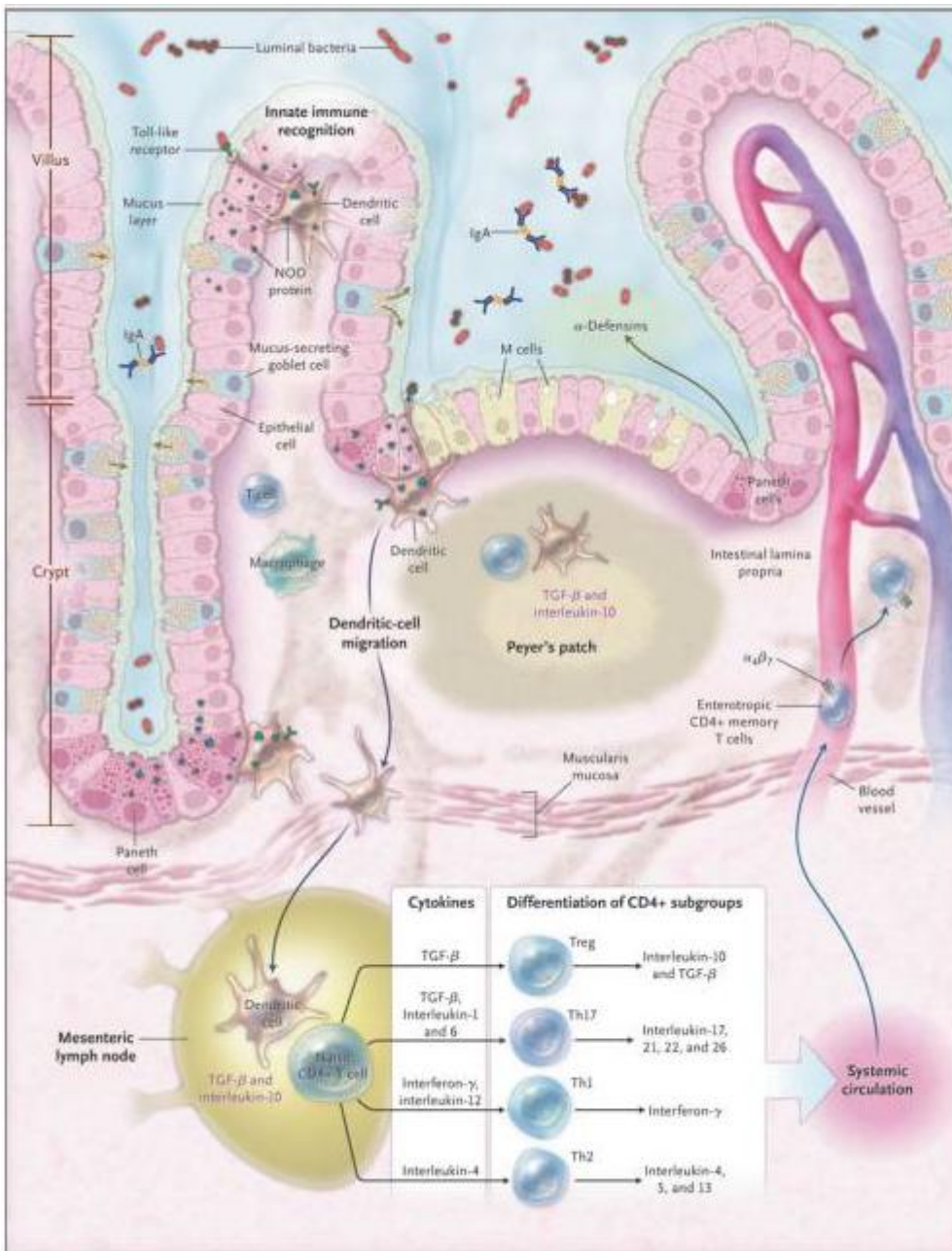


Figure 8: the intestinal immune system in healthy state (Abraham et al., 2009).

Interestingly, although no functional or numerical defects of Tregs have been detected in CD or UC patients (Valatas et al., 2015), IL-10 production by DCs obtained from CD patients is strongly impaired, whilst higher amounts of IL-23 are produced, resulting in a stronger Th1 immune response (Sakuraba et al., 2009). In addition, biopsies from IBD patients showed Th1- and Th17-cells infiltrating within the intestinal mucosa (Fujino et al., 2003; Ilan, 2016).

Hence, IBD is associated with an imbalance between effector (Th1, Th2, Th17) and regulatory (Treg) T-cells (figure 9): in particular, phenotype characterization of mesenteric lymph nodes

(MLN) T-cells derived from CD patients showed a Th1 and Th17 feature (Sakuraba et al., 2009), whereas in UC the phenotype is markedly Th2 (Abraham et al., 2009).

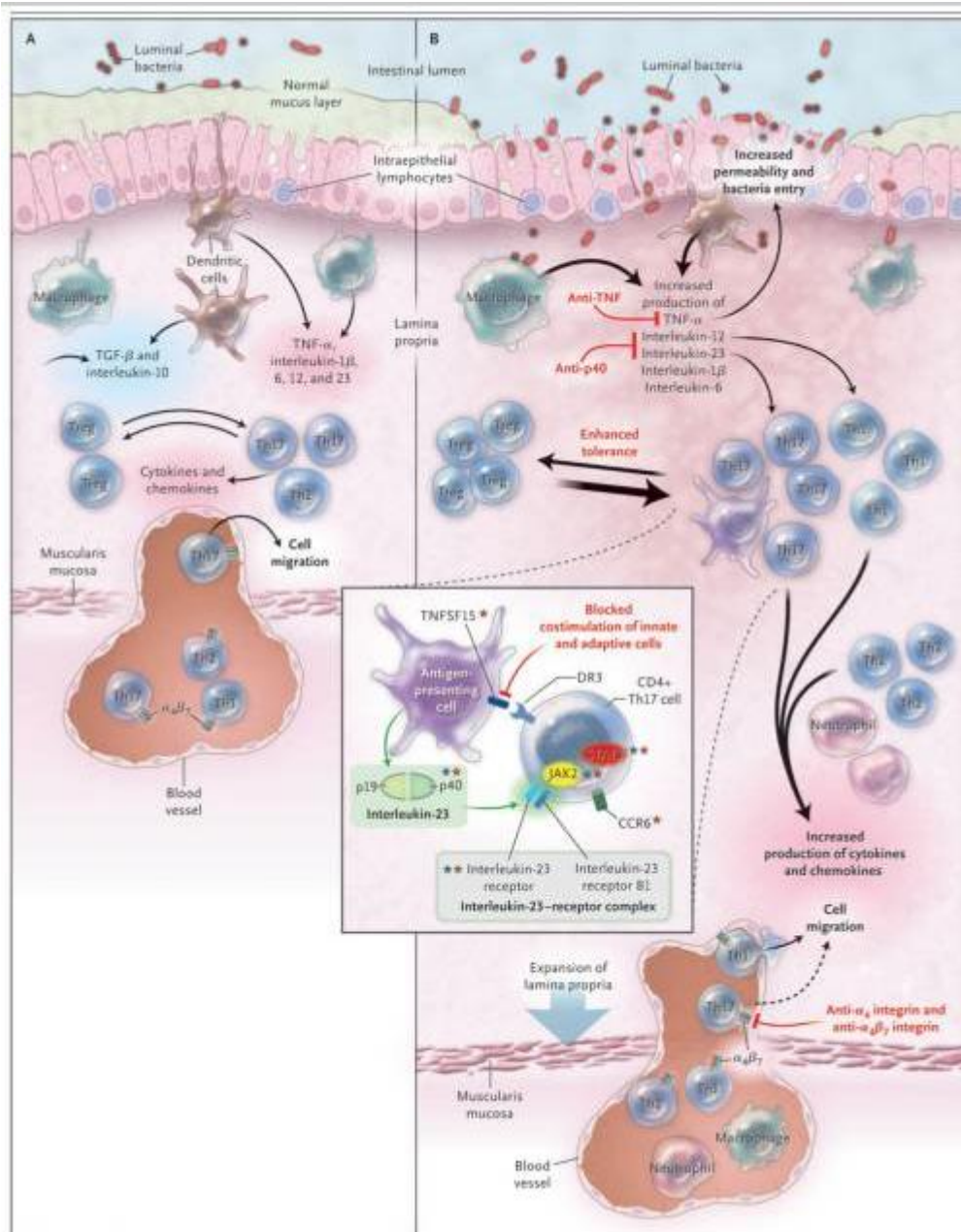


Figure 9: the intestinal immune system in health and disease (Abraham et al., 2009).

An impaired Treg function, due to a decreased production of IL-10 by DCs, is probably a critical factor for the development of intestinal inflammation, nevertheless, which subtype of DCs induces differentiation in regulatory T-cells in human intestinal mucosa remains to be elucidated (Baumgart et al., 2007).

CURRENT PHARMACOLOGICAL THERAPIES FOR IBD

Despite IBD etiology being still unknown, it is now clear that its pathogenesis results from complex interactions between host-derived (e.g. immune system, microbial flora and genetic composition) and environmental factors. IBD is still incurable but remarkable advances in medical therapies have been made in the last decades, thus significantly improving patients' quality of life. Currently available pharmacological therapies are aimed at preventing relapses in quiescent disease and at inducing remission during "flares" (i.e. active phase of the disease) (Talley et al., 2011).

Medical therapies for IBD have been recently reviewed by the *American College of Gastroenterology IBD Task Force* (Lichtenstein et al., 2009; Kornbluth et al., 2010) and by the *European Crohn's and Colitis Organization (ECCO)* (Dignass et al., 2010; Dignass et al., 2012).

Anti-inflammatory agents:

Sulfasalazine was the first molecule to show efficacy for the treatment of IBD: upon oral administration, sulfasalazine reaches the colon, where it is hydrolysed by microbial flora to sulfapyridine and 5-aminosalicylic acid (5-ASA), which inhibits NF- κ B transcriptional activity, leukocytes chemotaxis and modulates prostanoids metabolism (Hoult et al., 1986). 5-ASA-based therapies are still widely used for the treatment of IBD, as they are very effective at inducing remission in mild to moderately active disease, as well as at preventing relapses in quiescent UC, whereas their use is not recommended in CD patients (Talley et al., 2011). The use of 5-ASA-based products (e.g. PentasaTM) is limited by side effects, including drug-induced hypersensitivity syndrome, blood dyscrasia, infertility in women and other rare adverse reactions (e.g. hepatitis, pancreatitis, pericarditis and nephritis) (Nielsen et al., 2007).

Glucocorticoids are another class of anti-inflammatory agents that are extensively used for the treatment of IBD, as well as other chronic inflammatory diseases (e.g. rheumatoid arthritis). The interaction between glucocorticoids and their nuclear receptor triggers a wide range of effects leading to the suppression of the inflammatory response: these effects include the reduction of cell adhesion molecules (CAMs) expression, induction of neutrophils apoptosis and inhibition of cytokines production (Goulding et al., 2004). Systemic administration of standard corticosteroids

(e.g. hydrocortisone or methyl-prednisolone) is effective at inducing remission in active UC and CD, but because of the well-described (Seow et al., 2009) harmful effects, their use in the maintenance therapy is strongly limited. Hence the efficacy of oral Budesonide, a semi-synthetic glucocorticoid with low bioavailability per os, has been evaluated in CD patients; although budesonide was not as effective as standard corticosteroids at resolving active disease (Talley et al., 2011), it was less harmful. As reported by the *ECCO Guidelines for the management of Crohn's Disease* (Dignass et al., 2010), budesonide is associated with steroid side-effects (e.g. reduction in bone mineral density) at a lower or similar frequency (Campieri et al., 1997), although less severe than prednisolone. However, budesonide is not recommended at preventing relapses in quiescent CD, although it may be considered an alternative in patients who have become dependent on systemic corticosteroids (Talley et al., 2011).

Immunosuppressants:

Severe side effects associated with a long-term treatment with glucocorticoids shifted IBD therapy towards a new class of compounds defined as immunosuppressants: among these the most commonly used are thiopurines (azathioprine and 6-mercaptopurine), methotrexate and calcineurin inhibitors (tacrolimus and cyclosporine). Each class of immunosuppressants has different mechanisms, but collectively these drugs directly or indirectly affect immune cells number or function (Talley et al., 2011). Thiopurines analogues are recommended for preventing relapse in both UC and CD, whilst methotrexate is effective at inducing remission as well as at preventing relapse in CD. Cyclosporin might be used only in hospitalized patients with severe active UC, not responding to other therapies (Talley et al., 2011). The range of side effects varies between the different molecules, even though all these drugs are responsible for an increased risk of infection, due to their action on the immune system. Beyond this, thiopurines are associated with bone marrow suppression (Aberra et al., 2005), nausea and allergic reactions, methotrexate can induce hepatotoxicity and myelosuppression, whilst cyclosporine is associated with renal toxicity.

The *ECCO Guidelines* reported that immunosuppressants should be started only in steroids refractory or steroids-dependent patients (Dignass et al., 2010).

Biological therapies:

In 1998 biological therapies were introduced in the United States, and subsequently worldwide, for the treatment of IBD: these therapies have been incorporated into the recent guidelines for therapy of CD and UC by *ECCO* (Dignass et al., 2010; Dignass et al., 2012), the *American Gastroenterological Association* (AGA) (Lichtenstein et al., 2006) and *The American College of Gastroenterology* (Lichtenstein et al., 2009; Kornbluth et al., 2010). These recombinant products are mostly chimeric or humanized monoclonal antibodies against pro-inflammatory mediators (e.g. cytokines), able to neutralize the action of their target. Among biological drugs anti-TNF agents (infliximab, adalimumab, golimumab and certolizumab pegol) are the most relevant class (Danese S, 2012): anti-TNF agents are currently recommended for moderate to severe CD in patients that do not respond or tolerate conventional therapies. Infliximab (Remicade™) significantly ameliorated clinical parameters in 60% of CD patients, and in 40% of these patients succeeded in keeping remission (Hanauer et al., 2002) and similar results were provided by adalimumab (Humira™) (Hanauer et al., 2006).

Beneficial effects of anti-TNF agents have been reported also in patients with UC, but the long-term efficacy in maintaining the quiescent phase has still to be evaluated (Talley et al., 2011).

Data from clinical trials suggest that biological therapies increase the risk of opportunistic infections (Irving et al., 2007) and there are also concerns that the biological therapies may increase the risk of lymphoma (Hansen et al., 2007); however, these adverse effects are observed also with corticosteroids and immunosuppressant therapies.

As stated by *ECCO* all anti-TNF therapies share similar efficacy and adverse effects, so the choice among the different drugs depends on availability, route of delivery, patient preference, cost and national guidance.

Besides anti-TNF agents many other biological therapeutics have reached the market in recent years: the most promising novel class of agents for the treatment of CD are selective anti-adhesion drugs targeting integrins (anti-integrins agents). Natalizumab is a humanized monoclonal antibody against $\alpha4\beta1$ and $\alpha4\beta7$ integrins that inhibits leukocyte adhesion and migration into the inflamed tissue. Despite its efficacy in patients with CD, natalizumab treatment is associated with the risk of progressive multifocal leukoencephalopathy (PML), an opportunistic brain infection that is caused by JC (John Cunningham) polyomavirus. Because of this rare but extremely severe side effect natalizumab was withdrawn from the market in 2005 (Bloomgren et al., 2012) but then re-introduced when approved by FDA against multiple sclerosis and IBD (Danese et al., 2015).

Similarly, vedolizumab, a humanized monoclonal antibody that selectively targets intestinal $\alpha4\beta7$ integrins, was approved by FDA for the treatment of IBD in 2014. Besides providing a significant reduction of inflammation in IBD patients, vedolizumab is generally well tolerated (Cherry LN et al., 2015).

In summary, biological therapeutics provided a significant step forward in ameliorating the quality of life in patients affected by IBD, but the huge costs related to their production makes such agents not easily accessible.

