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Eph/ephrin system: investigation of its role in intestinal inflammation

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SUMMARY

INTRODUCTION

THE EPH/EPHRIN SYSTEM

The term Eph was coined by Hirai and co-workers in 1987, when they isolated for the first time the cDNA of a receptor with similarities for Tyrosine Kinases receptors (RTKs) in an *erythropoietin-producing-hepatocellular* carcinoma cell line (Eph) (Hirai et al., 1987).

Since then the Eph family has been widely studied: crucial roles in tissue organization during embryogenesis, in tissue homeostasis in adulthood as well as in tumour progression and other pathologies have been described (Barquilla et Pasquale, 2015).

The Eph receptors, the largest family of RTKs in mammals, comprise fourteen human Eph receptors that are subdivided in two different classes: nine EphA and five EphB receptors.

The distinction between EphA and EphB receptors was initially based on sequence differences within the extracellular ligand binding domain, but it depends also on binding preferences for the five ephrinA ligands, *Eph receptors interacting proteins*, or the three ephrinB ligands respectively *[Figure 1]* (Coulthard et al., 2012).

The receptor-ligand interactions are promiscuous within each A or B class, with differences in binding affinities. There are some exceptions: EphB4 only binds ephrin-B2, EphA4 binds ephrin-B2/3 and EphB2 binds most A-type ephrins *[Figure 1]* (Coulthard et al., 2012).

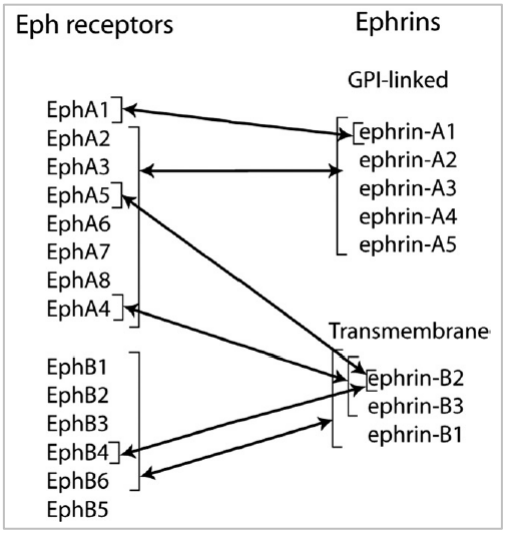


Figure 1. Eph receptor and ephrin ligand binding preferences (Coulthard et al., 2012)

STRUCTURE AND BIDIRECTIONAL SIGNALLING

EphA and EphB receptors have conserved structures and domains *[Figure 2]*. The N-terminal extracellular region consists of a *Ligand Binding Domain*, LBD, an epidermal growth factor-like motif within a *Cysteine-Rich Domain*, CRD, and two *fibronectin-type III repeats*, FN III1 and FN III2. The receptors pass through the membrane via a single *transmembrane domain*, TM. The intracellular C-terminus starts with a *juxtamembrane region*, JM, followed by a *tyrosine kinase domain*, TKD, *sterile α motif*, SAM, and a *postsynaptic density protein 95 discs large-1 zonula occludens-1*, PDZ-binding motif (Perez White et Getsios, 2014). The LBD contains a high affinity binding site that regulates receptor-ephrin interaction between cells (Pasquale, 2008). Two additional low-affinity sites have been identified in LBD and cysteine domain, which are involved in the formation and stabilization of clustered Eph receptor-ephrin complexes (Pasquale, 2005).

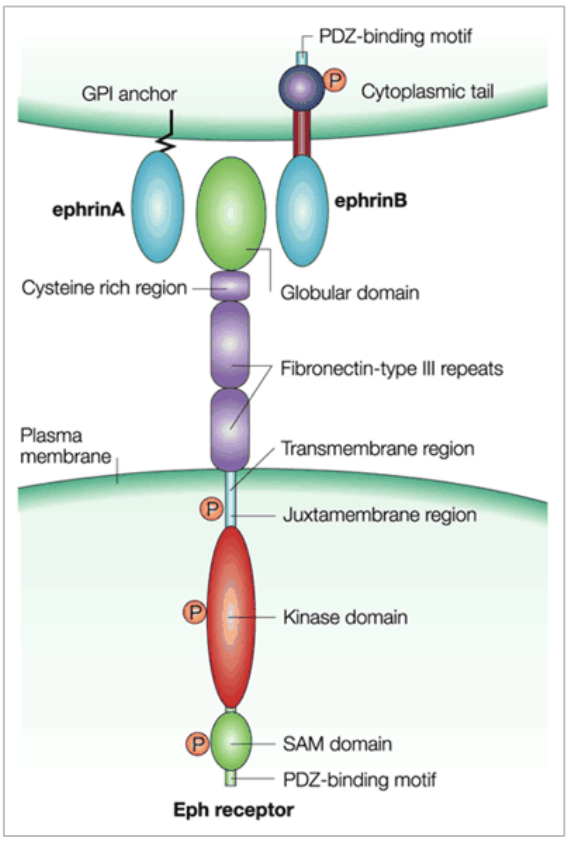


Figure 2. The structure of Eph receptors and ephrin ligands (adapted from Kullander et Klein., 2002)

The ephrin ligands share a conserved extracellular, N-terminal *receptor binding domain*, RBD, but they differ in the C-terminal region and mechanism of membrane anchorage. EphrinA ligands are attached to the cell membrane with a *glycosylphosphatidylinositol*, GPI, anchor. In contrast, ephrinB ligands have a C-terminal tail that extends into the cytoplasm of the ligand-bearing cell and contains a PDZ binding motif *[Figure 2]* (Perez White et Getsios, 2014).

Since both receptors and ligands are anchored to plasma membrane, their interaction is based on cell-cell contact, although it has been demonstrated that EphA receptors are responsive also to the soluble forms of ephrinA ligands and they can elicit a signal independent from the membrane-linked ligand (Beauchamp et al., 2012).

The signalling can therefore propagate bidirectionally into both the Eph-receptor-expressing cells, in a process known as *forward signalling*, and in the ephrin-expressing cells, *reverse signalling [Figure 3]* (Kania et Klein, 2016).

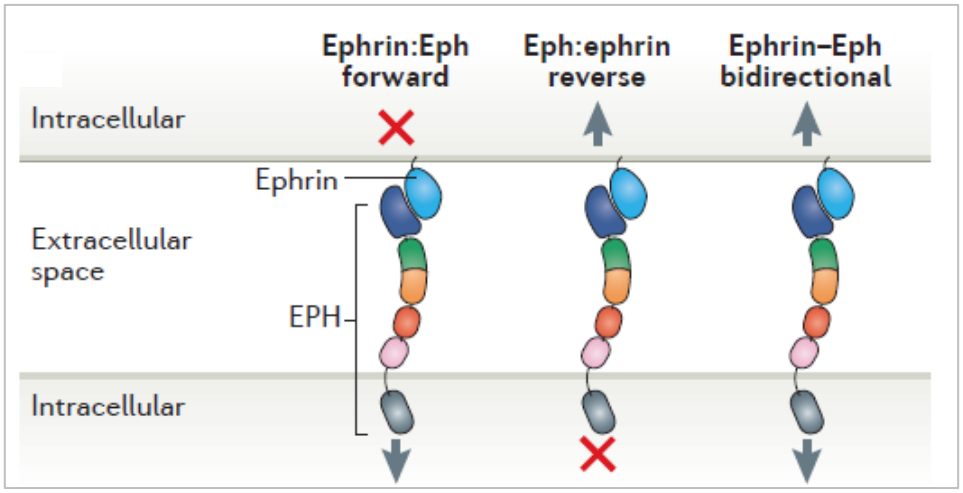


Figure 3. Eph/ephrin *forward* and *reverse* signalling (adapted from Kania et Klein, 2016)

After the binding of ephrin ligands to the receptor, its dimerization and the formation of clusters, the *forward signalling* relies on the autophosphorylation of tyrosine residues present in the juxtamembrane domain and on the consequent activation of effector proteins *[Figure 4]* (Kania et Klein, 2016). Differently, the *reverse signalling* depends on the two subtypes of ligands: for ephrinBs it involves the SRC-mediated

phosphorylation of tyrosine and serine residues, while for ephrinAs it requires the interaction with other transmembrane partners (Barquilla et Pasquale, 2015).

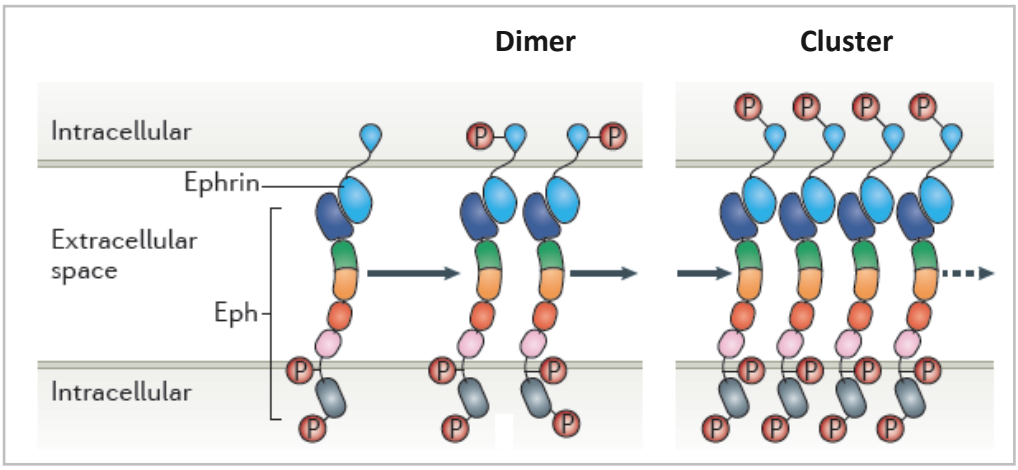


Figure 4. Dimer and Cluster formation (adapted from Kania et Klein, 2016)

The physiological shutdown of the signal is mediated by recruitment of phosphatases, which dephosphorylate the tyrosine kinase domain, and adaptor proteins like c-Cbl responsible for receptor internalization and degradation. Moreover, different proteases such as *metalloproteases*, MMPs, *α-disintegrin and metalloproteinase*, ADAMs, *γ-secretase and caspases*, can cleave Eph receptors in their extracellular, transmembrane and intracellular regions. These cleavages are important not only to stop the signal, but also because they lead to proteolytic fragments that can have signalling functions in the extracellular space, in the cytoplasm or in the nucleus *[Figure 5]* (Barquilla et Pasquale, 2015).

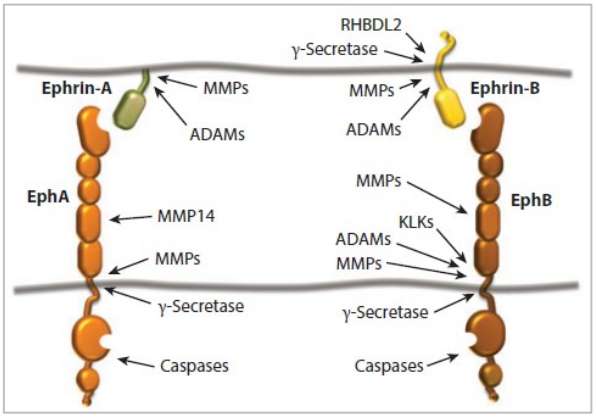


Figure 5. Modulation of Eph/ephrin signalling by proteases
(Barquilla et Pasquale, 2015)

EPH/EPHRIN SYSTEM IN PHYSIOLOGY AND PATHOLOGY

The Eph/ephrin system plays a key role in various stages of human life. However, limited by the complexity of adult tissues, our understanding of Eph-ephrin signalling in adults is still poor. The ability of this system to control the cytoskeleton, and thus cellular morphology, and its capacity to affect cell-cell signalling, makes it particularly suitable for controlling the adult stem cell niche, the stability of neuronal synapses and the balance between bone resorption and deposition, as well as energy metabolism (Kania et Klein, 2016).

CENTRAL NERVOUS SYSTEM

In adult brain the Eph/ephrin system is present particularly in areas where neuronal circuits are continuously remodelled in response to environmental changes (Yamaguchi et al., 2004). For instance, Eph signalling controls neuronal plasticity in the hippocampus by regulating synapse number and size, and consequently taking part in learning and memory processes (Murai et Pasquale, 2011). Alteration in the Eph/ephrin system can lead to mental retardation and dendritic spine abnormalities. Moreover, it has been found that EphB2 down-regulation, consequent to the presence of the soluble-amyloid protein, can contribute to neuronal degeneration and memory loss in Alzheimer's disease (Lacor et al., 2007). On the other hand, up-regulation of the Eph/ephrin system has been observed in injured areas of the nervous system, suggesting a role in nerve regeneration (Du et al.,2007).

BONE HOMEOSTASIS

Multiple findings over the years have highlighted the role of the Eph-ephrin system in bone remodelling and maintenance (Zhao et al., 2006; Pasquale, 2008). Bones are continuously remodelled by the activity of osteoblasts and osteoclasts, which are able to produce or degrade bone, and a proper balance has to be maintained in order to avoid diseases like osteoarthritis or cancer. Eph receptor/ephrin knockout mice show bones abnormalities and defects in skeletal development (Davy et al., 2006; Pasquale et al., 2005).

Eph-ephrin signalling controls bone homeostasis in adult mice. Initially, it was shown that ephrin-B2 *reverse* signalling inhibits osteoclast differentiation; however, conditional deletion of ephrin-B2 resulted in more efficient osteoclast formation *in vitro* (Zhao et al., 2006). Subsequent work found that ephrin-B1 is the key player in this system (Cheng et al., 2012). Recently, EphA-ephrinA signalling was shown to enhance osteoclastogenesis and to suppress osteoblastogenesis suggesting that osteoclasts and osteoblasts can be regulated separately, but also may signal to each other via Eph-ephrin interactions (Irie et al., 2009).

INSULIN SECRETION

A newly identified function for Eph-ephrin signalling is the mediation of cell-cell communication between pancreatic islet β-cells and the regulation of insulin secretion. EphA-ephrinA *forward* signalling between β-cells inhibits insulin secretion, whereas *reverse* signalling stimulates it. Thus, bidirectional signalling between β-cells seems to control a fundamental process underlying energy metabolism. This suggests that Eph-ephrin signalling regulation could be potentially exploited to find new treatments for type 2 diabetes (Kostantinova et al, 2007).

BLOOD AND LYMPHATIC VESSELS DEVELOPMENT

The development of blood vessels, *angiogenesis*, and of the lymphatic vasculature, *lymphangiogenesis*, depends on the pair EphB4-ephrin-B2 and on EphA-ephrin-A signalling.

During angiogenesis the Eph/ephrin system controls blood vessel sprouting, assembly, remodelling and stabilization by regulating endothelial cells and their supporting mural cells. EphA2 in concert with ephrin-A1 regulates angiogenesis and vascular permeability through the interplay with vascular endothelial growth factor, VEGF. Moreover, expression of ephrin-B2 in arterial vessels and of EphB4 in venous vessels helps establishing borders between these two compartments, while their absence in mice causes severe defects in blood vessel growth and remodelling (Adams et al., 1999; Wang et al., 1998; Gerety et Anderson, 2002).

In the lymphatic vasculature the unidirectional fluid flow is controlled by the intraluminal valves, ephrin-B2 and EphB4 are expressed by lymphatic vessels and are

required for valve formation and maintenance (Bazigou et Makinen, 2013; Makinen et al., 2005).

IMMUNE FUNCTION

Many Eph receptors and ephrins are expressed in lymphoid organs and lymphocytes, suggesting that they can have immunoregulatory properties (Wu et Luo, 2005). The mRNA of most Ephs and ephrins can be detected in the thymus or spleen: among them, EphB6 mRNA is expressed at high levels in the thymus and its function in immune regulation has been best characterized (Wu et Luo, 2005).

EphB/ephrinB system has three main functions in the immune compartment. On one side, it is important for the structural organization of the thymus and for guiding the movement of thymocytes through the different thymic compartments allowing their maturation into T cells (Wu et Luo, 2005). Several studies have shown that perturbing Eph-ephrin interactions in thymic organ culture with Fc-fusion proteins interferes with thymocyte survival and maturation (Alfaro et al., 2007; Munoz et al., 2006; Wu et Luo, 2005). Defects in thymocyte maturation have also been observed in EphA4 knockout mice, which have strongly decreased numbers of peripheral T cells. Moreover, preliminary observations suggest that EphB6, EphB2 and EphB3 knockout mice also have a disorganized thymic architecture and decreased numbers of thymocytes (Pasquale, 2008).

On the other side, a prominent Eph function in T cells relates to T-cell co-stimulation: EphB receptors can, in fact, modulate responses mediated by the T cell receptor (TCR) and, therefore, represent a class of co-stimulatory receptors. EphB receptors can cluster with activated TCR in aggregated lipid rafts and their clustering with anti-EphB6 antibodies or ephrinB-Fc ligands can lower the activation threshold of T cells responding to suboptimal TCR ligation. Moreover, EphB activation can promote T cell proliferation, production of interferon γ (but not interleukins 2 and 4), and cytotoxic T cell activity. Additionally, EphB receptor and TCR clustering can promote positive thymocyte selection and T cell responses to antigen-presenting cells or can blunt the effects of high TCR signalling leading to the negative selection of self-reactive thymocytes (Wu et Luo, 2005).

Finally, EphB receptors in T cells are activated through interactions with ephrinB ligands expressed by other T cells as well as other cell types, such as thymic epithelial cells and antigen-presenting cells. These Eph interactions may facilitate T cell responses in lymphoid organs to promote differentiation of naive T cells into effectors (Wu et Luo, 2005).

EphA receptors and A-type ephrins are also expressed in thymocytes and T cells (Wu et Luo, 2005) and have been reported to modulate TCR signalling as well. Similarly to EphB receptors, EphA receptors can modulate negative selection of self-reactive thymocytes, which depends on apoptosis triggered by strong TCR stimulation. Furthermore, the EphA/ephrinA system has been proposed to modulate thymocyte and T cell migratory responses to chemokines and integrin-dependent adhesion, which guide thymocyte movements within the thymus and T cell trafficking between the blood, lymphoid tissues, and sites of extravasation (Sharfe et al., 2008; Wu and Luo, 2005).

Eph receptors and ephrins are also expressed in B lymphocytes, but their effects in these cells have not been characterized.

Clearly, more work is needed to refine our knowledge of Eph bidirectional signalling in the immune system.

INTESTINAL ARCHITECTURE

The stem cell compartment of the intestinal epithelium is one of the best understood system with respect to Eph-ephrin signalling. Intestinal stem cells are in the base of the crypts, where they divide and form progenitor cells that continue to divide as they migrate up the crypt axis towards the villus. While leaving the crypt and approaching the villus, cells stop dividing and differentiate. Eph-ephrin signalling controls cell positioning along the crypt-villus axis and the proliferation of progenitor cells. Canonical Wnt signalling promotes mitogenesis and the transcription of EphB2 and EphB3 in intestinal stem cells through β-catenin-Tcf (the transcriptional effector complex of the Wnt pathway). The same pathway also negatively regulates the transcription of ephrin-B1 and ephrin-B2, which are expressed by differentiated cells. The opposite expression of Eph receptors and ephrin ligands maintains the

organization of proliferating progenitor cells in the crypt and differentiated cells in the villus. Proliferation of stem cells in intestinal niches is regulated by EphB2 in a kinase dependent pathway via Abl and cyclin D1, while migration is mediated by the PI3-kinase activity *[Figure 6]* (Genander et Frisén, 2010).