Modulation of gut microbiota:

Many environmental factors have been associated with an increased risk of developing IBD: among them, diet and medications, such as antibiotics, may affect the composition of gut microbiome, thus leading to intestinal dysbiosis, a condition that, by modifying the communication between the local and the systemic immune system, may increase the risk of chronic inflammatory disorders (Ilan Y., 2016).

Since the key role of gut microbiome in the pathogenesis of IBD was discovered, scientists have been developing many therapeutic strategies to modulate luminal flora composition and function through the administration of probiotics, antibiotics or, more recently, through the *Fecal Microbiota Transplant* (FMT). Probiotics-based products contain live microorganisms (e.g. *Bifidobacterium*, *Lactobacillus*, *Saccharomyces*) able to restore the intestinal barrier, supporting epithelial barrier integrity and function (Andrade et al., 2015) and to reduce inflammation by affecting both innate and adaptive immunity (Ramakrishna BS, 2009). Evidence coming from clinical trials showed a beneficial effect by *E. coli* Nissle 1917 in maintaining remission in patients intolerant or resistant to 5-ASA, but, nevertheless, the protective effect of probiotics in either CD or UC is still unproven.

A different approach is to modulate gut microbiome through the administration of antibiotics: an example is represented by rifaximin, a wide spectrum antibiotic with a very low intestinal absorption rate. Oral rifaximin was able to reduce mucosal adhesion of pathogenic bacteria, to decrease NF- κ B levels (Prantera et al., 2012), and to increase *Bifidobacteria*, commensal bacteria with beneficial properties for the host (Prantera et al., 2012), considered as microbial biomarkers for IBD (Duranti et al., 2016). All these mechanisms suggest a potential protective role for rifaximin in reducing intestinal inflammation and restoring eubiosis, although, up to now, according to the results of the clinical trials, antibiotics are recommended only for septic complications of CD (Dignass et al., 2010).

The newest approach to modulate microbiota composition is *Fecal Microbiota Transplant* (FMT), a procedure by which fecal bacteria are collected from healthy individuals and transferred into IBD patients by colonoscopy or enema. Although the FMT has been shown to be very efficient for the treatment of *Clostridium difficile* colitis, its efficacy in IBD remains to be elucidated, as clinical trials performed in recent years showed conflicting results (Konturek et al., 2015).

ANIMAL MODELS OF IBD

The first model of IBD was described more than 50 years ago (Kirsner et al., 1957) and, since then, more than 60 different animal models have been developed (Mizoguchi A, 2012), using mainly rodents, because of their phylogenetic similarities with humans and easy handling (Dothel et al., 2013). Animal models of colitis are essential tools to evaluate the efficacy of potential novel therapeutics at preclinical level and, although none of these models perfectly reproduces all the features of human IBD, they provided further understanding of the complex pathogenic mechanisms underlying the disease, besides representing a crucial resource to discover new therapeutic targets (Strober et al., 2008).

Animal models of IBD can be divided in models of chemically-induced colitis, immune-mediated, transgenic and spontaneous models (Dothel et al., 2013): the most widely used models are based on Dextran Sodium Sulphate (DSS) and TriNitro- (or DiNitro-) BenzenSulfonic acid (TNBS/DNBS), chemical agents able to trigger an immune and/or inflammatory response in rodents, reproducing the conditions of human colitis.

TNBS-induced colitis:

TNBS is a haptening agent typically administered as enema in either mice or rats, dissolved in 40-50% ethanol (Wallace et al., 1995). Ethanol transiently increases intestinal epithelial permeability, thus allowing TNBS to reach the sub-epithelial region, where it generates immunogenic products by covalently binding tissue or microbial proteins. Immunogenic adducts trigger an intense immune response, which is mainly T-cells driven (Strober et al., 2008). Such a response provokes severe ulcerations within the colonic mucosa and infiltration of inflammatory cells within some days (Dothel et al., 2013). Intrarectal administration of TNBS might be preceded by a skin sensitization to the same agent, in order to trigger a more specific and intense immune response, involving also

adaptive immunity. Otherwise, TNBS administration by enema might be repeated to evoke a chronic inflammatory condition (Elson et al., 1995).

TNBS elicits a characteristically Th1-mediated immune response, associated with an increased expression of pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, IL-12 and IL-17 (Dothel et al., 2013). TNBS-induced inflammation spontaneously resolves within few weeks, hence this model is not suitable to investigate the long-term course of the disease, unless the administration of the haptening agent is repeated.

DSS-induced colitis:

In DSS-colitis rodents are exposed to 2-5% DSS, which is dissolved in the drinking water, and develop an acute colitis within 5-7 days. DSS exposure might be repeated in 4-7 cycles to reproduce a chronic inflammatory condition, which is more appropriated to study the course of the disease. However, the inflammation observed in this model spontaneously resolves within 14 days after DSS withdrawal (Perse & Cerar, 2012).

The mechanism by which DSS elicits colonic mucosa damage is not fully clarified yet, but is likely to be associated with DSS infiltration within epithelial cells through a vesicular delivery system and competition with ribosomes substrate for mRNA translation (Laroui et al., 2012). Upon mucosal damage, an increased infiltration of microbial agents within intestinal lamina propria triggers an acute immune and inflammatory response, initially characterized by high levels of cytokines involved in innate immunity and Th-1 adaptive responses, such as TNF- α , INF- γ , IL-1 β , IL-6, IL-12, IL-17 and IL-10. Notably, in the chronic DSS model a mixed Th-1/Th-2 mediated response is observed, with enhanced expression of IL-5, IL-13, IL-10, IL-5 and IL-6, thus mimicking some of the features of the inflammation observed in human UC (Valatas et al., 2015).

In conclusion, chemically-induced models represent a very versatile, reproducible and low-cost tool to study IBD, but, on the other hand, they do not completely resemble the pathogenesis of the human disease. The lack of the complexity of human etiopathogenesis remains the strongest limit for either DSS- and TNBS-induced colitis (Maxwell et al., 2009).

Other models of colitis:

Immune-mediated, gene knock-out and transgenic models are extremely useful tools to investigate pathways implicated in IBD pathogenesis and to discover novel potential therapeutic targets (Dothel

et al., 2013). Immune-mediated models are based on mice lacking T-cells function (i.e. genetically deficient SCID-mice) in which subsets of CD4⁺ effector T-cells are inoculated, thus triggering an immune response that leads to an inflammatory condition resembling the human CD, with increased levels of Th1-derived cytokines including TNF- α , INF- γ and IL-12 (Powrie et al., 1994).

In genetically engineered models of colitis (e.g. IL-10 knock-out mice or NOD2 knock-out mice) modifications of the genome, such as gene deletion, allow to investigate the role of specific genes in IBD pathogenesis or to achieve an inflammatory condition that reproduces the human disease.

Although these models have a very high reproducibility, they are limited by their high cost, and, moreover, the induction of the disease is obtained by the modification of a single gene, whilst it is well established that human IBDs are polygenic disorders (Kuhn et al., 1993).

Finally, spontaneous models of colitis are achieved through the crossbreeding between animals with a different genetic background, generating hybrids that spontaneously develop the disease.

However, the low standardization of these models is strongly limiting their use in preclinical research (Mizoguchi et al., 2012).

Table 1:

	Chemically induced	Immune-mediated	Genetically engineered
Applications	Acute, chronic and recurrent inflammation Intestinal mucosal impairment Innate immune response	Adaptive immunity Chronic inflammation	Specific gene-related mechanisms
Advantages	Cost saving, easily achievable, reproducible Acute, recurrent inflammatory episodes Suitable for testing drug candidates Large number of data available from previous studies Suitable to study T-cell mediated response (TNBS)	Reliable chronic models Commercially available Resemble mechanisms of specific immune response Suitable for testing target-specific drug candidates	Reliable chronic models Commercially available Symptoms derived from endogenous mechanism New updated models available Valuable means for aetiopathogenesis studies

Drawbacks

Standardization depending on procedural features

Self-limiting inflammation (spontaneously resolving)

Not suitable for studies on T-cell mediated immunity (DSS)

Laborious and cost-expensive

Differences between immune response of mice and humans

Cost-expensive

Phenotype assessment required

Often limited to pathophysiological studies

Validation for drug screening still under debate

Table 1: Applications, advantages and drawbacks of different IBD models

AIM OF THE STUDY

Several experimental evidences support a key role of the cholinergic anti-inflammatory pathway (CAP) in attenuating the inflammatory response through the activation of vagus nerve efferents (Borovikova et al., 2000a). In the last two decades the mechanisms involved in this vagal modulation of inflammatory and immune responses have been extensively studied, and most part of the studies concluded that CAP involves splenic immune cells and the activation of *nAChRs* (Tracey et al., 2002). Among the nicotinic receptors, the $\alpha 7$ subtype has emerged as the responsible for the anti-inflammatory activity (Wang et al., 2003), although preliminary evidence suggests the involvement also of other *nAChRs* subtypes, such as the $\alpha 4\beta 2$ receptor (Vishnu et al., 2011).

The aim of this study was to pharmacologically investigate the role of $\alpha 7$ and $\alpha 4\beta 2$ *nAChRs* subtypes in the regulation of the local and systemic inflammatory responses induced in a murine model of TNBS-induced colitis. To this end, the first part of the research was dedicated to evaluate the effects produced by application of various doses of highly selective agonists (AR-R 17779 and TC 2403) and antagonists (methyllycaconitine and dihydro- β -erythroidine) of $\alpha 7$ and $\alpha 4\beta 2$ *nAChRs* on the clinical and inflammatory markers (Disease Activity Index, macroscopic colonic mucosal damage, colonic thickening and lung and colonic granulocyte infiltration) increased in mice by TNBS exposure.

In the second part of the study, the contribution of the spleen to the protection afforded by treatment with $\alpha 7$ agonist AR-R 17779 was assessed by repeating the experiments in splenectomised mice.

Finally, given the key role played by T lymphocytes in the development and pathogenesis of human IBD, in TNBS-induced colitis and in the CAP as well, the phenotypic characterization of T cells subpopulations ($CD4^+$ and cytotoxic $CD8^+$) in the spleen and mesenteric lymph nodes was performed by flow cytometry in vehicle- and AR-R 17779-treated colitic mice, whether or not subjected to splenectomy. As regards $CD4^+$ subgroups, we focussed our attention in particular on $CD4^+ CD25^+ FoxP3^+$ regulatory cells (Tregs), a crucial population in limiting and resolving inflammation, and their cytokine IL-10, by investigating whether the beneficial effects showed by $\alpha 7$ agonist involved the modulation of their trafficking or activation.

METHODS

Animals:

Female CD/1 Swiss mice (7–12 weeks old) were housed and maintained under standard conditions at our animal facility. Food and water were available ad libitum. All animal experiments were performed according to the guidelines for the use and care of laboratory animals and they were authorized by Ministero della Salute (DL 26/2014).

Induction and assessment of colitis:

Six days before colitis induction (day -6) animals were subjected to cutaneous application of 50 μ L of a 10% (w/v) TNBS solution in 50% ethanol. Such skin sensitization was performed in order to trigger a more intense and specific immune response against TNBS upon the second exposure to the haptening agent. After 20 hours fasting with free access to water containing 5% glucose, colitis was induced in lightly anaesthetized mice by intrarectal (i.r.) administration of the same volume and concentration of TNBS applied during skin sensitization. TNBS instillation was performed using a PE50 catheter positioned 4 cm from the anus in mice kept in the head-down position for 3 minutes to avoid the leakage of intracolonic instillate. Sham animals were i.r. inoculated with 50 μ L 0.9% NaCl (saline solution). Three days after TNBS or saline instillation (day 4) mice were euthanized by CO₂ inhalation.

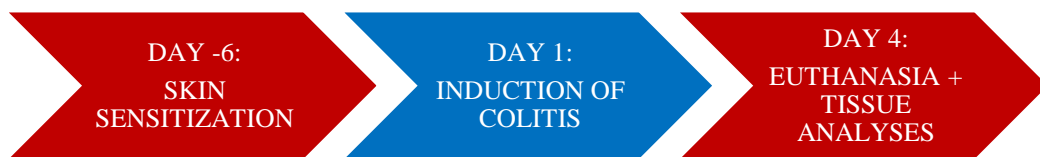


Figure 10: Schematic representation of the experimental protocol.

Body weight loss and reduction of stools consistency were determined daily in order to assess the Disease Activity Index (DAI). The macroscopic colonic damage was assessed as macroscopic score (MS). The wet weight and the length of each colon were recorded and weight/length ratio was considered as disease-related intestinal wall thickening (Bischoff et al., 2009).

Pharmacological treatments:

Pharmacological treatments started 8 hours after colitis induction (day 1) and were applied twice daily by subcutaneous (s.c.) injection. Control mice (TNBS) received 10 mL/kg 0.9% NaCl subcutaneously (b.i.d.), while positive control animals (SULF) were treated with 50 mg/kg/die of the standard drug sulfasalazine per os.

Animals were randomly divided in the following different experimental groups:

- SHAM: saline solution i.r. and s.c.
- TNBS: TNBS i.r. and saline s.c.;
- SULF: TNBS i.r. and sulfasalazine (50 mg/kg) per os;
- AR: TNBS i.r. and $\alpha 7$ agonist AR-R 17779 (0.5; 1.5; 5 mg/kg) s.c.;
- MLA: TNBS i.r. and $\alpha 7$ antagonist methyllycaconitine (0.1; 0.5; 1 mg/kg) s.c.;
- TC: TNBS i.r. and $\alpha 4\beta 2$ agonist TC 2403 (2; 5 mg/kg) s.c.;

- DBE: TNBS i.r. and $\alpha 4\beta 2$ antagonist Dihydro- β erythroidine (0.5; 1.5; 5 mg/kg) s.c..

Disease Activity Index (DAI):

DAI is a parameter that estimates the severity of the disease; it is based on the daily assignment of a total score, according to Cooper's modified method (Cooper et al., 1993), on the basis of body weight loss and stool consistency.

The scores were quantified as follows:

Stool consistency: 0 (normal), 1 (soft), 2 (liquid);

Body weight loss: 0 (<5%), 1 (5–10%), 2 (10–15%), 3 (15–20%), 4 (20–25%), 5 (>25%).

Colon macroscopic damage (MS):

After euthanasia, the colon was explanted, opened longitudinally, flushed with saline solution and MS was immediately evaluated through inspection of the mucosa: MS was determined according to previously published criteria (Wallace et al., 1989; Khan et al., 2002), as the sum of scores (max = 12) attributed as follows:

- Presence of strictures and hypertrophic zones (0, absent; 1, 1 stricture; 2, 2 strictures; 3, more than 2 strictures);
- Mucus (0, absent; 1, present);
- Adhesion areas between the colon and other intra-abdominal organs (0, absent; 1, 1 adhesion area; 2, 2 adhesion areas; 3, more than 2 adhesion areas);
- Intraluminal hemorrhage (0, absent; 1, present);
- Erythema (0, absent; 1, presence of a crimsoned area < 1 cm²; 2, presence of a crimsoned area > 1 cm²);
- Ulcerations and necrotic areas (0, absent; 1, presence of a necrotic area < 1 cm²; 2, presence of a necrotic area > 1 cm²).

Colonic length and thickness:

To evaluate muscular contraction and deposition of fibrotic material induced by a prolonged inflammatory state, the length of colon and its weight were measured, while weight/length ratio was calculated to estimate colon thickness (Bischoff et al., 2009).

Colonic and pulmonary myeloperoxidase activity (MPO):

Myeloperoxidase activity, marker of tissue neutrophil infiltration, was determined according to Krawisz's modified method (Krawisz et al., 1984). After being weighed, each colonic and lung sample was homogenized in ice-cold potassium phosphate buffer (100 mM, pH 7.4) containing aprotinin 1 µg/mL (1:10, v/v) and centrifuged for 20 min at 10,000 rpm at 4 °C. Pellets were re-homogenized in five volumes of ice-cold potassium phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethyl-ammoniumbromide (HTAB) and aprotinin 1 µg/mL. The samples were subjected to three cycles of freezing and thawing, and then centrifuged for 30 min at 12,000 rpm at 4°C. 100 µL of the supernatant was then allowed to react with 900 µL of a buffer solution containing o-dianisidine (0.167 mg/mL) and 0.0005% H₂O₂ .

Each assay was performed in duplicate and the rate of change in absorbance was measured spectrophotometrically at 470 nm (Jenway, mod. 6300, Dunmow, Essex, England). The sensitivity of the assay was 10 mU/mL, 1 unit of MPO being defined as the quantity of enzyme degrading 1 µmol of peroxide per minute at 25°C. Data were normalized with edema values [(wet weight-dry weight)/dry weight] and expressed as U/g of dry weight tissue.

Isolation of splenocytes:

Spleen was explanted immediately after euthanasia and mechanically dispersed through a 100 µm cell-strainer, washed with PBS containing 0.6 mM EDTA (PBS-EDTA). The cellular suspension

was then centrifuged at 1,000 rpm for 10 minutes at 4°C, the pellet re-suspended in PBS-EDTA and incubated with 2 mL of NH₄Cl lysis buffer (0.15 M NH₄Cl, 1mM KHCO₃, 0.1 mM EDTA in distilled water) for 5 minutes, at the dark, to induce the lysis of erythrocytes. Afterwards, samples were centrifuged at 1,000 rpm for 10 minutes at 4°C, the pellet was washed with PBS-EDTA and re-suspended in 5 mL cell staining buffer (PBS containing 0.5% fetal calf serum (FCS) and 0.1% sodium azide). The obtained cellular suspension was subjected to staining with fluorescent antibodies.

Isolation of mesenteric lymph-nodes:

The lymphoid tissue located in the middle of proximal colon's mesentery was explanted immediately after euthanasia and flushed with PBS, then mesenteric lymph-nodes (MLN) were separated from adherent adipose and vascular tissue, mechanically disgregated through a 100 µm cell-strainer and washed with *Hank's Balanced Salt Solution* (HBSS) containing 5% FCS. The cellular suspension was centrifuged at 1,000 rpm for 10 minutes at 4°C, the pellet was washed with HBSS+5% FCS and re-suspended in 3 mL cell staining buffer. The obtained cellular suspension was subjected to staining with fluorescent antibodies.

Immunofluorescent staining:

Before the incubation with fluorescent antibodies, 200 µL of cellular suspension were incubated with IgG1-Fc (1µg/10⁶ cells) for 10 minutes in the dark at 4°C, in order to block non-specific binding sites for antibodies.

The following antibodies were used: Phycoerythrin-Cyanine 5 (PE-Cy5) conjugated anti-mouse CD3ε (0.25 µg/10⁶ cells) emitting red fluorescence (FL-3), Fluorescein Isothiocyanate (FITC) anti-mouse CD4 (0.25 µg/10⁶ cells) emitting green fluorescence (FL-1), PE anti-mouse CD8a (0.25 µg/10⁶ cells) emitting yellow fluorescence (FL-2), Peridinin Chlorophyll Proteins-Cyanine5.5 (PerCP-Cy5.5) anti-mouse CD25 (1 µg/10⁶ cells) emitting red fluorescence (FL-3) and PE anti-mouse FoxP3 (1 µg/10⁶ cells) emitting yellow fluorescence (FL-2).

Cells were incubated with antibodies for 1 hour in the dark at 4°C, washed with PBS to remove excessive antibody and suspended in cell staining buffer to perform flow cytometry analysis.

Because of the intracellular localization of FoxP3, staining with PE anti-mouse FoxP3 was preceded by cells fixation and permeabilization: after staining with cell surface markers, cells were fixed with FOXP3 Fix/Perm Buffer and permeabilized with PBS containing 0.2% Tween 20. Cells were then incubated with PE anti-mouse FoxP3 for 30 minutes in the dark, at room temperature, washed with PBS to remove excessive antibody and suspended in cell staining buffer.

The viability of the cellular suspension was assessed through propidium iodide (PI) staining, a membrane impermeable fluorescent dye, excluded by viable cells, that binds to DNA emitting red fluorescence (FL-3), thus resulting as a suitable marker for dead cells. Cells were incubated with 10 µg/mL PI for 1 minute in the dark, at room temperature, and immediately subjected to flow cytometry analysis.

Flow cytometry:

Samples were analyzed using Guava easyCyte™ and InCyte™ software (Merck Millipore, Darmstadt, Germany). Lymphocytes were gated on the basis of their size in the *Forward Scatter* (FSC)-*Side Scatter* (SSC) plot (FSC low: SSC low), and T cells' percentage was determined by selecting CD3⁺ cells (FL-3). T-cells subpopulations were determined by measuring the percentages of CD4⁺ CD8⁻ and CD4⁻ CD8⁺ cells (FL-1; FL-2) within CD3⁺ (FL-3) lymphocytes (FSC low: SSC low). T-regs were determined by assessing the percentages of CD25⁺ FoxP3⁺ (FL-3; FL-2) cells within CD4⁺ (FL-1) lymphocytes (FSC low: SSC low). Cells viability was determined by assessing PI cells; all PI⁺ (FL-3) cells within lymphocytes gating (FSC low: SSC low) were excluded from the analysis.

Colonic IL-10 levels:

After euthanasia, colon segments were homogenized for 1 min in 750 µL of tissue lysis buffer containing 0.1 M Tris and 0.5% Triton X-100 (pH 7.4) and protease inhibitors cocktail (1 µg/mL aprotinin and 1 µg/mL leupeptin). Samples were then centrifuged for 30 min at 14000 g at 4°C and the supernatant was collected. Total protein concentration was quantified using Pierce BCA protein assay kit (ThermoFisher Scientific Inc., Waltham, MA). IL-10 colonic concentration was determined in duplicate in 100 µL aliquotes, using a commercially available ELISA kit (Mouse IL-

10 ELISA kit, Abcam™, Cambridge, UK) according to the manufacturer's protocol. The absorbances of the samples were measured spectrophotometrically at 450 nm (TECAN Sunrise™ powered by Magellan™ data analysis software, Mannedorf, Switzerland), subtracting readings at 550 nm to remove optical imperfections. The assay sensitivity was 0.03 ng/mL (linear range 0.031 - 2 ng/mL). Results were expressed as pg IL-10/mg protein.

Splenectomy:

15 days before the induction of colitis, the spleen was surgically removed from mice fasted for 16 hours and anaesthetised with intraperitoneal injection of 60 mg/kg Nembutal. Spleen was removed after laparotomy and ligation of blood vessels and, after the surgical intervention, mice were monitored daily to examine their health state and scar cicatrisation. Colitis induction and the whole experimental procedure was performed as previously described.

Splenectomized (SPX) mice were randomly divided in the following experimental groups:

- SPX/SHAM: saline solution i.r. and s.c.;
- SPX/TNBS: TNBS i.r. and saline s.c.;
- SPX/TNBS + AR: TNBS i.r. and AR-R 17779 1.5 mg/kg s.c.

Statistics:

All data were presented as means \pm SEM. Comparison among experimental groups were made using analysis of variance (one-way or two-way ANOVA) followed by Dunnett's or Bonferroni's post-test. Non-parametric Kruskal-Wallis analysis, followed by Dunn's post-test, was applied for statistical comparison of MS.

P<0.05, P<0.01, and P< 0.001 showed, respectively, statistically significant, highly significant, or extremely highly significant differences.

All analyses were performed using Prism 4 software (GraphPad Software Inc. San Diego, CA, USA).

Drugs, antibodies and reagents:

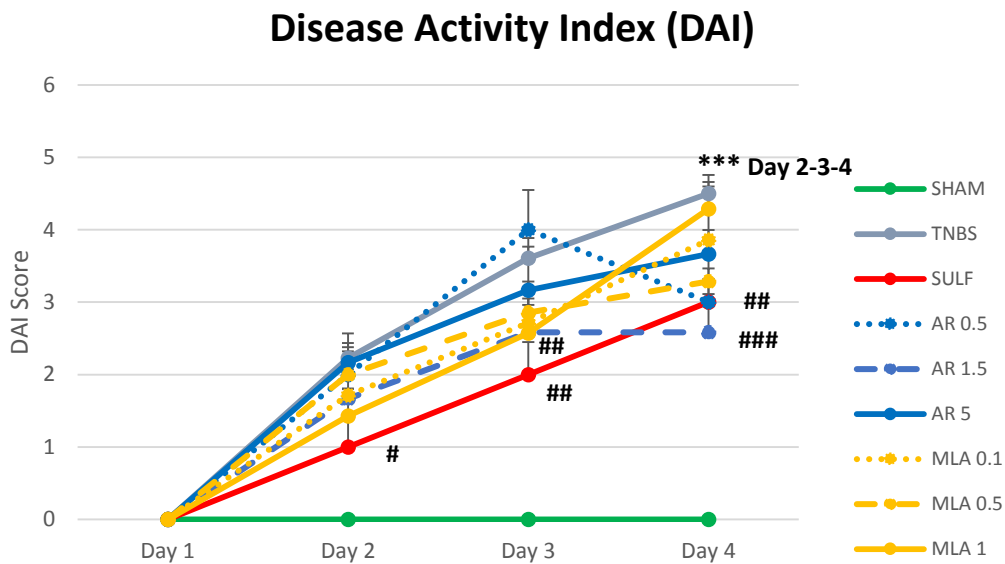
Sulfasalazine, MLA, TNBS, ethanol, HTAB and 30% hydrogen peroxide were purchased from Sigma Aldrich® (St. Louis, MO). AR-R 17779 and TC 2403 were purchased from Abcam Biochemicals® (Cambridge, UK), while dihydro-βerythroidine was purchased from Tocris Bioscience® (Bristol, UK). Fluorescent antibodies used for flow cytometry (FITC anti-mouse CD4, PE anti-mouse CD8, PE anti-mouse FOXP3, PerCP/Cy5.5 anti-mouse CD25), Propidium Iodide and FOXP3 Fix/Perm Buffer were purchased from BioLegend® (San Diego, CA), PE-Cy5 anti-mouse CD3ε from affymetrix eBioscience® (San Diego, CA) and Ig-G1-Fc from Millipore® (Merck, Darmstadt, Germany).

RESULTS (PART I)

In the first part of this work we analysed the effects of various doses of $\alpha 7$ *nAChRs* agonist AR-R 17779 and antagonist Methyllicaconitine (MLA) and of $\alpha 4\beta 2$ *nAChRs* agonist TC-2402 and antagonist Dihydro- β erythroidine (DBE) in TNBS-induced colitis. In particular, we evaluated the effects of these agents on clinical and inflammatory parameters to assess how they influence the animals' state of health and the inflammatory response elicited by TNBS instillation.

Disease Activity Index:

Graph 1:

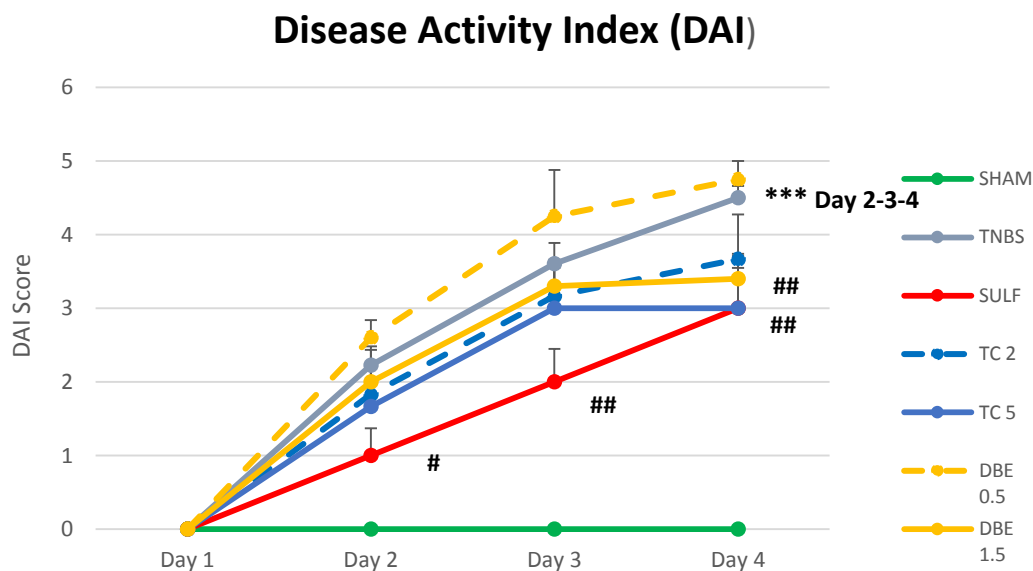


*** P<0.001 vs. SHAM; # P<0.05; ## P<0.01; ### P<0.001 vs. TNBS; two-way Anova + Bonferroni's post test

Disease Activity Index was measured daily since the day of colitis induction. DAI allows estimating the onset and the severity of the disease through the assignment of a score based on body weight loss and reduction of stools consistency. As shown in graph 1, [SHAM] mice scored 0 for the entire

duration of the experiment, whilst TNBS instillation provoked a remarkable increase in DAI ($p < 0.001$) as [TNBS] group scored 2.2 ± 0.2 (day 2), 3.6 ± 0.3 (day 3) and 4.5 ± 0.2 (day 4). Notably, DAI has raised throughout the entire duration of the experiment. Treatment with Sulfasalazine significantly decreased DAI ($P < 0.01$) in each day (1 ± 0.4 at day 2, 2 ± 0.5 at day 3, 3 ± 0.7 at day 4). Among $\alpha 7$ agents, on one hand AR-R 17779 provoked a reduction of DAI at 0.5 mg/kg (3 ± 0.3 at day 4, $P < 0.05$) and at 1.5 mg/kg (2.6 ± 0.4 at day 3; 2.6 ± 0.5 at day 4, $P < 0.001$), and, on the other hand 0.5 mg/kg MLA reduced DAI at day 4 (3.3 ± 0.2 , $P < 0.05$).

Graph 2:



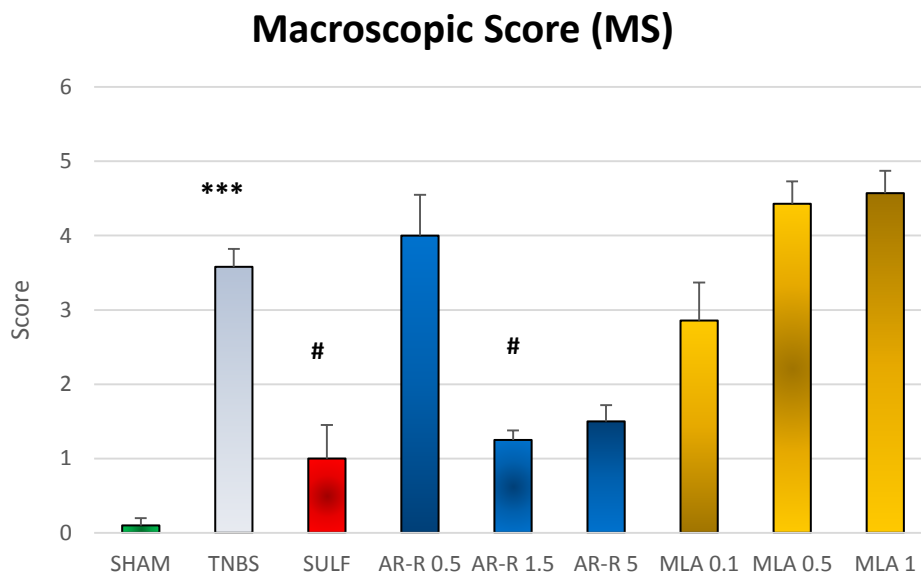
*** $P < 0.001$ vs. SHAM; # $P < 0.05$; ## $P < 0.01$ vs. TNBS; two-way Anova + Bonferroni's post test.