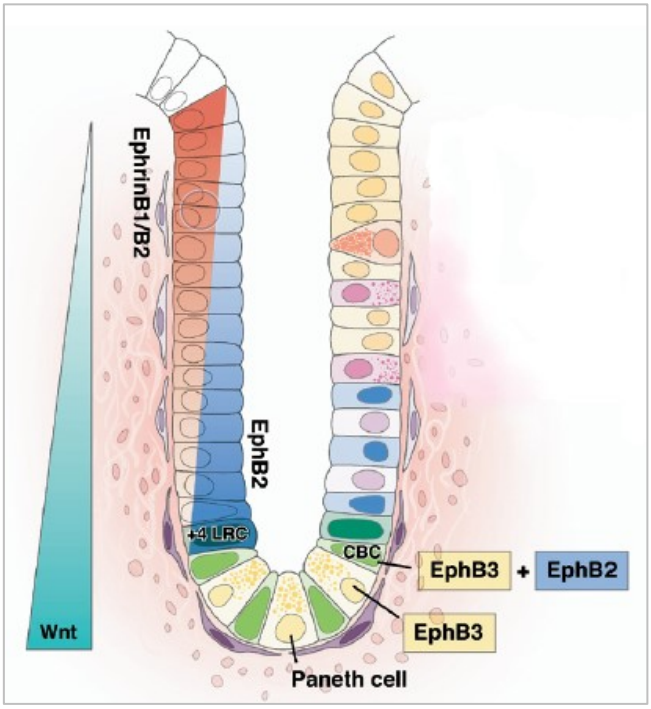


Figure 6. Eph/ephrin distribution along crypt-villus axis
(adapted from Scoville et al., 2008)

CANCER

First identified in the erythropoietin-producing hepatoma cell line, the role of Eph-ephrin signals in cancer has been validated by a robust line of research.

In addition to angiogenesis, in fact, Eph-ephrin signalling has a role in the control of tissue architecture and cell motility, processes that are both involved in cancer development (Pasquale, 2010). Members of this system are aberrantly expressed in tumours and they can affect malignancy through both bidirectional signalling and interplay with other signalling systems. EphA2 and EphB4 are the most widely overexpressed in tumours and their downregulation typically inhibits tumorigenicity, supporting a role in cancer malignancy (Barquilla et Pasquale, 2015).

For example, EphB limits cell migration and inhibits invasive growth signalling in intestinal adenoma, a type of colorectal cancer caused by constitutive activation of the

Wnt pathway (Batlle et al., 2007). In glioblastoma, the most prevalent type of primary brain tumour, EphA2 and EphA3 were found to promote the self-renewal of glioblastoma stem cells and to inhibit their differentiation (Day et al., 2013; Miao et al., 2015). Moreover, their down-regulation by RNA interference or administration of high doses of ephrin-A1-Fc drastically reduced glioblastoma xenograft tumorigenicity (Day et al., 2013).

VIRAL INFECTIONS

The involvement of certain Eph receptors and ephrins in viral infections is still under investigation. The best characterised role involves ephrin-B2 and ephrin-B3 as entry receptors for henipaviruses. Henipaviruses can bind to the RBD of the ephrinB protein to infect blood vessels and the nervous system, causing respiratory and encephalitic illnesses (Xu et al., 2012). Similarly, EphA2 is required for endothelial cell infection by Kaposi's Sarcoma-associated herpes virus, a causative agent of Kaposi's Sarcoma and B malignancies (Hahn et al., 2012). A better understanding of the role of this system in viral infections might open the door to new antiviral therapies.

EPH-EPHRIN SIGNALLING IN INFLAMMATION

Tissue injury or damage is followed by an acute inflammatory response characterised by the recruitment of inflammatory cells to the damaged site, the elimination of the causing agent and the restoration of a physiological equilibrium (Coulthard et al., 2012).

The initial evidence that Eph/ephrins proteins could play a crucial role in inflammatory response was the identification of ephrin-A1 as a *Tumor Necrosis Factor-alfa*, TNF-α, responsive gene in endothelial cells (Dixit et al., 1990). Subsequent *in vitro* studies found that the expression of other ephrins and of some of their receptors is affected by TNF-α and IL-1β (Ivanov et al., 2005). Those studies were then confirmed in vivo in an LPS-induced fever rat model: three ephrins (A1, A3, B2) and one receptor (EphA2) were up-regulated (Ivanov et al., 2005).

Since then, several studies have been conducted and Eph/ephrin system has demonstrated to participate in all the stages of the inflammatory process: vascular leakage, leukocyte homing and transmigration, and tissue repair.

VASCULAR LEAKAGE

The vascular endothelium plays a key role in orchestrating the response to injury or infection. The passage of fluid and inflammatory cells across the endothelium is regulated by both the shape of the endothelial cells and the permeability of gap junctions. The cell-cell junctional structures (which include the gap, adherens, and tight junctions, TJ) are linked to the actin and myosin filament of the cytoskeleton of the endothelial cells (Dejana, 2004). The actin filaments are tethered to membrane proteins, including VE-cadherin, and are regulated by the Rho family guanosine triphosphatases (Rho-GTPases), specifically RhoA, Rac1, and Cdc42, which are known targets of Eph-ephrin signalling (Beckers et al., 2010). Co-localization studies suggest that Eph receptors may regulate the permeability of the endothelial barrier (Ivanov et Romanovsky, 2006). Furthermore, during inflammatory processes a rapid up-regulation of EphA2 receptor and ephrin-A1 expression may lead to the re-organisation of actin cytoskeleton and thus to the formation of gaps in the endothelial

barrier allowing the passage of fluid into the interstitium and the migration of inflammatory cells into the tissue *[Figure 7]* (Ivanov et et Romanovsky, 2006).

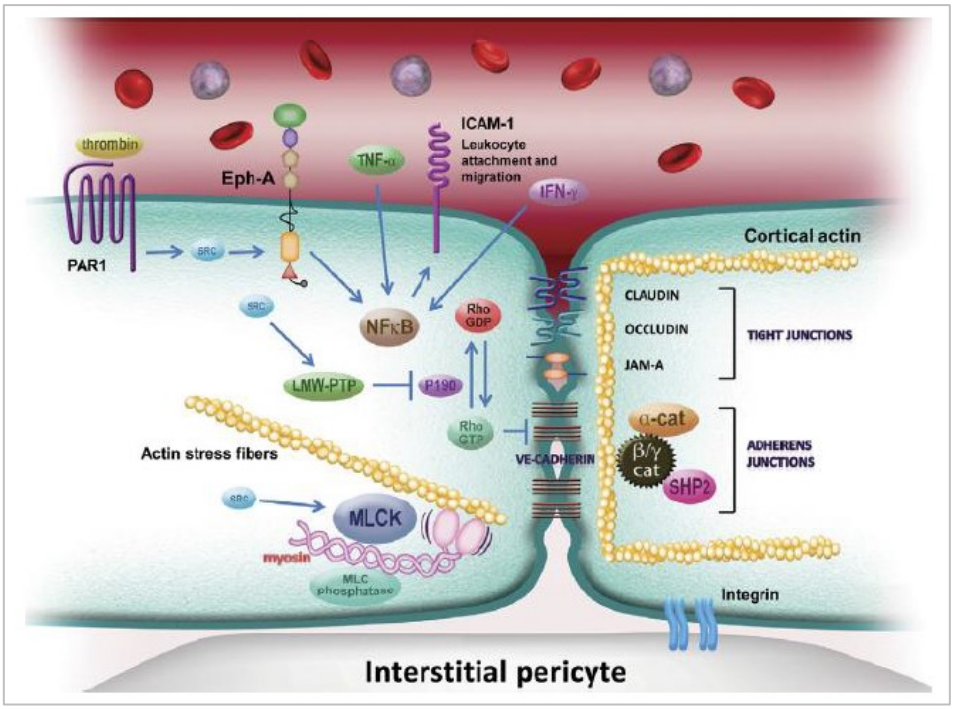


Figure 7. EphA signalling mechanism in vascular leakage and leukocytes recruitment (Coulthard et al., 2012)

LEUKOCYTES RECRUITMENT

Early during inflammation, the endothelial cell layer changes its inactive phenotype in an activated one characterised by the expression of several adhesion molecules, including P and E-selectin, *intercellular adhesion molecule 1*, ICAM-1, and *vascular adhesion molecule 1*, VCAM-1, that facilitate leukocyte stickiness (Galkina et Ley, 2007).

Eph and ephrin expression patterns change significantly during endothelial activation: several studies have demonstrated that EphA2, ephrin-A1 and ephrin-B2 are over-expressed and therefore implicated in the inflammatory process (Funk et al., 2013). In particular, EphA2 signalling, besides increasing endothelial permeability, can activate the pro-inflammatory transcription factor NF-kB and enhance the expression of ICAM-1, VCAM-1 and E-selectin *[Figure 7]* (Carpenter et al., 2012; Funk et al., 2013).

Activated endothelial cells promote leukocyte recruitment through a complex series of interactions between leukocytes and endothelial adhesion molecules. The first step is

the loose binding between endothelial selectin molecules and leukocyte selectin ligands that facilitates leukocyte tethering to the endothelium and the rolling along the vascular surface (Ley et al., 2007). The second step is the creation of high affinity interactions that enhance the firm adhesion of leukocytes to the endothelium through ICAM-1 and VCAM-1 binding. The third step is a highly coordinated and complex mechanism that allows the transmigration of leukocytes across the endothelial monolayer (Muller, 2003).