Disease Activity Index was significantly reduced at day 4 in mice treated with TC-2403 5 mg/kg ($P < 0.01$) with respect to [TNBS]. Notably, also the $\alpha 4\beta 2$ antagonist at 1.5 mg/kg decreased DAI in the final day of the study (3.4 ± 0.3 , $P < 0.01$ vs. [TNBS], graph 2).

Treatment with 5 mg/kg DBE (data not shown) was lethal in 100% animals.

Macroscopic Score:

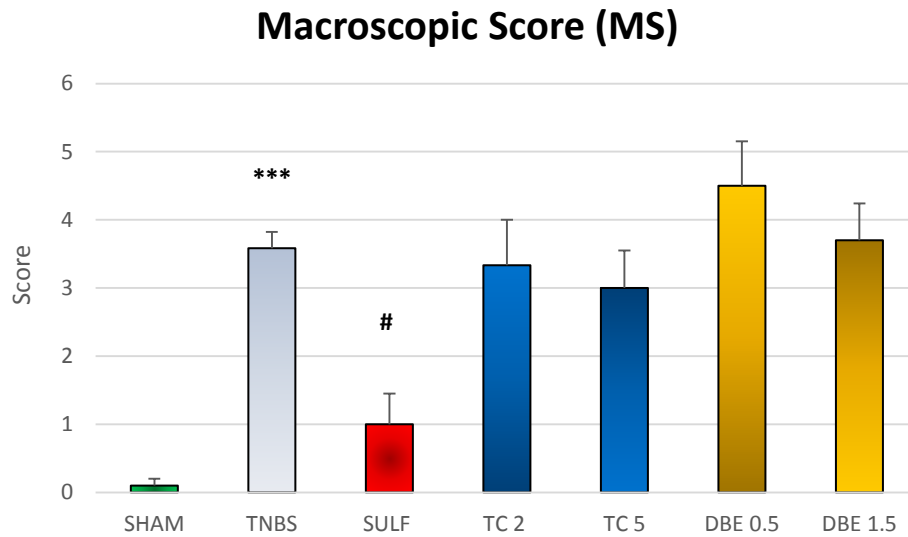
Graph 3:



***P<0.001 vs. SHAM; #P<0.05 vs. TNBS; Kruskal Wallis' test + Dunn's post test

MS was quantified to assess the colonic mucosal macroscopic damage (data shown in graph 3). [SHAM] mice scored 0, whilst [TNBS] group showed a significantly higher MS, as they scored 3.6 ± 0.2 ($P < 0.001$). Treatments with sulfasalazine and AR-R 17779 1.5 mg/kg provoked a significant reduction of MS, with respect to [TNBS] ($P < 0.05$), whilst MLA 0.5 and 1 mg/kg induced a moderate increase of MS compared to [TNBS], although not statistically significant.

Graph 4:

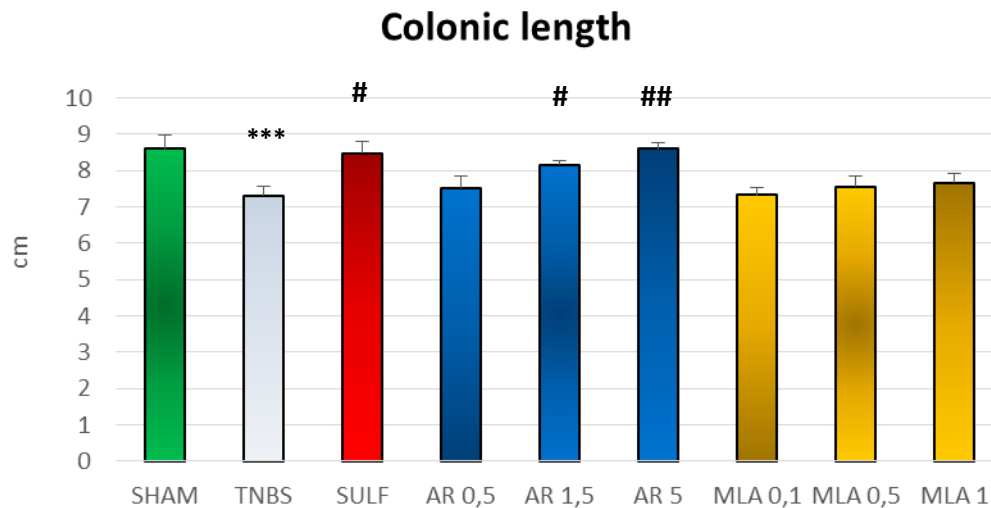


***P<0.001 vs. SHAM; #P<0.05 vs. TNBS; Kruskal Wallis' test + Dunn's post test.

The colonic macroscopic damage elicited by TNBS instillation was not counteracted by any of the treatments with $\alpha 4\beta 2$ agents. On the contrary, treatment with DBE 0.5 mg/kg slightly increased MS with respect to [TNBS] group, although not significantly (graph 4).

Colonic length:

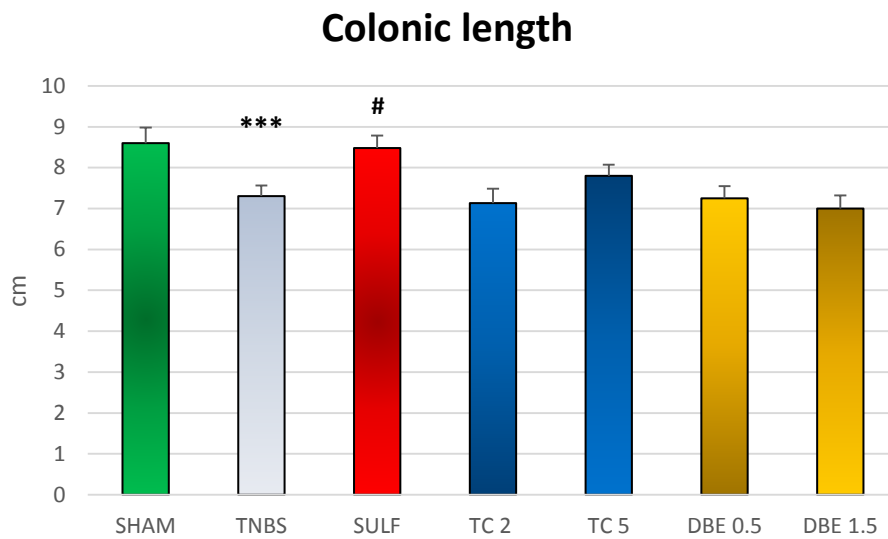
Graph 5:



***P<0.001 vs. SHAM; ## P<0.01; # P<0.05 vs. TNBS; one way Anova + Dunnett's post test.

The induction of colitis caused a notable shortening of the colon in [TNBS] mice with respect to [SHAM] group (P<0.001), as shown in graph 5. Administration of Sulfasalazine significantly counteracted the shortening of the colon provoked by TNBS (P<0.05). Similarly, [AR] mice showed a marked increase of colonic length, with respect to [TNBS], when treated with the $\alpha 7$ agonist at 1.5 mg/kg and 5 mg/kg (P<0.01). On the contrary, this parameter was not modified, compared to [TNBS], either by AR-R 17779 at the lowest tested dose or by MLA (graph 5).

Graph 6:

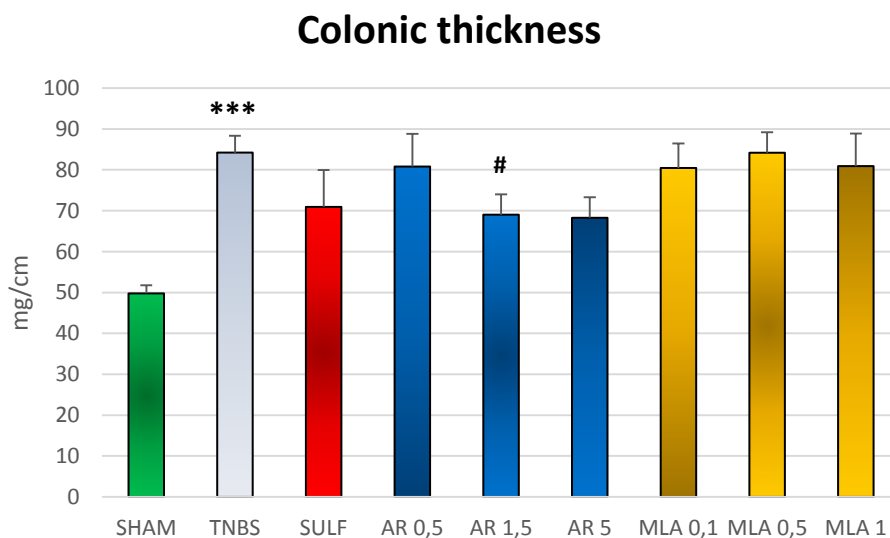


*** P<0.001 vs. SHAM; # P <0.05 vs. TNBS; one way Anova + Dunnett's post test.

As shown in graph 6, colonic shortening observed upon colitis induction in [TNBS] mice persisted in colitic mice treated with $\alpha 4\beta 2$ agents, as none of these treatments substantially modified colonic length with respect to [TNBS] group.

Colonic thickness:

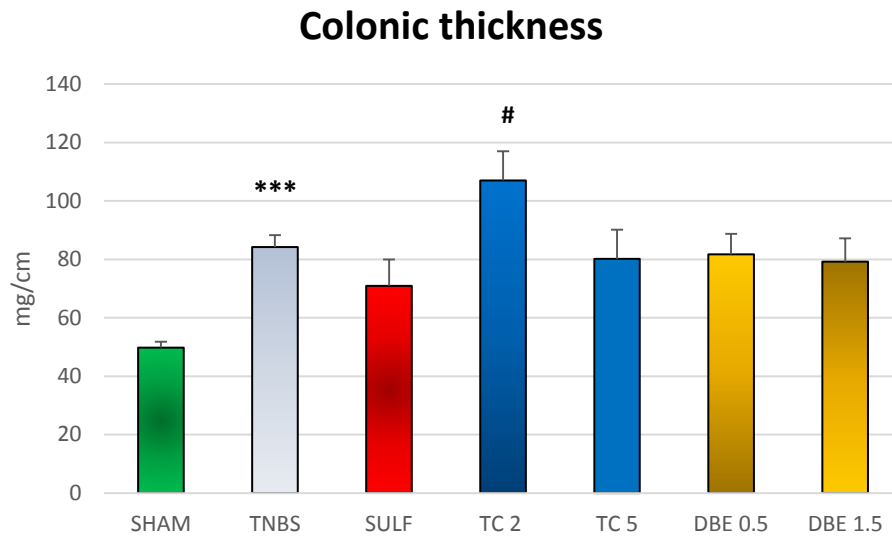
Graph 7:



*** $P < 0.001$ vs. SHAM; # $P < 0.05$ vs. TNBS; one way Anova + Dunnett's post test.

Upon colitis induction a massive thickening of the colonic wall was observed (graph 7); in fact [TNBS] mice, compared to [SHAM], showed an extremely significant increase of colonic thickness ($P < 0.001$). Treatment with 1.5 mg/kg AR-R 17779 counteracted colonic thickening ($P < 0.05$ vs. [TNBS]), and a reduction of colonic thickness, although not significant, was also observed upon treatment with 5 mg/kg AR-R 17779 and sulfasalazine.

Graph 8:

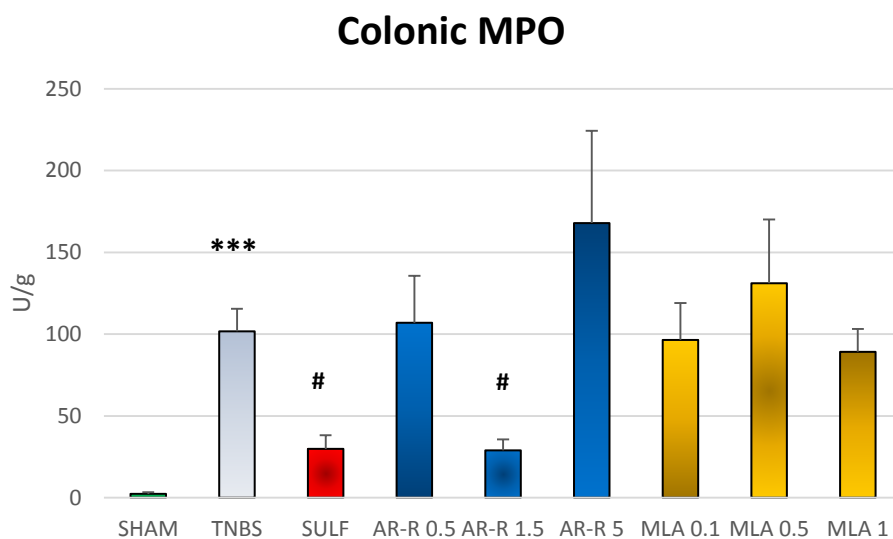


*** P<0.001 vs. SHAM; # P <0.05 vs. TNBS; one way Anova + Dunnett's post test.

The thickening of colonic wall provoked by colitis induction was not counteracted by any of the treatments applied (graph 8), whilst a significant increase of colonic thickness was observed in mice treated with TC-2403 2 mg/kg (P<0.05 vs. [TNBS]).

Colonic MPO:

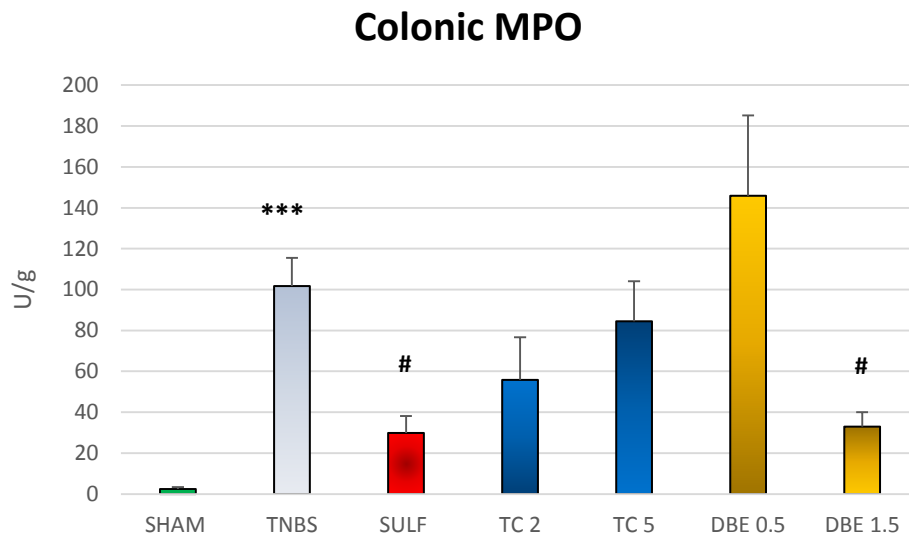
Graph 9:



*** P<0.001 vs. SHAM; # P<0.05 vs. TNBS; one way Anova + Dunnett's post test.

Colonic myeloperoxidase activity assay was performed to determine the granulocyte infiltration within the colon. Colitis induction triggered a remarkable increase in colonic MPO levels in [TNBS] mice compared to [SHAM] (P<0.001). Colonic granulocyte infiltration was significantly counteracted by the treatment with sulfasalazine and with AR-R 17779 1.5 mg/kg (P<0.05 vs. [TNBS] mice), as shown in graph 9.