Since both leukocytes and endothelial cells express a wide number of Eph receptor and ephrin ligands, Eph-ephrin interactions may modulate leukocyte trafficking. T cells, B cells and monocytes express EphA4 and ephrin-A4, whereas EphA2 expression is restricted to dendritic cells and monocytes. The EphB/ephrinB family appears to have a more ubiquitous expression pattern: EphB1, EphB2, EphB4, EphB6 and ephrin-B1/B2 are expressed on multiple leukocytes subtypes (Sakamoto et al., 2011). Interestingly several studies have demonstrated that leukocyte Eph/ephrin expression is not static but changes with phenotypic modulation (Funk et al., 2013).

EphA-ephrinA interactions seem to participate in integrin-mediated adhesion of lymphocytes to the activated endothelium: the so-called *homing* process. For example, the activation of ephrinAs on T cells by exogenous EphA2-Fc enhances T cells adhesion to integrin α4β1 and the transmigration through the endothelial monolayer *[Figure 8b]*. In contrast, the activation of EphAs receptor on T cells by ephrinAs-Fc ligands reduces the adhesion to integrin substrates *[Figure 8c]* (Sharfe et al, 2008).

EphB/ephrinB family has been involved in monocytes adhesion and transmigration: EphB receptors on monocyte surface can interact with endothelial ephrin-B2 to promote this process. Moreover, this interaction seems able to recruit the metalloproteinase ADAM-10 that might result in a remodelling of endothelial junctions during extravasation *[Figure 8a]* (Solanas et al., 2011).

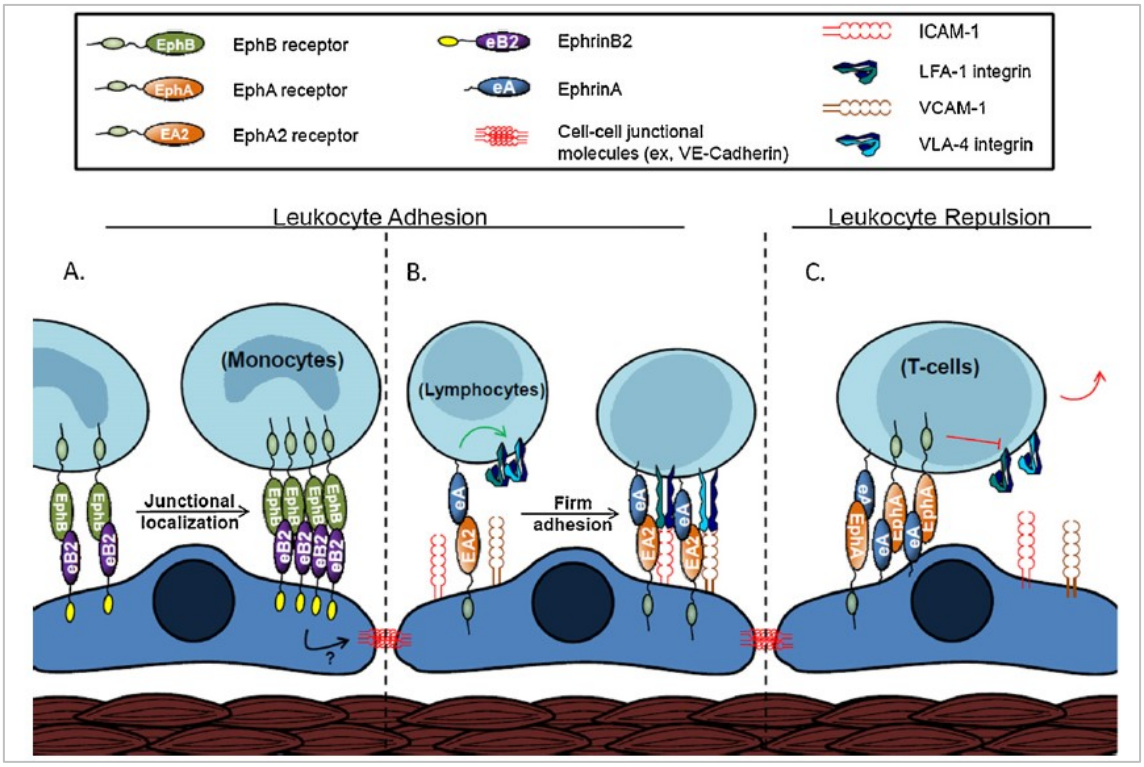


Figure 8. Examples of Eph/ephrin mediated adhesion or repulsion in leukocyte-endothelial interactions (Funk et al., 2013)

TISSUE REPAIR

The Eph/ephrin proteins are involved in angiogenesis and cell migration, both critical aspects of wound healing, thus it is possible to hypothesize that they can play a role in tissue repair and maintenance. Moreover, they participate in tissue development and adult homeostasis maintaining.

Vihanto and colleagues demonstrated that after hypoxic injury, in a flap skin mouse model, the expression levels of EphB4, ephrin-B2, EphA2 and ephrin-A1 were up-regulated. This supported the hypothesis of their involvement in re-vascularization after hypoxic injury (Vihanto et al., 2005). Other evidences come from models of spinal cord injury in both lower vertebrates and rodents: in those models both members of A and B families were transiently up-regulated showing an expression pattern similar to the one of the development phases, thus suggesting their potential role in axon recovery (Coulthard et al., 2012).

In the intestine, the fast epithelial healing is particularly important considering the necessity to protect the host against microbial threat and several immunogenic and toxic factors (Wilson et Gibson, 1997). Hafner and his group have studied the effects mediated by *reverse* ephrinB2 signalling in an *intestinal epithelial cell line 6*, IEC-6, in the scratch wound assay. The results of their studies demonstrated firstly an increased wound closure competency of IEC-6 intestinal cells consequent to *reverse* ephrin-B2 signalling activation, and, secondly, that this effect was mediated through the activation of a set of genes, downstream of ephrinB pathway, involved in wound healing, like ERK1-2, c-Fos, Egr1-2, but also in inflammation, like COX2 (Hafner et al., 2005a; Hafner et al., 2005b).

Despite the numerous investigations, the complexity of Eph-ephrin bidirectional signalling pathways must be further investigated in order to unveil their possible role in tissue repair mechanisms.

ACUTE INFLAMMATORY PATHOLOGIES

The involvement of the Eph/ephrin system in acute inflammatory conditions has been widely investigated.

Ischemia-reperfusion injury (IRI) is characterised by an intense inflammatory response mediated by pro-inflammatory cytokines, by endothelial cells activation and up-regulation of vascular adhesion molecules, and finally by the recruitment of inflammatory cells. Up to now, the role of Eph/ephrin system in IRI is debated.

EphA2 expression has been documented to be up-regulated in renal IRI (Baldwin et al., 2006) and in myocardial IRI together with EphA1 and EphA3 (Dries et al., 2011). Thundyl and colleagues demonstrated that KO mice for EphA2 had a reduced BBB permeability, an increased expression of TJ components and a reduction of leukocytes infiltration after brain ischemic injury (Thundyl et al., 2013). By opposite the local administration of ephrinA1-Fc, able to activate EphAs signalling, promoted tissue salvage following myocardial infarction in mice (Dries et al., 2011). In a model of intestinal IRI, the systemic EphA4-Fc treatment, purportedly blocking endogenous Eph-ephrin interactions, contrasted gut vascular leakage and neutrophil recruitment (Woodruff et al., 2016). Recently in my research group, the role of EphA-ephrinA

signalling has been investigated in a model of mesenteric IRI. We have observed that the activation of *forward* signalling by systemic administration of ephrinA1-Fc is able to counteract intestinal and lung damage, to prevent neutrophil infiltration and abolish EphA2 over-expression within the lungs (Vivo et al., 2017).

In a rat model of **lung viral infection**, Cercone and colleagues demonstrated that EphA2 is over-expressed in hypoxic infected rats leading to increased vascular permeability and proteins leakage. The blockade of EphA2 by EphA2-Fc or the use of specific monoclonal antibodies was able to reduce vascular permeability and oedema formation (Cercone et al., 2009).

Those evidences prove that EphA/ephrinA system is involved in acute inflammatory processes and, in future, a fine pharmacological tuning of its signalling pathway might be a target for the treatment of these pathological conditions.

CHRONIC INFLAMMATORY DISEASES

There is evidence for the involvement of Eph/ephrin proteins in a multitude of chronic inflammatory diseases (Funk et al., 2013).

Eph/ephrin signalling has been associated with **atherosclerosis**. Atherosclerosis consists in lipoproteins accumulation within the vascular wall activating a local inflammatory response. This response is sustained by monocytes that can differentiate into macrophages and dendritic cells; those cells start engulfing low density lipoproteins (LDL) and transforming into dysfunctional cells called foam cells. A systemic response is also risen against the atherosclerotic plaque leading to a continuous growth of the necrotic core and of the fibrotic cap. These phenomena terminate with progressive lumen occlusion and the reduction of blood flow to specific tissues (Coulthard et al., 2012). Genes encoding for several EphA/ephrinAs and EphBs/ephrinBs were found altered in tissues taken from human carotid. Moreover, EphB2 and ephrin-B1 were localised to plaque macrophages and T cells (Sakamoto et al., 2008). Coulthard and his research group demonstrated enhanced EphA2 and ephrin-A1 protein expression in both murine and human atherosclerotic endothelium and macrophages (Funk et al, 2012). Interestingly, the gene encoding for EphA2 is located within the murine atherosclerosis susceptibility locus, which is highly

homologous to human myocardial infarction susceptibility locus that similarly contains the EphA2 gene (Wang et al., 2004).