Graph 10:

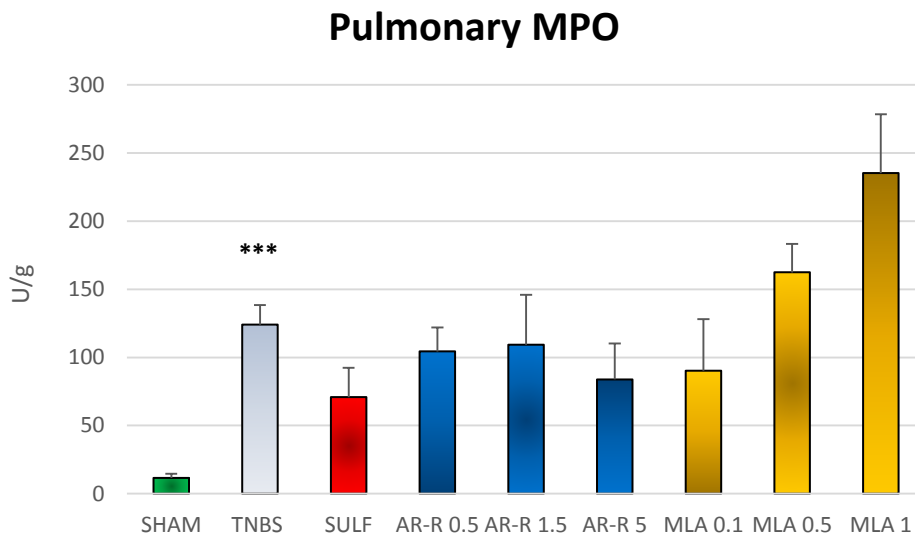


** P<0.001 vs. SHAM; # P<0.05 vs. TNBS; one way Anova + Dunnett's post test.

With respect to [TNBS] mice, colonic granulocyte infiltration was markedly decreased in animals treated with DBE 1.5 mg/kg (P<0.05 vs. [TNBS], data shown in graph 10). A moderate reduction of MPO levels was also observed upon treatment with TC-2403 either at 2 or 5 mg/kg, although no significant difference was observed between animals treated with any dose of $\alpha 4\beta 2$ agonist and [TNBS] group. On the other hand, a tendency to increase colonic myeloperoxidase activity was observed upon treatment with 0.5 mg/kg DBE with respect to [TNBS].

Pulmonary MPO:

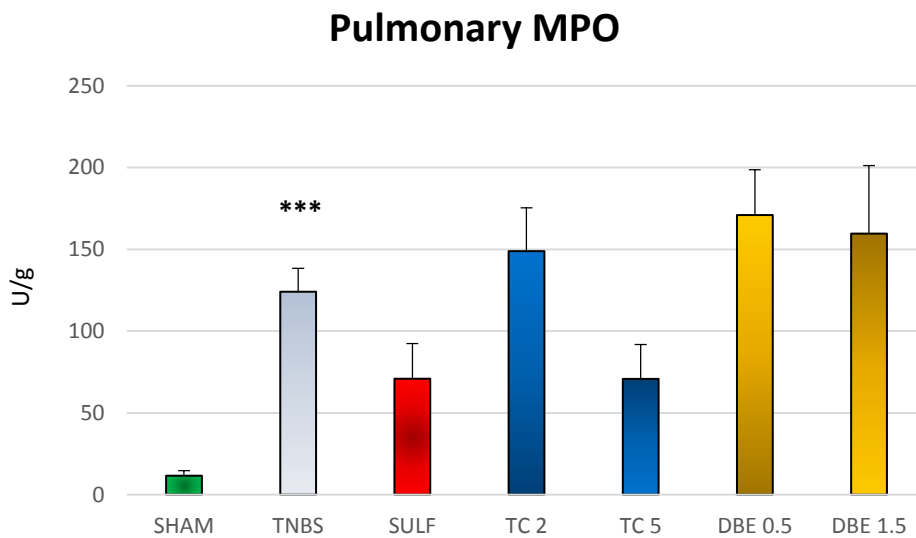
Graph 11:



*** P<0.001 vs. SHAM; one way Anova + Dunnett's post test.

Myeloperoxidase activity was determined also in the pulmonary tissue as a marker of systemic inflammation; as shown in graph 11, [SHAM] animals showed low levels of lung MPO (11.6 ± 0.3 U/g), whereas in [TNBS] group pulmonary granulocyte infiltration was severely augmented (124.1 ± 14.4 U/g, $P < 0.001$ vs. [SHAM]). In colitic mice none of the treatments applied significantly counteracted lung MPO levels, although a moderate reduction was observed upon treatment with sulfasalazine and 5 mg/kg AR-R 17779, whilst in animals treated with MLA 0.5 and especially 1 mg/kg myeloperoxidase activity was higher than in [TNBS] mice, although not significantly.

Graph 12:

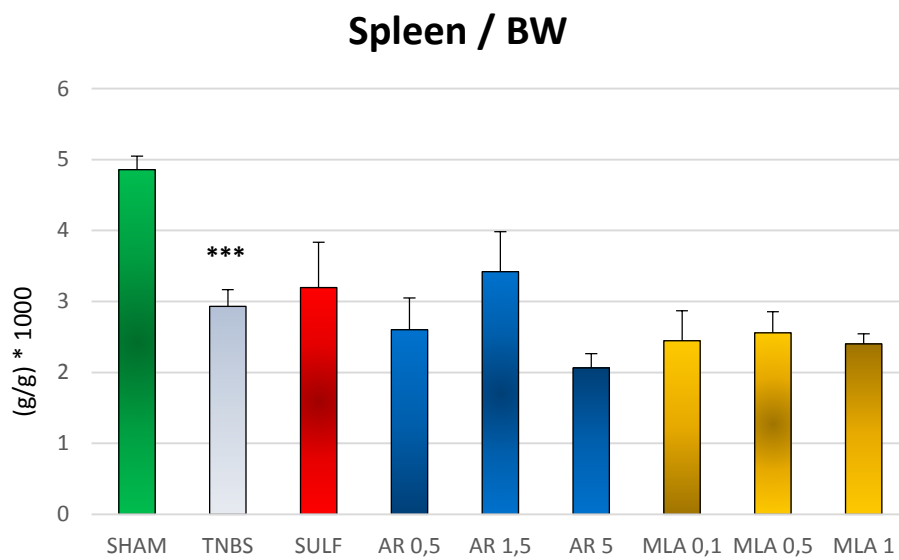


*** P<0.001 vs. SHAM; one way Anova + Dunnett's post test.

Lung MPO levels were moderately reduced in colitic animals treated with TC-2402 5 mg/kg, compared to vehicle-treated mice; such a reduction was of similar entity to the one provided by the standard drug sulfasalazine. On the contrary, administration of TC-2403 2 mg/kg and of DBE, either at 0.5 or 1.5 mg/kg, elicited a weak, not significant increase in pulmonary myeloperoxidase activity with respect to [TNBS] mice (graph 12).

Spleen / Body weight:

Graph 13:

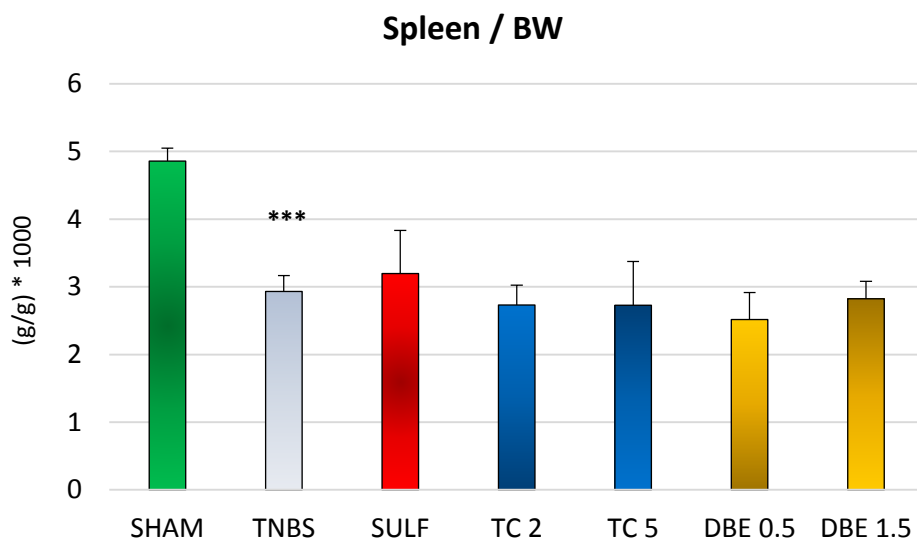


*** P<0.001 vs. SHAM; one way Anova + Dunnett's post test.

The ratio between spleen size and body weight was remarkably lower in [TNBS] mice compared to [SHAM] group (P<0.001). The decrease in spleen/BW was not counteracted by any of the treatments applied neither $\alpha 7$ nor $\alpha 4\beta 2$ ligands (graph 13 and 14). A further reduction of this

parameter has been observed upon the treatment with 5 mg/kg AR-R 17779, however the difference between [AR 5] and [TNBS] group was not statistically significant.

Graph 14:



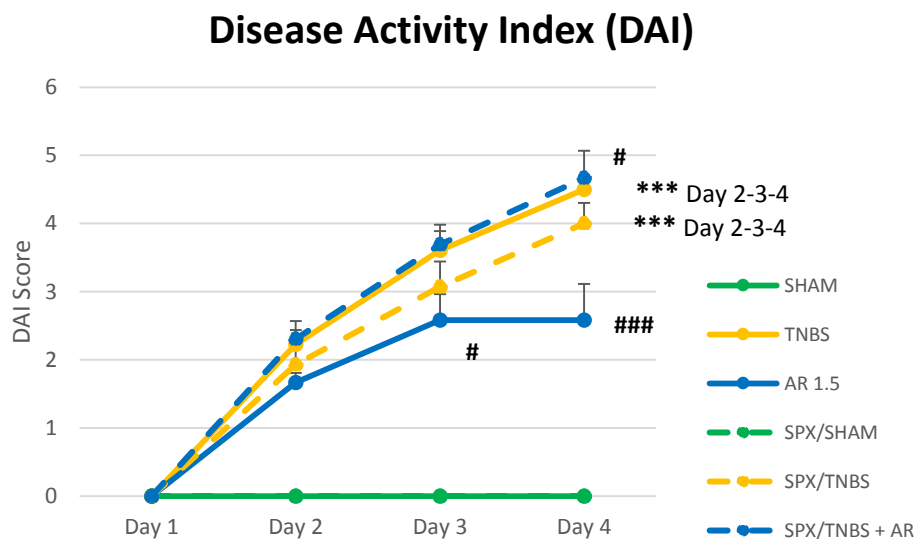
*** P<0.001 vs. SHAM; one way Anova + Dunnett's post test.

RESULTS (PART II)

In the second part of this work, we investigated the splenic contribution to the beneficial effects elicited by $\alpha 7$ agonist AR-R 17779 1.5 mg/kg by repeating the experiments in mice previously subjected to surgical splenectomy.

Disease Activity Index:

Graph 15:



*** P<0.001 vs. corresponding SHAM; ### P<0.001; # P<0.05 vs. corresponding TNBS;

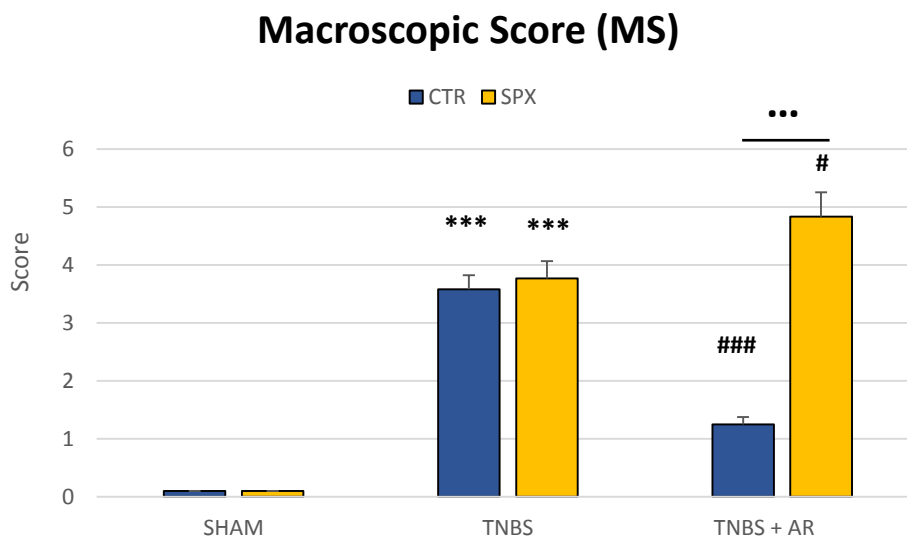
two-way Anova + Bonferroni's post test.

As expected, mice subjected only to laparotomy without induction of colitis ([SPX/SHAM]) scored 0 as regards DAI, similarly to [SHAM] mice. TNBS instillation caused a significant increase in Disease Activity Index during all days of experimentation in both [TNBS] and [SPX/TNBS] mice (P<0.001). Treatment with AR-R 17779 failed to reduce DAI in SPX mice during the entire

treatment period. Disease Activity Index was slightly higher in [SPX/TNBS+AR] than in [SPX/TNBS] group ($P < 0.05$, graph 15).

Macroscopic Score:

Graph 16:

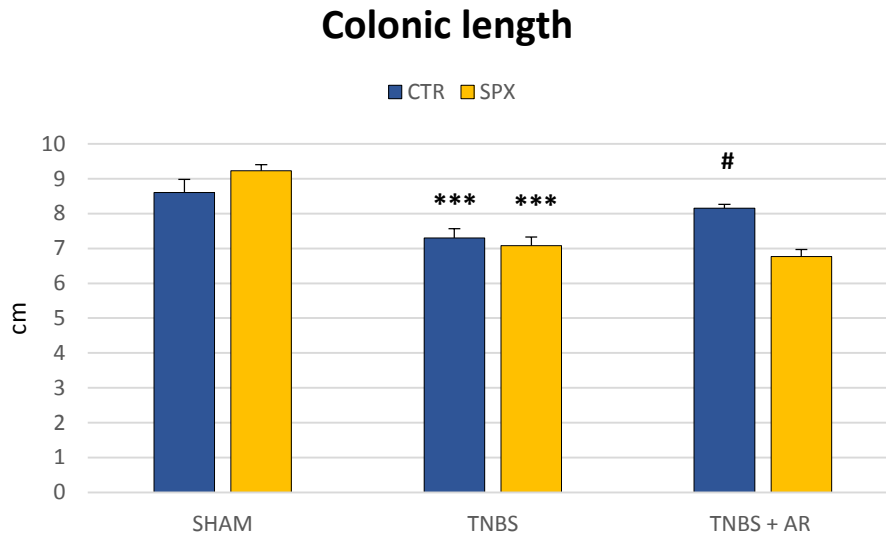


*** $P < 0.001$ vs. corresponding SHAM; ### $P < 0.001$; # $P < 0.05$ vs. corresponding TNBS; two-way Anova + Bonferroni's post test. ... $P < 0.001$ Unpaired T-test.

As shown in graph 16, TNBS inoculation in splenectomised mice elicited a remarkable mucosal damage ($P < 0.001$ vs. [SPX/SHAM]), comparable to the one observed in non-operated animals. On the other hand, in SPX animals, treatment with the $\alpha 7$ agonist provoked a remarkable increase of MS (4.83 ± 0.83) with respect to [SPX/TNBS] group ($P < 0.05$). In addition, colonic mucosal damage was prominently higher in [SPX/TNBS+AR] group compared to the animals treated with the same agent but not subjected to splenectomy ($P < 0.001$).

Colonic length:

Graph 17:

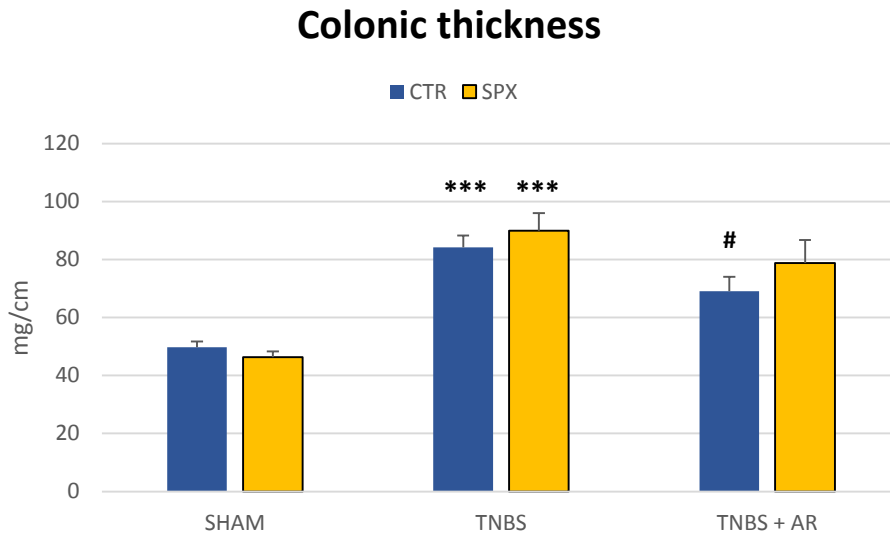


*** P<0.001 vs. corresponding SHAM; # P<0.05 vs. corresponding TNBS; two-way Anova + Bonferroni's post test.

As in non-operated mice, TNBS instillation produced a remarkable colon shortening also in splenectomised mice with respect to corresponding SHAM (P<0.001, graph 17). As regards the treatment with AR-R 17779 1.5 mg/kg, in SPX animals the $\alpha 7$ agonist lost its efficacy in counteracting the reduction in colon length.

Colonic thickness:

Graph 18:

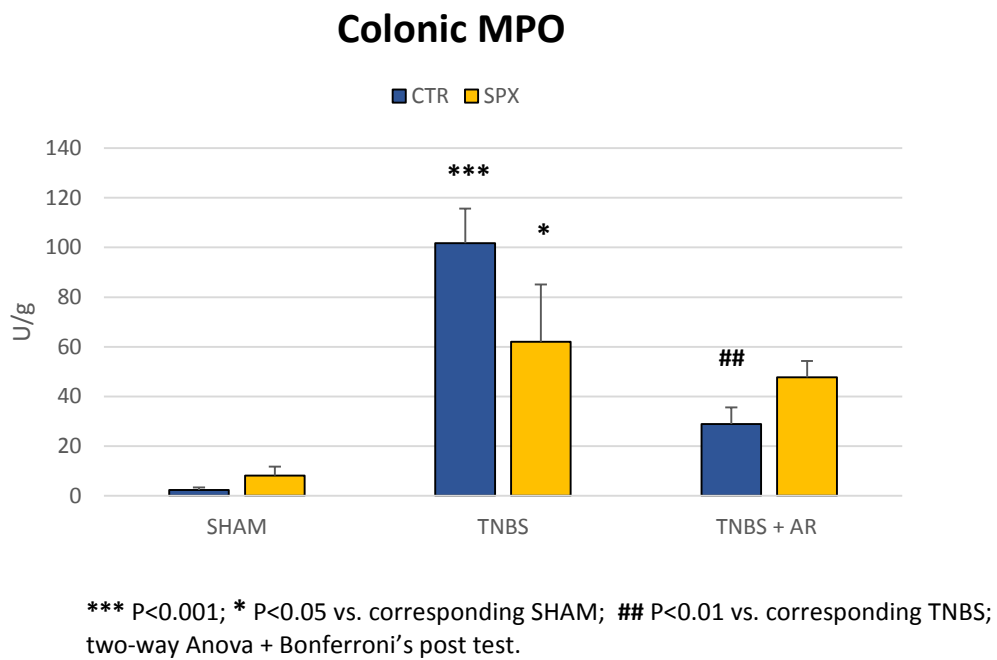


*** P<0.001 vs. corresponding SHAM; # P<0.05 vs. corresponding TNBS; two-way Anova + Bonferroni's post test.

Splenectomy did not influence TNBS-triggered colonic wall thickening, as colonic thickness was significantly higher in [SPX/TNBS] group compared to [SPX/SHAM] ($P<0.001$) (46.3 ± 2 mg/cm). If, one hand, AR-R 17779 significantly counteracted colonic thickening in [TNBS] mice, on the other hand the $\alpha 7$ agonist provoked only a mild reduction of colonic thickness in SPX animals (data shown in graph 18).

Colonic MPO:

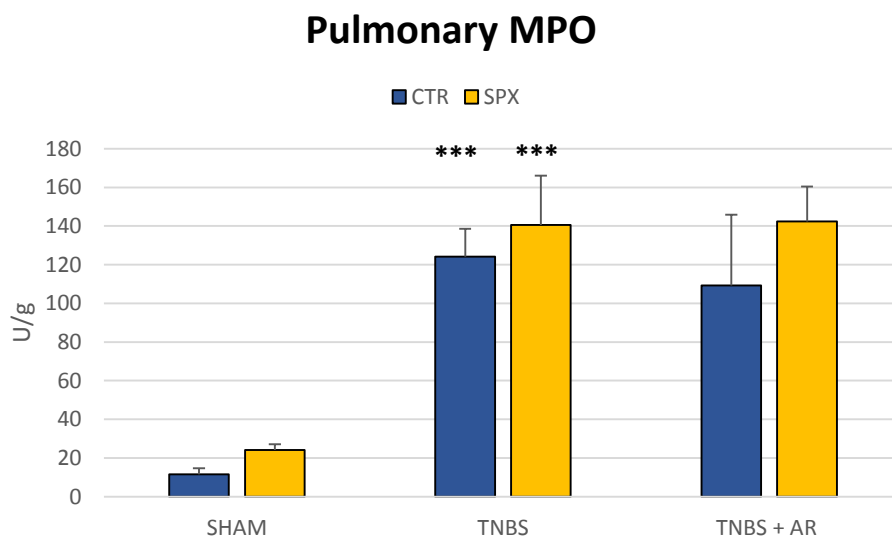
Graph 19:



In SPX animals, TNBS instillation triggered a prominent increase in colonic MPO levels (P<0.05 vs. [SPX/SHAM], graph 19), although the granulocyte infiltration was moderately lower than the one observed in non-operated mice. AR-R 17779 lost its efficacy in decreasing colonic myeloperoxidase activity in mice subjected to splenectomy.

Pulmonary MPO:

Graph 20:



*** P<0.001 vs. corresponding SHAM; two-way Anova + Bonferroni's post test.

As shown in graph 20, splenectomy did not significantly influence lung myeloperoxidase activity; in fact SPX mice subjected to colitis induction had markedly higher MPO levels than [SPX/SHAM] (P<0.001). Treatment with AR-R 17779 did not significantly influence pulmonary granulocyte infiltration either with or without splenectomy.

RESULTS (PART III)

In the final part of this study, we characterized T-cells subpopulations in the spleen and mesenteric lymph nodes (MLN) by flow cytometry in vehicle- and AR-R 17779 (1,5 mg/kg) -treated sham and colitic mice, whether or not subjected to splenectomy. T-cells and relative subpopulations were identified as previously described.

Splenic T-cells:

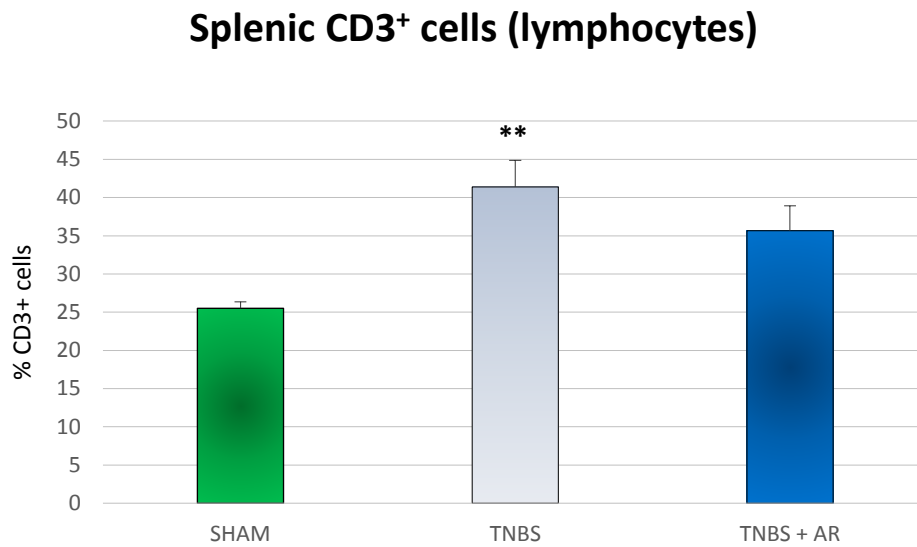
The amount of splenic T-cells and total lymphocytes in the different experimental groups is indicated in table 2; both CD3⁺ cells and total lymphocytes were markedly augmented upon colitis induction, whilst treatment with the $\alpha 7$ agonist counteracted the increase of these populations.

Table 2:

	n. CD3 ⁺ cells (*10 ⁶)	Total lymphocytes (*10 ⁶)
SHAM	0,85 ± 0,2	3,7 ± 0,9
TNBS	3,33 ± 0,8 **	8,02 ± 1,9
TNBS + AR	1,33 ± 0,4 #	4,12 ± 1,2

Table 2: splenic T-cells and total lymphocytes in different experimental groups (** P<0.01 vs. SHAM; # P<0.05 vs. TNBS; one-way Anova + Dunnett's post test).

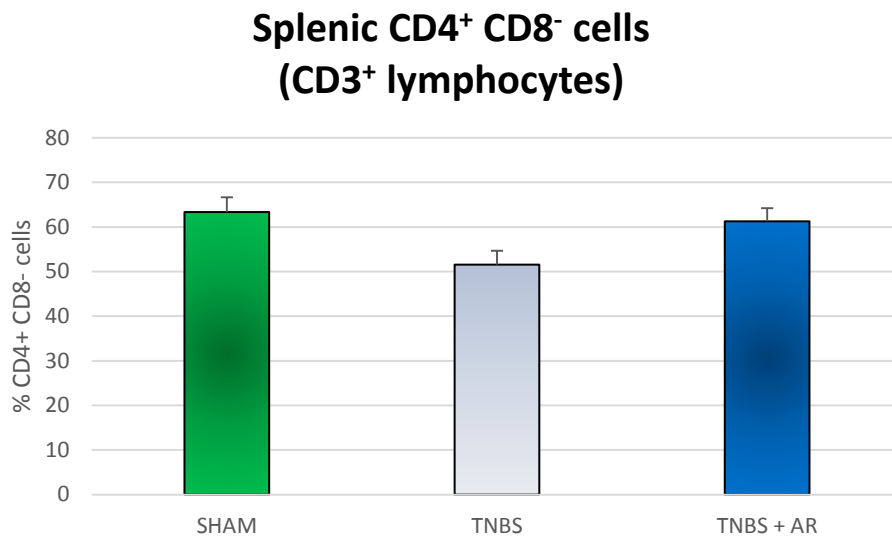
Graph 21:



** P<0.01 vs. SHAM; one way Anova + Dunnett's post test.

As shown in graph 21, induction of colitis provoked a significant increase in splenic CD3⁺ lymphocytes in [TNBS] group with respect to [SHAM] (P<0.01). Animals treated with AR-R 17779 1.5 mg/kg showed lower percentages of T-cells with respect to vehicle-treated mice, although not reaching statistical significance.

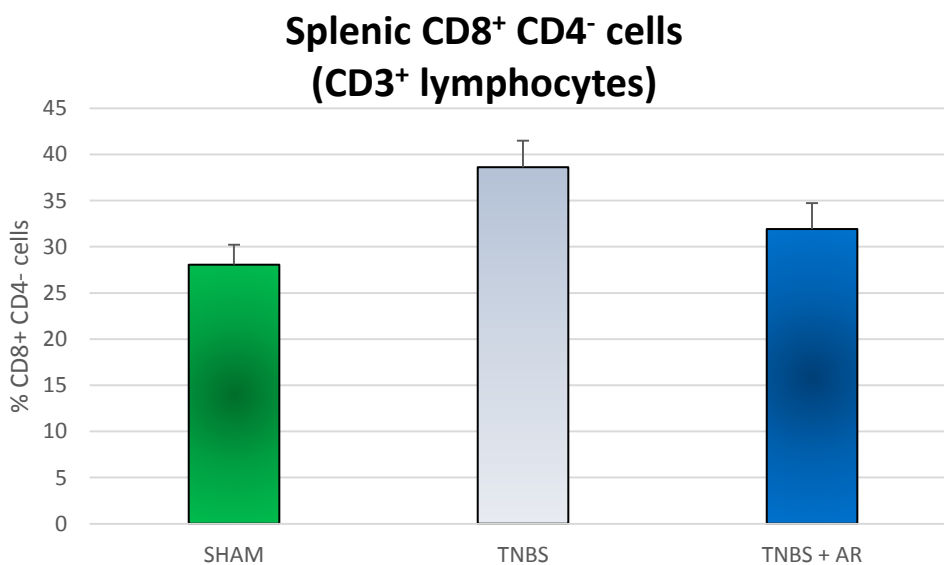
Graph 22:



One way Anova + Dunnett's post test.

With respect to helper T-cells, TNBS inoculation evoked a moderate decrease in splenic CD4⁺ CD8⁻ cells (gated on CD3⁺ lymphocytes), whilst the percentage of CD4⁺ T-cells slightly augmented upon AR-R 17779 administration. No statistically significant differences were observed among the various groups (graph 22).

Graph 23:

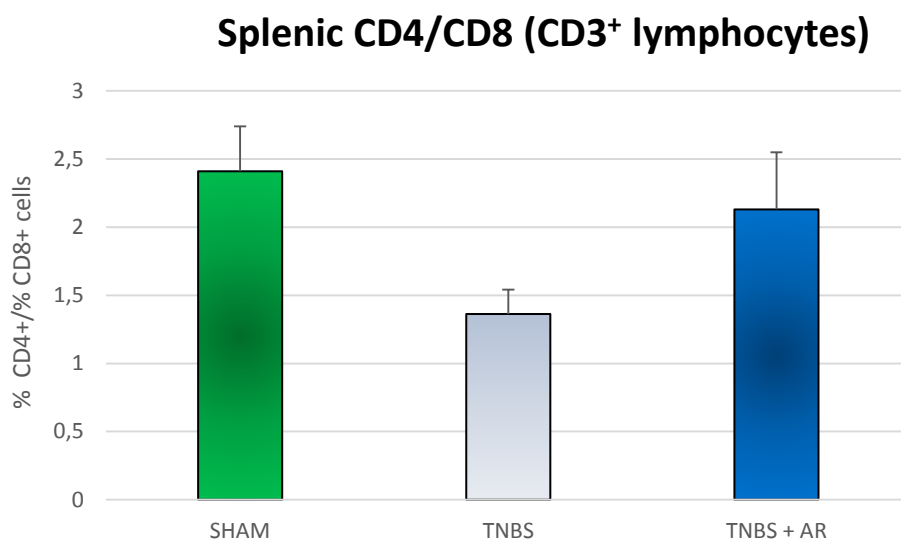


One way Anova + Dunnett's post test.

In mice subjected to TNBS inoculation, cytotoxic T-cells were moderately augmented compared to [SHAM] mice (graph 23). In [TNBS+AR] group, a mild reduction in CD8⁺ T-cells was observed with respect to [TNBS] mice.