Taken together, these studies demonstrate that the Eph/ephrin expression profile is significantly altered during the atherosclerotic plaque formation, suggesting these proteins may affect the atherosclerotic plaque development (Coulthard et al., 2012).

Although the aetiology of **rheumatoid arthritis**, RA, is still elusive, anomalies in T cells homeostasis and hyperproliferation of synovial-lining cells seem to be involved in its development. Because of the role displayed by Eph/ephrin system in inflammatory processes, Kitamura and co-workers focused on ephrinB molecules in T cells and synovial cells derived from RA patients (Kitamura et al., 2008). They found that ephrin-B1 expression levels were high in synovial $CD3^+$ T cells, in synovial fibroblasts and in peripheral blood lymphocytes (PBLs) of RA patients. Moreover, they tested the effects of ephrinB1-Fc administration in a mouse model of RA and they observed an increased migration of PBLs and increased TNF-α production. Ephrin-B1 seems thus to play an important role in the inflammatory states of RA, especially by affecting the population and function of T cells. Inhibition of the ephrinB/EphB system might be a novel target for the treatment of RA (Kitamura et al., 2008).

Finally, since Eph/ephrin proteins are expressed during small intestine development and members of the A and B classes have been respectively described in the maintenance of intestinal barrier and in the regulation of intestinal epithelial cell positioning and differentiation within the crypts, this system has been studied in the context of **inflammatory bowel diseases** (IBD).

Together with the findings that ephrin-B2 *reverse* signalling apparently promotes wound healing and pro-inflammatory genes transcription in IEC-6, Hafner and colleagues determined through Real-time PCR and cDNA microarray analysis that EphA2, ephrin-A1, EphB2, and ephrin-B1/B2 expression was up-regulated in the intestinal epithelial cells of mucosal lesions in patients with inflammatory bowel disease compared to healthy donors (Hafner et al., 2005a). These findings represent the proof of concept for the study of Eph/ephrin system in IBDs.

STRATEGIES FOR THE INHIBITION OF EPH-EPHRIN SIGNALLING

The altered expression and functional involvement of Eph receptors and ephrins in many diseases offers the opportunity for therapeutic strategies based on modulating the activities of the pertinent family members (Barquilla et Pasquale, 2015). Various therapeutic strategies have been proposed during the years to target the Eph-ephrin signalling; among them we can identify two classes: kinase inhibitors or protein-protein interactions inhibitors, PPI. While the formers are able to inhibit only Eph *forward* signalling, the second one can prevent the binding of the ephrin ligand to the Eph receptor thus blocking the bidirectional signalling *[Figure 9]* (Tognolini et al., 2014).

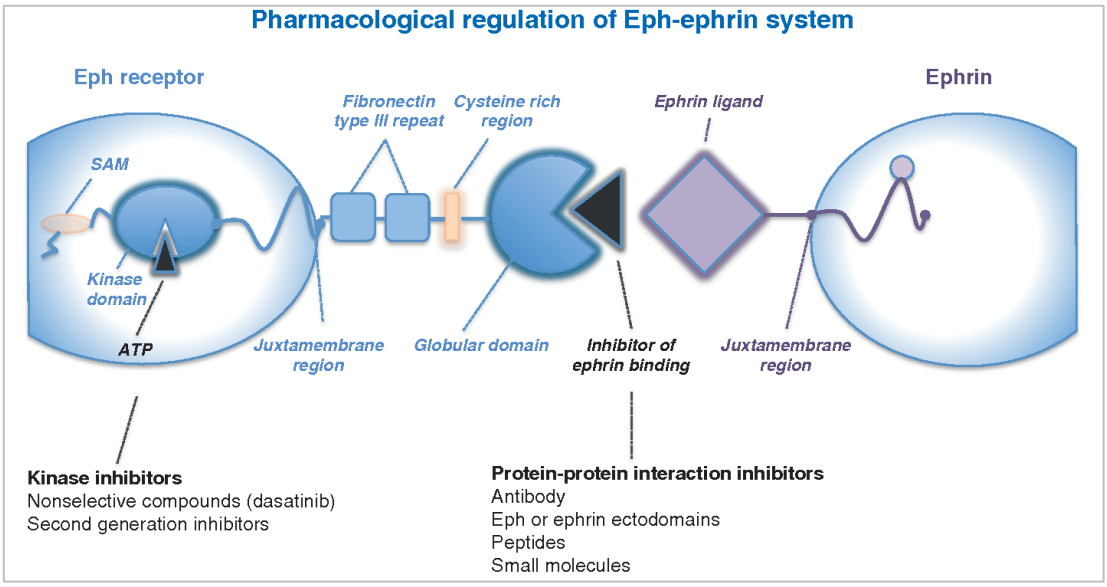


Figure 9. Pharmacological regulation of Eph/ephrin system (Tognolini et al., 2014)

KINASE INHIBITORS

Kinase inhibitors bind with nanomolar affinity to the ATP-binding pocket of Eph receptors. Among them, some have been developed as selective inhibitors of EphB4 receptor (Baron et al., 2010), while others are able to block Eph receptors activity although they were in first instance designed as inhibitors for other targets. An example is dasatinib, a compound able to bind Src and Abl kinases but also to inhibit various Eph receptors such as EphA2 (Chang et al., 2008).

PROTEIN-PROTEIN INTERACTION INHIBITORS

PPIs can be divided in antibodies, Eph or ephrin recombinant molecules, peptides and small molecules.

Antibodies are particularly suitable for modulating the Eph/ephrin system, given their high binding affinity, their specificity and their long in vivo half-life. Both activating and inhibitory monoclonal antibodies can recognize Eph/ephrin extracellular domains (ECDs) and they have been developed for applications against cancer and angiogenesis, with particular focus on EphA2, EphA3, EphB4, and ephrin-B2 (Pasquale, 2010).

Eph or ephrin recombinant molecules are widely used as soluble alternatives for their membrane-bound counterparts to activate, as dimers, as well as inhibit, as monomers, *forward* signalling, *reverse* signalling, or both (Pasquale, 2010). These recombinant proteins bind with high affinity and can have a long in vivo half-life, particularly when coupled to an Fc domain or albumin.

Peptides have also showed their potential for modulating Eph-ephrin signalling with high selectivity and binding affinity. A series of dodecapeptides that can selectively target the ephrin-binding pocket of single Eph receptors, or subsets of receptors, and antagonize ephrin binding were initially identified by phage display (Noberini et al.,2012) and their affinity was improved to low nanomolar by optimization (Duggineni et al., 2013). Most of the identified peptides act as antagonists, except for peptides targeting EphA2, which function as agonists promoting receptor activation and internalization through an unknown mechanism (Mitra et al., 2010).

The ephrin-binding site of Eph receptors presents favourable features for high-affinity binding of **small molecules**, allowing the discovery of a few classes of ligands of EphA and EphB receptors. These include: lithocholic acid (LCA) derivatives such as cholanic acid and L-Trp conjugates, Eph receptor antagonists with a moderate preference for the EphA receptor subfamily; salicylic acid derivatives, which inhibits EphA2 and EphA4 receptor subtypes, and doxazosin, a marketed α_1-adrenoceptor antagonist that has been recently shown to bind EphA2 and EphA4 receptor subtypes (Tognolini et al.,

2014). Some of these Eph/ephrin inhibitors are now in clinical trials for the treatment of cancer.

INFLAMMATORY BOWEL DISEASE- IBD

IBD is an idiopathic disease driven by a dysregulated immune response to gut microbiota in a genetically susceptible host causing defects in epithelial barrier function and response to pathogens *[Figure 10]* (Coskun, 2014).

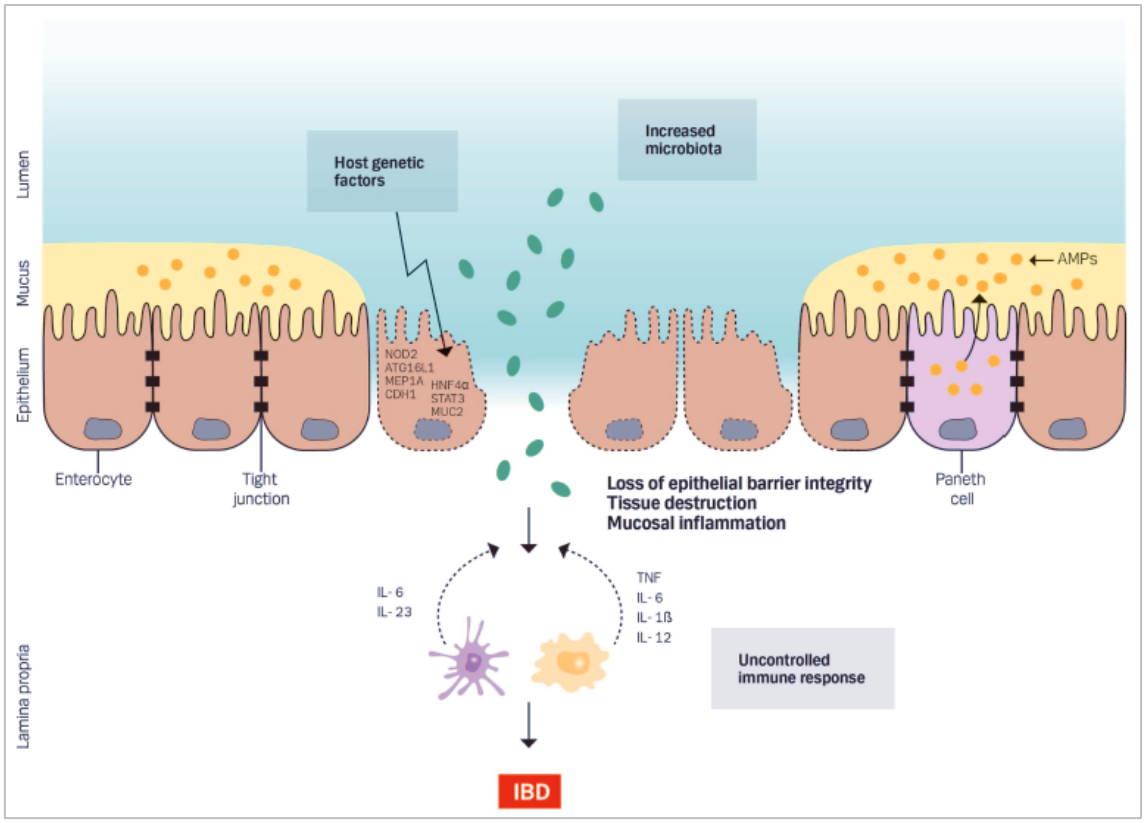


Figure 10. Scheme representing IBD pathogenesis (Coskun, 2014)

Two major types can be distinguished: ulcerative colitis (UC), confined to the mucosal surface of the colon, and Crohn's disease (CD), spread along the whole gastrointestinal tract with transmural inflammation. IBD affects millions of people around the world and its incidence is constantly increasing making this pathology among the most worrying ones.

IBD PATHOGENESIS

In the gastrointestinal tract, the host defense against the harmful luminal microenvironment relies primarily on the protective barrier represented by intestinal epithelium: on one side, it allows selective permeability and absorption of nutrients and drugs, and, on the other side, it separates the intestinal lumen from the underlying lamina propria, so to control the crosstalk between microbiota and immune cells (Coskun, 2014). The balanced interaction between the microbiome and the immune system contributes to intestinal homeostasis. Intestinal barrier disfunctions play therefore a central role in IBD pathogenesis: the increased permeability of the intestinal epithelial layer contributes to an over reactivity of the immune cells that harms the mucosal barrier and leads to a dangerous chronic inflammatory state (Maloy et Powrie, 2011).

The major players in IBD pathogenesis are therefore the microbiota, the intestinal epithelium and the innate and adaptive immune system.

MICROBIOTA

Intestinal microbiota consists of the microorganisms that inhabit our gut: they provide key nutrients, modulate energy metabolism and help developing our immune system (Bäckhed et al., 2005). Host-microbiome interactions can be mutually beneficial or can be deleterious arousing intestinal inflammation. Observations in IBD patients and in animal models have pointed out the role of bacteria in this pathology. Indeed, it is true that several pathogens have been blamed for the development of IBD, none of them being confirmed as causal; however, alterations in gut microbiota appear pivotal. In fact, the bacterial profile of IBD patients is completely different from that of healthy subjects, showing a strong decrease in Firmicutes and Bacteroidetes, two mucosal associated phyla (Frank et al., 2007), and antibiotics, together with aminosalicylates and topical corticosteroids, still represent the first line of therapy against IBD; moreover, most of IBD murine models require gut colonisation by bacteria for inflammation to occur (Elson et al., 2005).

THE INTESTINAL EPITHELIUM

The intestinal epithelium is a physical barrier against the excessive entry of bacteria and luminal antigens into the circulation. Tight junctions represent a key element in controlling the sealing of the paracellular space of intestinal epithelium. However, in IBD those junctions are larger and the permeability of the barrier increased: this can be due to intrinsic defects of barrier function or can be a consequence of inflammation.

Additional defenses against luminal harmful agents are represented by other specialized intestinal epithelial cells (IECs) subtypes: Goblet cells and Paneth cells. Goblet cells produce mucus and factors that contribute to epithelial repair, whereas Paneth cells secrete antimicrobial peptides as α-defensin. In IBD, the mucus layer, important in controlling bacteria-epithelium interactions, is damaged: the result is an increased exposure of epithelium to microbiota and the amplification of the inflammatory response.

Interestingly, defects of genes related to mucus production or to other intestinal protective factors have been implicated in increased susceptibility to IBD in both humans and animal models (Coskun 2014). Examples are represented by polymorphism of the gene encoding for receptor 4 of prostaglandin E, which contributes to mucosal repair and barrier function (Kabashima et al., 2002) and by the variants in genes encoding for mucin components as muc2 or muc19 (Van der Sluis et al., 2006; Barrett et al., 2008). Another example can be the famous NOD2 polymorphism, the first acknowledged genetic susceptibility locus for CD.

NOD2 is an intracellular receptor that recognises bacterial muramyl dipeptide and induces autophagy and bacterial clearance (Hoefkens et al., 2013). Epithelial cells that do not have a perfectly functional NOD2 or that carry sequence variants for other autophagy-related genes, such as ATG16L1, display, therefore, an increased susceptibility to bacterial-induced inflammation.

Also mutations of STATs, signal transducers and activators of transcription pathway responsible of orchestrating appropriate cellular responses to cytokines signalling (Coskun et al., 2013), are reported as IBD-related susceptibility genes (Jostins et al., 2012).

IMMUNE RESPONSES IN IBD

The hallmark of active IBD is a huge infiltration of immune cells, both innate - neutrophils, macrophages, dendritic cells, natural killer T cells- and adaptive -B, T lymphocytes- into the lamina propria. In healthy conditions, the intestinal lamina propria contains a complex population of immune cells that coordinates the need for immune tolerance of luminal microbiota with the defense against pathogens and the excessive entry of luminal antigens (Abraham et Cho, 2009).

In the early phases of IBD, characterised by acute inflammation, innate immune responses predominate. Pattern recognition receptors are fundamental components of the innate immune system; among them, toll like receptors (TLRs) are crucial in maintaining tolerance towards the microbiota (Arsenau et al, 2007). TLR4 expression is significantly up-regulated in CD and UC patients (Cario et Podolsky, 2000), while TLR9 polymorphism has been associated to CD (Torok et al., 2004). Similarly to NOD2, defects in TLRs signalling expression could impair the ability of the host to recognise bacteria and pathogens and mount an appropriate immune response.

Adaptive immune responses are extremely complex. In normal conditions, T helper cells ($CD4^+$ T cells) are tightly regulated by TGF-β and IL-10, the so called immune suppressive cytokines. According to the stimulus they receive, naïve $CD4^+$ T cells can differentiate into different effector subgroups: Th1, Th2 and Th17 cells. Those subpopulations are critical for defenses against pathogens, but their uncontrolled expansion and over-reactivity can lead to chronic intestinal inflammation *[Figure 11]* (Izcue et al., 2006). Phenotype characterization of mesenteric lymph nodes (mLN) T-cells derived from CD patients showed a Th1 and Th17 feature (Sakuraba, 2009), whereas in UC the phenotype is markedly Th2 (Abraham et Cho, 2009).

In recent years the Th17 family and IL-23 pathway have gained a lot of attention and reports of genetic associations between IL-23 receptor and IBD have emerged leading to novel therapeutic molecules (Abraham et Cho, 2009).

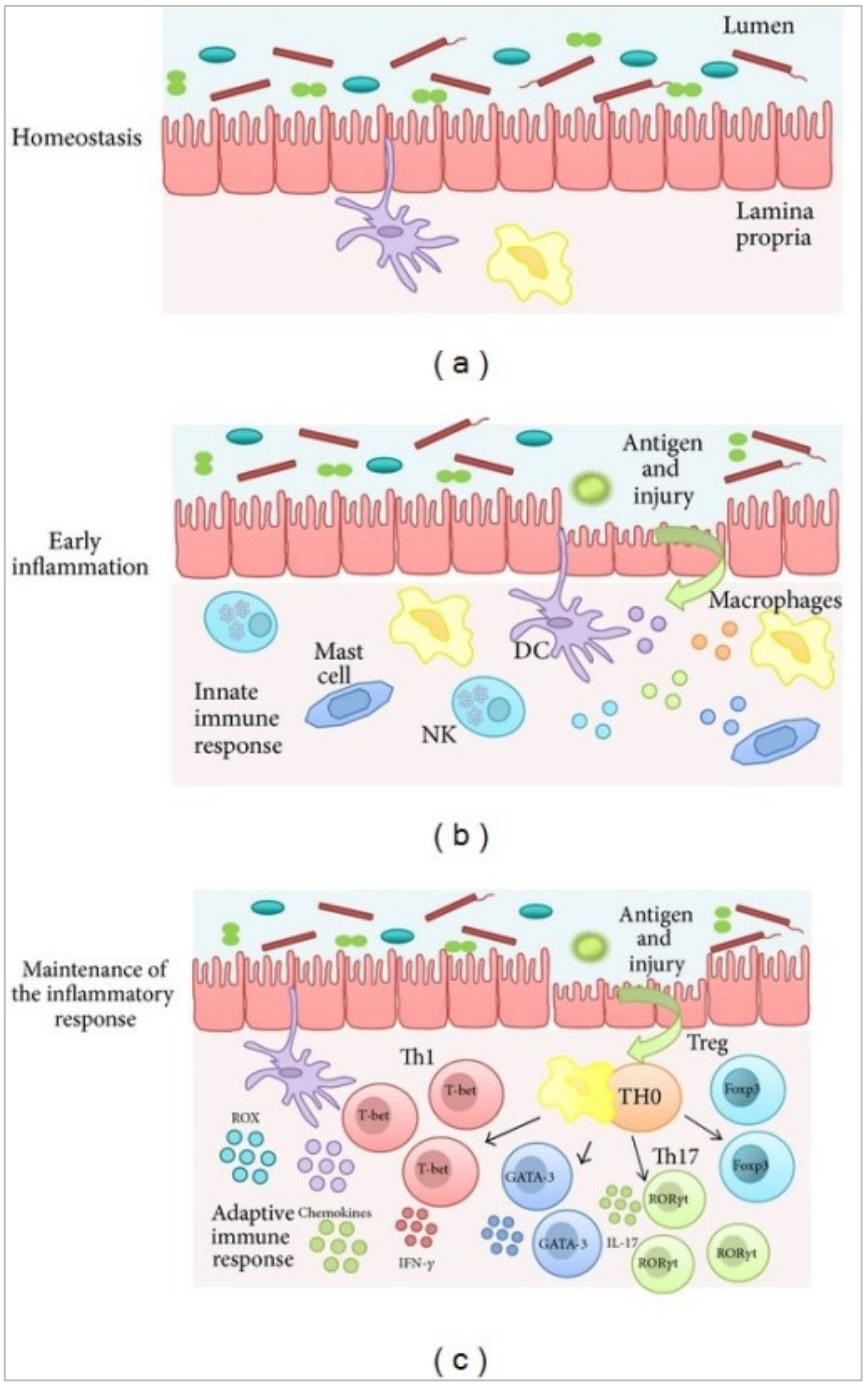


Figure 11. Intestinal immune system in health (a), early (b) and late inflammation (c) (Gálvez, 2014)

Increased numbers and activation of T cells in the intestinal mucosa are associated with high local levels of tumor necrosis factor α (TNF-α), interleukin-1β, interferon-γ, and cytokines of the interleukin-23-Th17 pathway (Abraham et Cho, 2009).