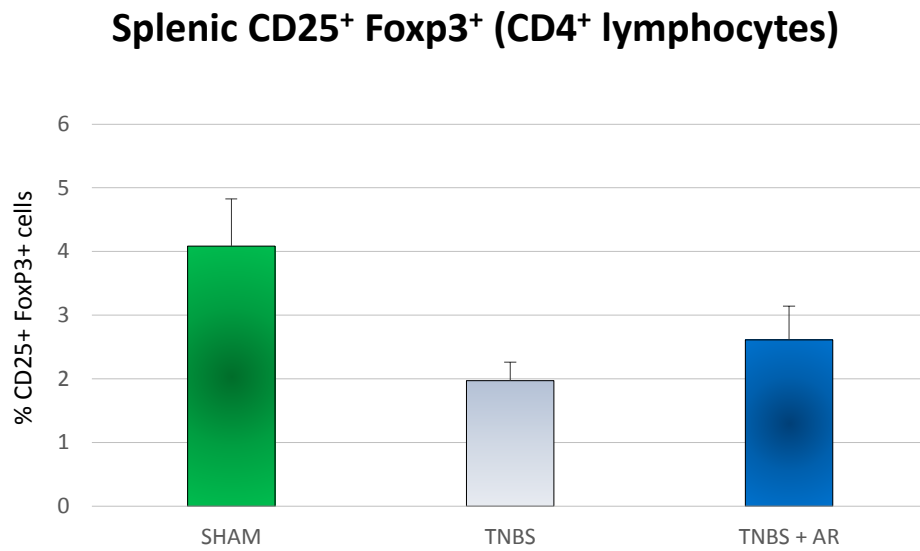
As a result of the changes in helper and cytotoxic T-cells, a marked tendency in decreasing splenic CD4/CD8 ratio was observed upon TNBS instillation (graph 24). The $\alpha 7$ agonist counteracted the TNBS-induced reduction of CD4/CD8 ratio, but no statistically significant differences were observed among the groups.

Graph 24:



One way Anova + Dunnett's post test.

Graph 25:



One way Anova + Dunnett's post test.

When we focussed our attention on the changes in splenic regulatory T-cells, in [TNBS] mice a reduction in CD4⁺ CD25⁺ FoxP3⁺ T-cells was observed with respect to [SHAM] mice, although statistical significance was not reached. AR-R 17779 elicited only a mild increase in splenic Tregs with respect to [TNBS] (graph 25).

Mesenteric Lymph Nodes (MLN) T-cells:

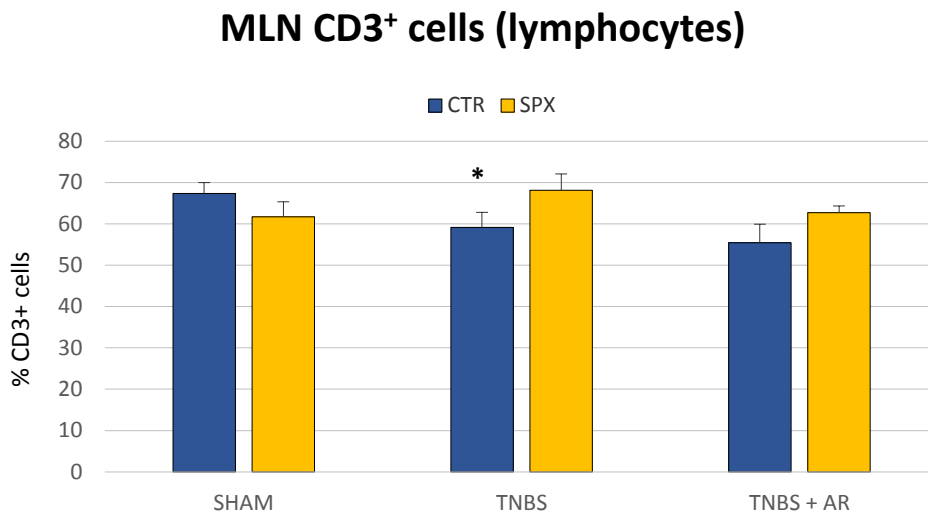
As specified in table 3, either in non-operated or splenectomised mice, the induction of colitis reduced the amount of MLN T-cells and total lymphocytes, whilst treatment with AR-R 17779 did not substantially modified these cellular populations. Furthermore, splenectomy provoked a marked reduction of CD3⁺ cells and total lymphocytes in all experimental groups, with respect to their corresponding group of non-operated mice.

Table 3:

	n. CD3⁺ cells (*10⁶)	Total lymphocytes (*10⁶)
SHAM	1,62 ± 0,5	2,73 ± 1
SPX/SHAM	0,72 ± 0,1	1,18 ± 0,2
TNBS	0,84 ± 0,3	1,29 ± 0,5
SPX/TNBS	0,37 ± 0,1	0,58 ± 0,2
TNBS + AR	0,83 ± 0,3	1,46 ± 0,5
SPX/TNBS + AR	0,27 ± 0,04	0,44 ± 0,06

Table 3: MLN T-cells and total lymphocytes in different experimental groups.

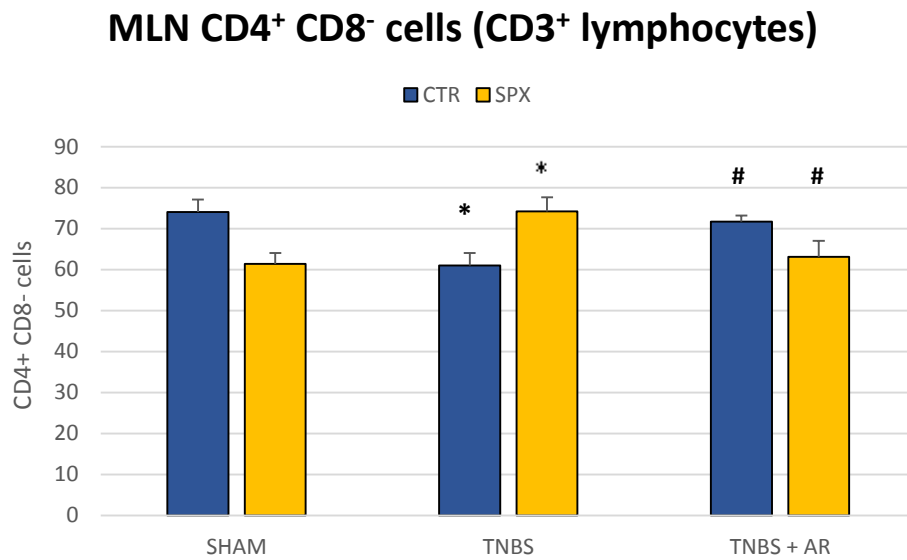
Graph 26:



* P<0.05 vs. SHAM; two way Anova + Bonferroni's post test.

In non-operated mice, TNBS inoculation evoked a significant decrease in MLN T-cells percentage with respect to [SHAM] (P<0.05, graph 26), whilst treatment with the $\alpha 7$ agonist did not substantially modify T-cells profile compared to [TNBS]. On the contrary, in mice subjected to splenectomy, the percentage of T lymphocytes was not significantly modified by colitis induction, either with or without AR-R 17779 treatment.

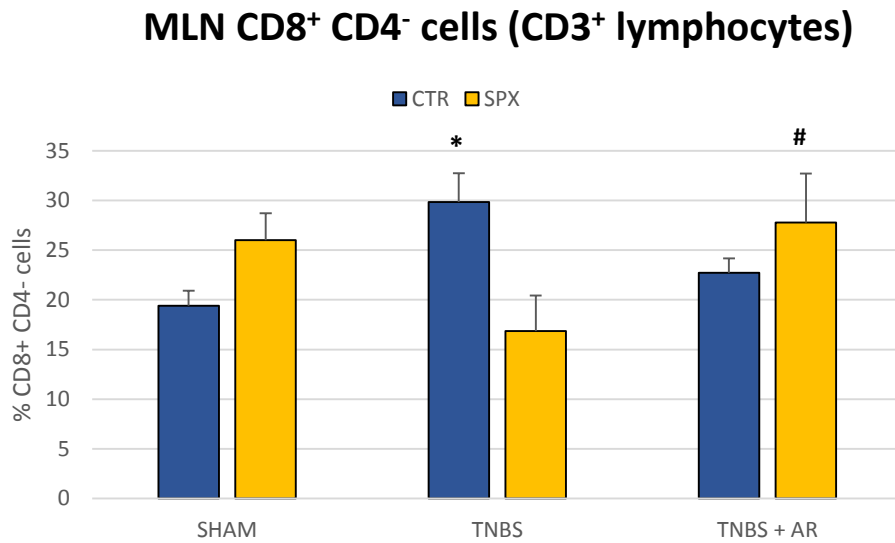
Graph 27:



* P<0.05 vs. corresponding SHAM; # P<0.05 vs. corresponding TNBS; two-way Anova + Bonferroni's post test.

As illustrated in graph 27, in non-operated mice subjected to TNBS inoculation, percentage of MLN CD4⁺ T-cells was significantly reduced with respect to [SHAM] animals (P<0.05) and treatment with $\alpha 7$ agonist at the dose of 1.5 mg/kg increased the percentage of T helper cells compared to [TNBS] (P<0.05). Conversely, CD4⁺ T cells percentage was augmented upon colitis induction in SPX conditions (P<0.05): pharmacological stimulation of $\alpha 7$ nAChRs by AR-R 17779 counteracted the change in the percentage of MLN helper-T cells provoked by colitis (P<0.05 vs. [SPX/TNBS]).

Graph 28:



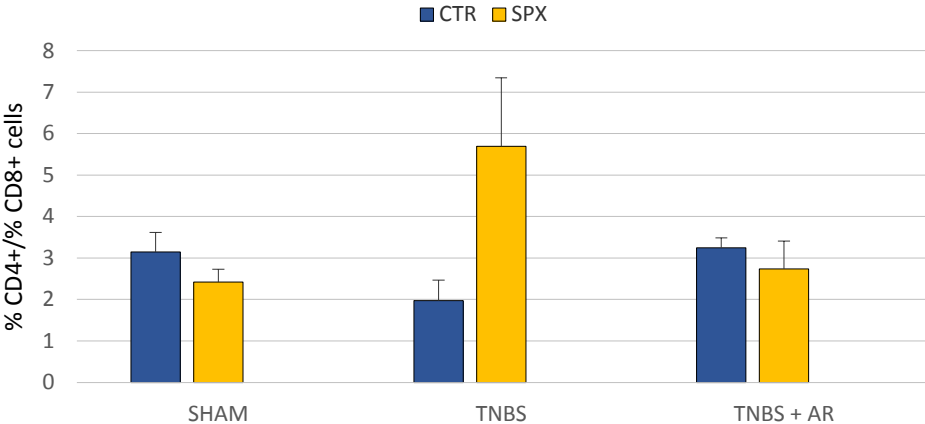
* P<0.05 vs. SHAM; # P<0.05 vs. SPX/TNBS; two-way Anova + Bonferroni's post test.

As regards CD8⁺ T-cells in the mesenteric lymph nodes, their percentage was higher in [TNBS] mice than in [SHAM] animals (P<0.05, data shown in graph 28), whilst in [TNBS+AR] group CD8⁺ T-cells percentage was reduced with respect to [TNBS], although not significantly. A different response was observed in SPX mice, as in [SPX/TNBS] group percentage of MLN cytotoxic T-cells was lower than in [SPX/SHAM] mice, whereas treatment with AR-R 17779 significantly increased CD8⁺ CD4⁻ percentage with respect to vehicle-treated colitic mice (P<0.05 vs. [SPX/TNBS]).

As a result of these changes, the induction of colitis, in mice not subjected to splenectomy, moderately decreased CD4/CD8 ratio in MLN (graph 29), a reduction counteracted by the treatment with AR-R 17779. On the other hand, SPX mice subjected to TNBS inoculation showed an augmented CD4/CD8 ratio compared to corresponding SHAM due to both an increase in CD3⁺ CD4⁺ helper T-cells and a decrease in CD3⁺ CD8⁺ cytotoxic T-cells. AR-R 17779 treatment reverted MLN CD4/CD8 ratio toward the [SHAM] condition both in non-operated and in splenectomised animals.

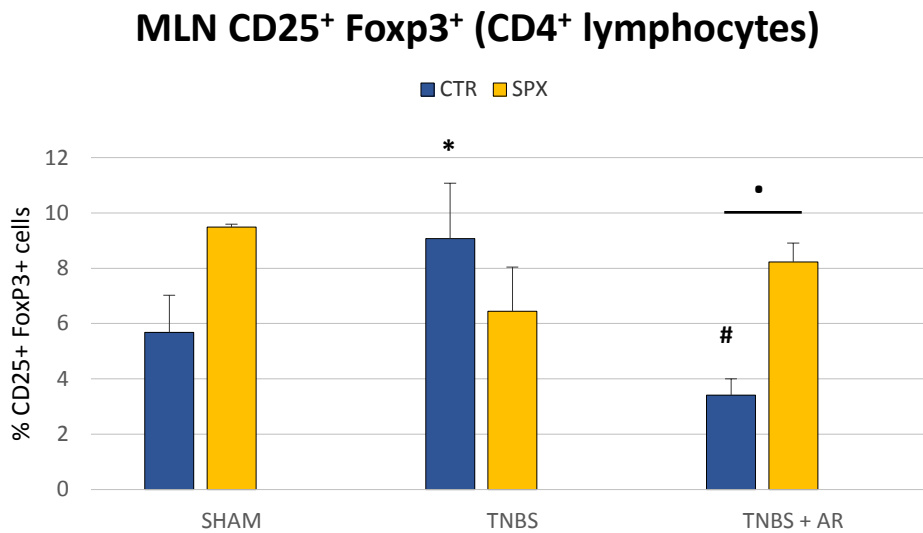
Graph 29:

MLN CD4/CD8 (CD3⁺ lymphocytes)



Two way Anova + Bonferroni's post test.

Graph 30:



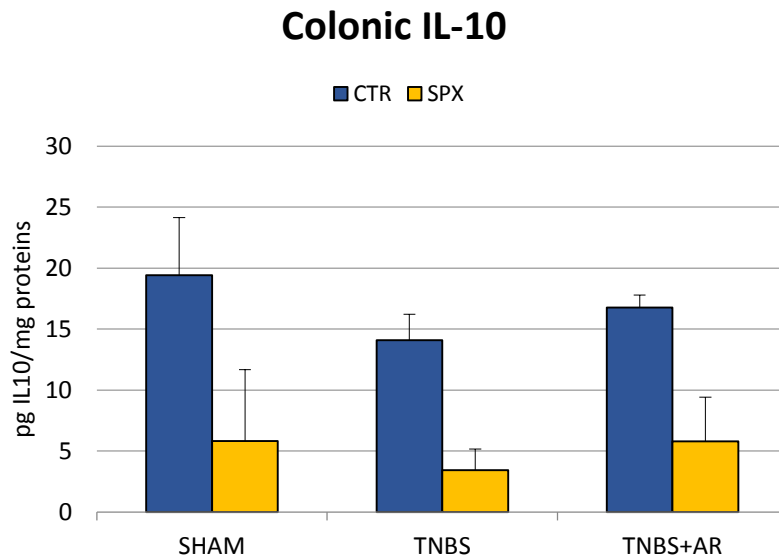
* P<0.05 vs. SHAM; # P<0.05 vs. TNBS; two-way Anova + Bonferroni's post test;

• P<0.05; Unpaired T-test.

Finally, when we evaluated the percentage of MLN regulatory T-cells, we noticed that it was significantly increased upon colitis induction in CTR mice (P<0.05, graph 30), whilst treatment with AR-R 17779 markedly decreased CD4⁺ CD25⁺ FoxP3⁺ lymphocytes with respect to [TNBS] (P<0.05). In splenectomised mice, instead, in basal condition Treg population was higher than in CTR mice, TNBS inoculation caused a weak reduction in MLN Tregs percentage with respect to corresponding SHAM, partially counteracted by the treatment with $\alpha 7$ agonist. As a result, MLN Tregs percentage appeared significantly increased by AR-R 17779 in splenectomised mice when compared to corresponding non-operated ones (P<0.05).