All those pro-inflammatory molecules will perpetuate the inflammatory-induced damage and will spread the inflammation systemically.

PHARMACOLOGICAL MANAGEMENT OF IBD

The complex mechanisms and multifactorial pathogenesis of IBD make its pharmacological management extremely challenging. The current goals of therapy are induction of remission and then the maintenance of remission. Several different approaches have been tried in the last years: they can be distinguished as based on biological or non-biological approaches. Besides medicines, surgery is another therapeutic option, especially for patients who do not respond to medical managements or develop complications (Kaistha et Levine, 2014). Moreover, in addition to medical managements, nutritional status should be monitored to prevent macronutrient deficiencies and psychosocial support should be considered to improve quality of life and depression (Kaistha et Levine, 2014).

NON-BIOLOGICAL THERAPIES

Non-biological therapies are defined as the therapies used before the introduction of Infliximab, a chimeric monoclonal antibody against TNF-α, in 2006. Those therapies can be divided in anti-inflammatory drugs, immunosuppressants and gut microbiota modulators.

Among **anti-inflammatory agents** 5-ASA-based therapies and corticosteroids can be listed. Aminosalicylates are a group of drugs containing the 5-ASA moiety effective in the maintenance of remission in mild CD and moderate UC; mesalazine and sulfasalazine are the 5-ASA derivatives of choice. Their use is limited by side effects. Glucocorticoids are extensively used in IBD treatment especially for inducing remission. They are usually administered for two weeks on full dose and then, due to their well-known side effects, there is a gradual tape over for 4-8 weeks. Budesonide represents an alternative to classical corticosteroids treatments because has local effects on the bowel and the high first-pass hepatic metabolism reduces the systemic side effects (Kaistha et Levine, 2014).

Immunosuppressants 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. Cyclosporine might be used only in hospitalized patients with severe active UC, not responding to other therapies (Talley et al., 2011). They usually represent the second line treatment for steroid-dependent or steroid-refractory patients. The common side effects include myelosuppression and the increased risk of developing infections.

In recent years increasing attention has been paid to the role of **microbiota** in IBD pathogenesis, since dysbiosis is a key feature of this disease. Therefore, the use of probiotics, antibiotics and more recently the *Fecal Microbiota Transplant* (FMT) have been proposed. However, while the use of several antibiotics as metronidazole, ciprofloxacin and rifaximin have been proved to be effective, the efficacy of probiotics and FMT is still unproven (Dignass et al., 2010).

BIOLOGICAL THERAPIES

Since the introduction of biologic agents, the traditional therapeutic dogma has shifted from non-specific immunomodulators and anti-inflammatories, toward a anti-inflammatory approach. Although the introduction of TNF-α inhibitors has initiated a new therapeutic era, they are clinically effective only in a subgroup of patients with IBD, 30% of patients being non-responders (Olesen et al., 2016). This evidence indicates that other inflammatory, non-TNF-driven, pathways may be pivotal in these patients.

Therefore, new targets have been identified and new monoclonal antibodies (mAb) or selective small molecules have been developed *[Table 1]*.

Drug	Formulation	Target	Admin.	Clinical status[b]	
				CD	UC
PF-04236921	Fully human mAb	IL-6	s.c.	–	–
Ustekinumab	Fully human mAb	IL-12/IL-23 (p40)	i.v./s.c.	Approved[c]	Phase III
AMG-139	Fully human mAb	IL-23 (p19)	i.v./s.c.	Phase II	–
BI-655066	Fully human mAb	IL-23 (p19)	i.v./s.c.	Phase II	–
LY3074828	Humanized mAb	IL-23 (p19)	i.v./s.c.	Phase II	Phase II
Tofacitinib	Small molecule	JAK1/JAK3	Oral	–[d]	Phase III
Filgotinib	Small molecule	JAK1	Oral	Phase III	Phase III
Peficitinib	Small molecule	JAK1/JAK3	Oral	–	–
Mongersen	Antisense oligonucleotide	*SMAD7*	Oral	Phase III	Phase II
Laquinimod	Small molecule	?	Oral	–	–
Natalizumab	Humanized mAb	α_4-Integrin	i.v.	Approved[e]	–
AJM300	Small molecule	α_4-Integrin	Oral	–	–
Vedolizumab	Humanized mAb	$\alpha_4\beta_7$	i.v.	Approved[f]	Approved[f]
AMG 181	Fully human mAb	$\alpha_4\beta_7$	s.c.	Phase II	Phase II
Etrolizumab	Humanized mAb	β_7-Integrin	i.v./s.c.	Phase III	Phase III
PF-00547659	Fully human mAb	MAdCAM-1	i.v./s.c.	–	Phase II
Ozanimod	Small molecule	$S1P_1/S1P_5$	Oral	Phase II	Phase III
Etrasimod	Small molecule	$S1P_1$	Oral	–	Phase II
Amiselimod	Small molecule	$S1P_1/S1P_5$	Oral	Phase II	–

Table 1. Emerging targeted therapies in IBD (Coskun et al., 2017)

IL-6 contributes significantly to the pathogenesis of various diseases, including IBD, because it is involved in the differentiation of Th17 cells. Among all the antibodies entered in clinical trials against IL-6, only Tocilizumab and PF-04236921 completed the trials (Coskun et al., 2017).

IL-12 and IL-23, heteromeric cytokines that share the p40 subunit, are expressed by dendritic cells and play a key role in T cell immune responses: in particular, they induce Th1 and Th17 differentiation, respectively (Teng et al., 2015). Ustekinumab was approved by US Food and Drug Administration (FDA) in September 2016 for the treatment of patients with CD, refractory to conventional and/or anti-TNF therapy. The drug was subsequently approved by the European Medicines Agency (EMA) in November 2016. IL-23-specific mAbs are currently under investigation in clinical trials for CD and UC (Coskun et al., 2017).

An alternative emerging approach is the use of intracellular small molecule JAK inhibitors that target and inhibit several pro-inflammatory signalling pathways simultaneously. Given the essential role of JAKs in inflammatory signalling, several inhibitors have been developed. Tofacitinib is an oral JAK inhibitor that mainly antagonises JAK1 and JAK3, thus blocking the downstream effects of a large subset of pro-inflammatory cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-15, IL-21, and interferon-gamma INF-γ (Coskun et al., 2013).

Finally, the idea of restoring the TGF-β1 signalling pathway has been the core in the search for novel therapeutic compounds. TGF-β1 is highly produced in IBD, but its activity is reduced by the high levels of Smad7, an intracellular protein that inhibits TGF-β1 signalling (Marafini et a., 2013). An intracellular antisense oligonucleotide, Mongersen, has been developed. It is able to facilitate RNase H-mediated degradation of SMAD7 mRNA, and to inhibit the production and activity of Smad7. Thus, Mongersen restores the production of TGF-β1, which, in turn, induces anti-inflammatory signalling (Monteleone et al., 2015).

As mentioned before, migration and infiltration of leukocytes from the blood to the site of inflammation in the intestinal mucosa is an important aspect of IBD pathogenesis. Thus, also agents targeting leukocyte trafficking have been developed.

Natalizumab is a recombinant humanized IgG4 monoclonal antibody directed against the α4-integrin subunit on leukocytes: it was the first anti-integrin molecule introduced and proven effective in the induction and maintenance of remission in patients with CD (Sandborn et al., 2015). Natalizumab blocks the interaction of α4β1 and α4β7 integrins with VCAM-1 and MAdCAM-1, respectively, adhesion molecules highly expressed on endothelial cells of the intestinal lamina propria during inflammation. Unfortunately, given VCAM-1 expression also in the central nervous system, targeting α4β1 also limits immune surveillance of the central nervous system, thus possibly leading to serious and lethal infections (Van et al., 2005). The use of natalizumab for CD patients has been approved only in the USA and some other countries, but not in Europe (Coskun et al., 2017). Therefore, gut-selective α4β7 inhibitors with a more acceptable safety profile have been developed: Vedolizumab is

an IgG1 humanized monoclonal antibody that specifically inhibits integrin α4β7 on T cells and blocks the interaction between α4β7 and MAdCAM-1. This is the first anti-integrin antibody approved for treatment of patients with UC and CD who are refractory to conventional and/or anti-TNF therapy (Feagan et al., 2013; Sandborn et al., 2013); overall, a favourable benefit-risk profile makes vedolizumab a useful option for the long-term treatment of IBD (Colombel et al., 2016).

Other inhibitors with a similar mechanism are currently under investigation: etrolizumab can inhibit β7-integrins and therefore it is thought to decrease lymphocytes trafficking to the gut and their retention in the intraepithelial compartment (Coskun et al., 2017).