Colonic IL-10:

Graph 31:



Two-way Anova + Dunnett's post-test.

As shown in graph 31, colonic levels of IL-10 were reduced upon colitis induction, whilst treatment with AR-R 17779 augmented IL-10 concentration, although no significant difference between the different groups was observed. Animals subjected to splenectomy had lower levels of IL-10 compared to corresponding groups in non-operated mice. Neither TNBS instillation nor $\alpha 7$ stimulation affected IL-10 levels in SPX mice.

DISCUSSION AND CONCLUSIONS

In our study animals subjected to hapten-induced colitis showed a marked decrease of body weight and reduction of stools' consistency (as indicated in disease activity index). Locally TNBS instillation, besides eliciting a thickening and shortening of the colon, provoked a substantial macroscopic damage, represented mainly by ulcerations, luminal hemorrhages and erythemas (as summarized in macroscopic score) and a remarkable granulocytic infiltration (determined as MPO levels).

Colitic mice were repeatedly treated (five times in a three days period) with subcutaneous injections of selective $\alpha 7$ *nAChRs* agonist AR-R 17779. Among the three different doses tested (0.5, 1.5 and 5 mg/kg) the middle one, 1.5 mg/kg, evoked the most prominent effects: in fact, in [AR 1.5] DAI was ameliorated since day 3, reflecting an attenuation of both body weight loss and diarrhea. As far as the local damage elicited by TNBS concerns, treatment with 1.5 mg/kg $\alpha 7$ agonist counteracted both colonic thickening and shortening, decreased macroscopic score and colon myeloperoxidase activity. On the contrary, the highest and the lowest doses tested were mostly ineffective; however, also treatment with 0.5 and 5 mg/kg AR-R 17779 sporadically showed protective effects, as Disease Activity Index improved at day 4 in [AR 0.5] mice and colonic macroscopic damage, thickening and shortening were counteracted by 5 mg/kg of $\alpha 7$ agonist (although only attenuation of colonic shortening was statistically significant).

As far as systemic inflammation concerns we observed a massive granulocyte infiltration within the lung upon TNBS administration. In this case, none of the tested doses of AR-R 17779 evoked substantial effects.

On the whole AR-R 17779 showed a non linear dose-dependence: frequently nicotinic agonists show bell-shaped dose-response curves and this has been associated with the particular nature of ligand-gated ion channels, such as *nAChRs*. In fact, it is well established that excessive stimulation of *nAChRs* can lead to receptor desensitization, thus resulting in opposite effects, as previously

mentioned in this work (see figure 4). Moreover, among nicotinic receptors, the $\alpha 7$ subtype is particularly subjected to fast desensitization (Yu & Role, 1998).

Surprisingly, administration of a selective $\alpha 7$ antagonist such as MLA did not evoke prominent effects in the parameters we evaluated, with the exceptions of DAI, which was ameliorated at day 4 by 0.5 mg/kg methyllycaconitine, and MS and pulmonary MPO, that were augmented (although not significantly) in [MLA 0.5] and [MLA1] mice. These findings seem to indicate that the endogenous $\alpha 7$ nicotinic tone does not play a substantial role in the modulation of TNBS-induced colitis; on the other hand, we cannot exclude that, in our experimental conditions, a further exacerbation of the colonic alterations and damage elicited by TNBS might be of difficult detection. Noteworthy, mice treated with the highest dose of MLA had a lower survival rate compared to vehicle-treated animals (data not shown).

Administration of $\alpha 4\beta 2$ agents in colitic mice, despite eliciting weak responses in the majority of the parameters we analyzed, evoked some controversial effects: in particular, both the agonist and the antagonist significantly attenuated DAI at day 4 and decreased colonic granulocytic infiltration, although only DBE reduced MPO levels in a statistically significant manner.

Administration of 5 mg/kg DBE was lethal in 100% animals: we consider that at this dose DBE might have lost its selectivity to $\alpha 4\beta 2$ *nAChRs* thus blocking other nicotinic receptors, such as the muscular receptor, exerting a curaro-mimetic action.

The similar response elicited by both TC-2402 and DBE supports the impression that, when analyzing the responses evoked by repeated administration of molecules targeting *nAChRs*, discrimination between agonists and blockers can be elusive. In fact, it has been reported not only that acute administration of $\alpha 4\beta 2$ agonists can lead to receptor desensitization resulting in antagonist-like effects (Anderson & Brunzel, 2015), but also that $\alpha 4\beta 2$ antagonists can induce receptors up-regulation (Hurst et al., 2003).

As regards the mechanisms by which $\alpha 4\beta 2$ *nAChRs* ligands can affect granulocytes infiltration, they remain unclear; further investigations aiming at elucidating the expression and function of this particular *nAChRs* subtype in enteric neurons and intestinal immune cells would be required.

Moreover, our data evidenced that, despite being inactive in the most of colonic parameters, both $\alpha 4\beta 2$ agents improved animals' state of health (as reflected by DAI). This can be explained by the multiple responses that nicotinic modulation incites *in vivo*: for instance, $\alpha 4\beta 2$ *nAChRs* ligands can

affect pain perception (Hurst et al., 2013), leading to an improvement of animals' state of health, increasing sense of hunger and body weight. Such a centrally-mediated effect could likely be evoked both by TC-2403 and DBE, reported to access the central nervous system (Lippiello et al., 1996; Martin et al., 2015), where $\alpha 4\beta 2$ *nAChRs* are widely expressed.

On the whole, however, effects evoked by $\alpha 4\beta 2$ agents did not significantly impact the severity of TNBS-induced colitis; for this reason, we then focussed our attention on the $\alpha 7$ subtype and on AR-R 17779.

Seen the crucial contribution of the spleen to the *Cholinergic Anti-inflammatory Pathway* we investigated whether protective effects evoked by the $\alpha 7$ agonist required spleen integrity. We found that in splenectomised animals colitis severity was not substantially different from non-operated mice. Instead, AR-R 17779 lost its protective effects in SPX mice in terms of colonic length, thickness and granulocytic infiltration, whilst it worsened local macroscopic damage and increased DAI. Our results are in line with previous evidence showing that vagus nerve stimulation exerts its anti-inflammatory activity only in mice with an intact spleen (Huston et al., 2006; Ji et al., 2013) and expand them indicating that the presence of the spleen is decisive also when the protection is directly mediated by $\alpha 7$ receptors stimulation.

Although the neuro-immune interactions involved in the vagal modulation of the spleen is still debated, the most widely held theory proposes that stimulation of $\alpha 7$ *nAChRs* located on splenic nerve terminals induces the release of norepinephrine, that, in the spleen, stimulates T-cells to release ACh, that in turn activates *nAChRs* suppressing cytokines production (Rosas-Ballina et al., 2011). Our data show that $\alpha 7$ *nAChRs* can be pharmacologically exploited to modulate intestinal inflammation triggered by TNBS with a small molecule like AR-R 17779, and that among $\alpha 7$ *nAChRs*, those located in the spleen are particularly critical for the anti-inflammatory action of the agonist.

In the final part of this work we focussed our attention on T-cells, in view of their pivotal role in both TNBS-colitis and human IBD as well as in the vagal modulation of inflammation through the spleen. Among T-cells we further focussed on Tregs to determine the impact of $\alpha 7$ pharmacological stimulation in the resolution of inflammation. In the spleen, colitis provoked a marked increase of T-cells and a decrease in Tregs population; treatment with the $\alpha 7$ agonist counteracted the augmentation of T-cells and also provoked a mild increase in Tregs. Conversely, in mesenteric lymph nodes of colitic mice we observed an impairment in total CD3⁺ cells, not attenuated by $\alpha 7$

stimulation, and an increase in Tregs, which was abolished by AR-R 17779. Notably, in splenectomised mice, differences between different experimental groups in terms of both total T-cells and Tregs were strongly limited.

Analyzing T-cells and Tregs within colonic tissue would have been crucial to complete the study of their profile in our experimental conditions. However, isolation of lamina propria mononuclear cells from colonic mucosa for subsequent flow cytometry analysis represented a strong technical issue, as the yield of the process was so low that Tregs were undetectable. Hence, to complete our study, we measured the levels of IL-10, the regulatory cytokine typically produced by Tregs, within colonic lysates. In line with splenic Tregs profile, colonic IL-10 levels were reduced in colitic mice compared to controls and such a reduction was attenuated upon treatment with AR-R 17779. Also in this case, differences between different groups in SPX mice, were not observed.

The decrease of T-cells number and percentage observed in MLN upon colitis induction, and their simultaneous increase within the spleen, corroborates a recently proposed model of vagal modulation of immune response (Martelli et al., 2014); in fact, our data support the hypothesis that, upon inflammation, T-cells are mobilized from peripheral depots (i.e. MLN) to the spleen. Accordingly, reduction of MLN T-cells upon colitis induction is abolished when the spleen is removed. However, this process does not seem to be mediated by $\alpha 7$ *nAChRs*: in fact treatment with AR-R 17779 did not affect the reduction of MLN T-cells, and weakly reduced T-cells in the spleen. Whether the migration of T-cells towards the spleen might be vagus-mediated remains to be elucidated, even though it can not be ruled out that other cholinergic receptors elicit this response when ACh is released in inflammatory conditions (Ji et al., 2013; Martelli et al., 2014). We reason that the reduction of splenic T-cells evoked by AR-R 17779 might be a consequence of the attenuated inflammatory response, which is clearly modulated by the $\alpha 7$ agonist at the splenic level. We found that this small molecule targeting $\alpha 7$ *nAChRs* can incite a moderate increase in colonic IL-10 levels and in splenic CD4⁺ CD25⁺ FoxP3⁺ regulatory T-cells, partly confirming the potential of $\alpha 7$ nicotinic agents in enhancing regulatory mediators of the inflammatory response implicated in the resolving phase. However, further investigations are required to fully assess the impact of $\alpha 7$ *nAChRs* in the resolution of inflammation, encompassing the analysis of the profile of pro-resolving lipid mediators (e.g. lipoxins, resolvins and protectins) whose production has been reported to be enhanced by vagus nerve stimulation (Mirakaj et al., 2014).

Surprisingly, Tregs were augmented in mesenteric lymph nodes upon colitis induction, and AR-R 17779 attenuated this increase, whilst in SPX mice this response was abolished. This apparently

counterintuitive observation is consistent with the findings reported in a recent study (Boschetti et al., 2016), showing that colonic inflammation triggers proliferation of MLN Tregs to incite their suppressive function. Accordingly, we see this data as the result of a compensatory mechanism perpetuated to limit the ongoing inflammatory response.

Overall, we showed that T-cells, and T-regs in particular, are involved in the modulation of the immune response mediated by $\alpha 7$ *nAChRs*, however, to further elucidate their role in our experimental settings, analyzing other subsets of CD4⁺ T-cells (e.g. Th1 and Th17 cells) on the basis of their cytokines pattern (e.g. INF- γ and IL-17) would be of particular interest.

Many authors focussed their attention on other immune cell populations, such as macrophages; however, our particular model of colitis is not the most appropriate to study innate immune cells, for which DSS-colitis, for instance, would be more suitable. Bringing together the knowledge coming from different animal models would be of great importance to fully understand the mechanisms underlying the vagal modulation of immune response.

Our study points to make a further step towards the elucidation of the mechanisms by which the vagus nerve can modulate inflammation through $\alpha 7$ *nAChRs* and the spleen, a very fertile area of research in physiology, immunology, gastroenterology and pharmacology. To confirm the vivacity of this research area, translational attempts are occurring, as a clinical trial evaluating vagus nerve stimulation (VNS) as a treatment in Crohn's Disease is currently recruiting patients for participation (NCT01569503, Principal Investigator: Bruno Bonaz, University of Grenoble, France). VNS has been studied in TNBS-induced colitis in rats (Bonaz et al., 2016), and it improved multiple aspects of the inflammatory response. However, such an approach is able to incite all the responses evoked by ACh, encompassing activation of muscarinic, muscular and neuronal nicotinic receptors, peripherally as well as in the central nervous system. On the contrary, administration of a selective $\alpha 7$ *nAChRs* agonist will possibly evoke more selective effects, attenuating inflammation without influencing heart rate variability, secretions, food intake and other cholinergic responses. We experienced issues in terms of selectivity in our group when we tested choline, reported to be a selective $\alpha 7$ agonist, in our experimental settings: choline was totally ineffective (data not shown) and did not influence any of the parameters we analyzed. If in vitro choline might bind preferentially to $\alpha 7$ *nAChR*, in vivo it is likely converted to acetylcholine, thus eliciting a wide range of responses, that, in our case, did not produce anti-inflammatory effects.

Results provided by AR-R 17779 in our experimental conditions are quite promising and confirm the anti-inflammatory properties that this compound showed in models of post-operative ileus and arthritis (The et al., 2007; Van Maanen et al., 2010). Moreover, the spleen has been confirmed to be a suitable target for this compound and for the modulation of the inflammatory response. Interestingly, in human IBD, appendectomy, but not splenectomy, is associated with an increased risk to develop IBD (Kaplan et al., 2008); accordingly, also in our model splenectomy did not substantially influence colitis severity, although some immunological aspects (i.e. MLN CD4+ T-cells, MLN T-regs and colonic IL-10) were clearly altered. In particular, surgical removal of the spleen influenced the levels of T-cells' subsets and IL-10 in basal conditions, thus attenuating the alterations evoked by colitis induction and pharmacological treatment. Although some authors investigated the impact of splenectomy on peripheral T-cells upon inflammatory stimuli (Hendrickson et al., 2009; Higashisjima et al., 2009), the effects of splenic removal in basal conditions were not reported. We consider that elucidating the mechanisms by which the spleen modulates the amount and activation of immune cells located in different secondary lymphoid organs, in both healthy state and inflammatory conditions, would be of great importance.

In recent years other research groups studied the involvement of $\alpha 7$ *nAChRs* in intestinal inflammation and some of them came to conclusions that differ from ours: in fact, AR-R 17779 did not improve clinical parameters and moreover increased the levels of colonic pro-inflammatory cytokines in DSS-colitis in female C57BL/6 mice (Snoek et al., 2010). In addition, GSK 1345038A (another selective $\alpha 7$ agonist) was ineffective in TNBS-colitis (Snoek et al., 2010), but no data were reported about AR-R 17779 in such model. Furthermore, nicotine has been shown to increase the severity of TNBS-colitis in balb/c mice and this has been associated with a nicotine-induced alteration of T-regs function (Galitovskiy et al., 2011).

On the whole, we reason that when studying molecules with such a complex pharmacological profile in such multifaceted conditions, many factors, including the animal model chosen, the strain and gender of animals, could deeply impact the final experimental evidences. In fact, effects evoked by the same agent can be markedly distinct when applied in different animal models (e.g. TNBS or DSS colitis) (Galitovskiy et al., 2011), and susceptibility to develop inflammation and response to drugs can significantly vary in a strain and/or gender-dependent manner: for instance, we observed a markedly different response to TNBS when applying the same animal model to either male or female C57/BL6 or balb/c mice (in the present study female CD1/Swiss mice were used) and also, response to treatments changed in function of gender or strain.

Concluding, in our experimental settings selective $\alpha 7$ nAChRs agonist AR-R 17779 showed protective and beneficial effects against the inflammatory response elicited by TNBS; these effects were spleen-dependent and involved changes in T-cells and T-regs populations distribution. However, the mechanisms by which the spleen might modulate T-cells and relative subpopulations in different secondary lymphoid organs, such as mesenteric lymph-nodes, and how alterations in such cell populations relate to disease severity, remain to be clarified. Hopefully ongoing and future studies will succeed in elucidating those aspects, which we consider as very critical to advance our knowledge about the role played by vagus nerve, T-cells and spleen in the modulation of inflammatory and immune responses.

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