Another interesting tool is ozanimod, a novel orally-acting small-molecule agonist selective for S1P receptor 1 (S1P1), which is expressed on the surfaces of lymphocytes. This drug blocks lymphocyte egress from lymph nodes into the systemic circulation by inducing S1PR1 internalization and degradation and, consequently, prevents lymphocyte trafficking to the sites of inflammation (Degagne et Saba, 2014; Sandborn et al., 2015).

ANIMAL MODELS OF IBD

A significant amount of data supporting the various hypotheses on IBD pathogenesis is derived by animal experimental models of intestinal inflammation. Since the description of the first model by Kirsner and Elchlepp almost 60 years ago, more than 50 different murine models have been generated by genetic engineering guided partly by human genome wide association studies in IBD (Kirsner et Elchlepp, 1957). Murine experimental colitis models have helped to identify the key elements of mucosal immune homeostasis such as the integrity of the epithelial barrier, the innate immune responses and the tight regulation of adaptive immune responses (Valatas et al., 2015). In recent years, there has been growing attention about the ethical acceptability of the animal models to study human diseases and their treatment. The major concern regards the scientific validation of the disease model in terms of similarity and transferability to humans, a disease model should, in fact, respect three obligatory conditions:

-*face validity*: mimic the clinical disease condition as much as possible;

-*construct validity*: investigation of pathological features towards the confirmation or negation of a mechanistic relationship;

-*predictivity*: assure a reliable development of new potential therapeutic options (Mayer et Collins, 2002).

Animal models provide an invaluable means to study complex physiological and biochemical interactions, which, up to now, cannot be completely simulated in silico and/or in vitro (Dothel et al., 2013).

In general, the majority of animal models of IBD are generated by chemical induction, by immunologic manipulation or through gene targeting.

TNBS-INDUCED COLITIS

TNBS, *Trinitrobenzene Sulfonic acid*, is a hapten administered as enema in rats or mice in combination to ethanol to disrupt the epithelial barrier. The intrarectal administration makes colonic protein immunogenic to the host immune system and thereby stimulates $CD4^+$ T cells responses responsible of intestinal inflammation. The hapten-induced immune response provokes CD-like severe ulcerations of the mucosal

barrier characterised by granulocytic and lymphocytic infiltrates and dominant Th1 responses, with high levels of TNFα, IL-1β, IL-12, IL-17, IL-18 and IL-6 (Dothel et al., 2013). Susceptibility to TNBS colitis and the type of cytokine response, induced by TNBS administration, vary significantly among mouse strains (Kiesler et al., 2015).

As some of the characteristics of this model resemble features of CD, TNBS colitis has been widely used in the study of immunological aspects relevant to this disease including cytokine secretion patterns, mechanism of oral tolerance, mechanisms underlying intestinal fibrosis and spontaneous resolution (Kiesler et al., 2015).

DSS-INDUCED COLITIS

Dextran sulfate sodium, DSS, is a sulphated polysaccharide that induces one of the most thoroughly studied model of intestinal inflammation. It has been widely used to study the events occurring after failure of mucosal homeostasis after epithelial destruction and loss of barrier integrity, as well as the mechanisms that lead to mucosal healing (Kiesler et al., 2015). The mechanism by which DSS damages the epithelial layer is still unclear: according to some authors, this might be due to a direct toxicity to colonic epithelium (Kiesler et al., 2015), while the ability to compete with the substrates of ribosomes for mRNA translation (Laroui et al., 2012), or the loss of zonula occludens, main component of TJ, due to chelation of divalent cations (Poritz et al., 2007), are other possible mechanisms as well.

Usually rodents fed with DSS solution (2-5% w/V), added to drinking water for a short period of time, develop an acute form of colitis characterised by bloody diarrhoea, weight loss and granulocytes infiltration, while an inflammatory condition reminiscent of human chronic inflammation can be induced by repeated DSS cycles. In mice, the severity of inflammation depends on the strain used (Perse et Cerar, 2012). The acute phase of inflammation shows a Th1-mediated response (Hall et al., 2011), this turns into a Th2-polarized response in the chronic DSS model, resembling features of human UC (Perse et Cerar, 2012). Moreover, the acute model is characterised by the involvement of the innate immune compartment (macrophages and neutrophils), whereas the chronic one by the recruitment of adaptive immune cells: therefore, this

model is useful to study the different contribution of the two branches of the immune system (Bento et al., 2012).

T-CELL TRANSFER MODEL OF COLITIS

The T-cell transfer model of IBD was pioneered by Powrie and colleagues, who observed that reconstitution of immune deficient C.B-17 scid mice with naïve $CD4^+CD45RB^{high}$ T cells isolated from normal BALB/c mice led to the development of colitis (Powrie et al., 1993). Disease is driven by the differentiation and expansion of pathogenic $CD4^+$ T cells that react toward components of the normal intestinal commensal flora (Powrie et al., 1994). By opposite, injecting $CD4^+CD45RB^{low}$ T cells did not elicit colitis and, indeed, even suppressed the development of disease in immune deficient mice that also received pathogenic $CD4^+CD45RB^{high}$ T cells (Powrie et al., 1993). Further experiments demonstrated that this suppressive activity was due to the $CD4^+CD25^+$ T cell population, showing that $CD25^+$ regulatory T cells (Treg) play a crucial role in the maintenance of self-tolerance and regulation of inflammatory responses (Coombes et al., 2005).

In this model, colon inflammation develops within 6-8 weeks after transfer and is characterized by transmural inflammation, ulcerations, loss of mucosal architecture and dense polymorphonuclear and mononuclear leukocyte infiltrations, features partly resembling CD (Valatas et al., 2015). Initial studies classified the T-cell transfer model as IL-12 dependent, Th1-mediated (Powrie et al.,1994). Subsequent studies however identified a mixed Th1/Th17 response (Hue et al.,2006; Kullberg et al.,2006).

This model has been largely used for the study of various aspects of mucosal T cell activation: one of the major contributions was the recognition of the crucial role of IL-23-dependent Th17 pathway in intestinal inflammation (Hue et al., 2006; Kullberg et a., 2006).

KO MODELS OF COLITIS

Genetically engineered models represent precious tools to study specific pathways involved in IBD pathophysiology and find novel potential therapeutic targets. In this case, the mice genome is modified in order to assess the role of a specific gene or to obtain a disease phenotype resembling the human condition, through gene deletion

and/or by modulating its expression (Dothel et al., 2013). The main limitations to the application of these models is the lack of insight into diverse mechanisms produced by the initial genetic modification as well as the lack of knowledge of compensatory mechanisms activated by the organism (Wood et al., 2000).

One of the earliest models was represented by mice with IL-10 deficiency: in humans, genetic polymorphisms of the IL-10 locus confer increased risk of both UC and CD (Kuhn et al., 1993; Franke et al., 2008 and 2010). Targeted deletion of IL-10 (*Il10*$^{-/-}$) induced spontaneous inflammation of the colon characterised by the presence of an inflammatory infiltrate of lymphocytes, macrophages and neutrophils (Kuhn et al., 1993). The inflammation is initially driven by pro-inflammatory Th1 responses, while during disease progression there is an increased production of Th2 cytokines (Berg et al., 1996; Spencer et al., 2002).

Concluding, even though experimental models can mimic various aspects of human IBD, no single colitis model can fully recapitulate IBD complexity; therefore, up to now, the concomitant use of different experimental models represents the only way to increase the translational value of preclinical studies and to identify therapeutic agents active in humans as well.

AIM

Since the discovery that several Eph receptors and ephrin ligands are TNF-α responsive genes, lots of studies have been performed to clarify the role of Eph/ephrin system in inflammation. Thanks to Ephs/ephrins expression in tissues like blood and lymphatic vessels, on immune cells and on epithelial barriers, and to their ability to signal with a broad spectrum of partners, this pathway seems to represent a central player of inflammatory processes.

However, despite the knowledge that the Eph/ephrin system is expressed at the intestinal level and the findings by Hafner demonstrating the increased transcriptional levels of some members of this family in mucosal lesions of IBD patients (Hafner et al., 2005), little research has been performed in the field of intestinal inflammation. Moreover, data obtained by Kitamura on rheumatoid arthritis have proved that Ephs/ephrins can modulate the activation of recruited T cells, thus representing a potential target for the treatment of a chronic inflammatory disease like RA (Kitamura et al., 2008).

Based on these premises and with the final goal of identifying a new therapeutic target for IBD treatment, the aim of this project was to investigate the role of Eph/ephrin system in intestinal inflammation through the application of three different experimental models of murine colitis. That would allow to mimic more precisely the human pathological condition and to identify the specific mechanism of action underlying the effects produced in vivo by the pharmacological modulation of the Eph/ephrin system.

In the first part of the study, an already well characterised model of TNBS colitis was used (Grandi A et al., 2017), and the role of the B class of the Eph/ephrin system was evaluated. Colitic mice were administered with equimolar doses of agonists, EphB1-Fc, activating B-*reverse* and blocking B-*forward* signalling, ephrinB1-Fc, activating B-*forward* and conversely blocking B-*reverse* signalling (Barquilla and Pasquale, 2015), or with monomeric protein EphB4, supposedly able to block both B-signalling pathways (Kertesz et al., 2006).

In the second part of the project, DSS models of acute and chronic colitis were developed: the sex of mice to be used, the dosage and the period of DSS

administration as well as the number of DSS and wash-out cycles to be performed were investigated to obtain two reproducible models of moderate intestinal inflammation in acute and chronic conditions. Moreover, a drug, currently used in therapy for the treatment of IBDs, was selected as positive control for acute DSS colitis. Subsequently, the effects produced by the pharmacological manipulation of the Eph/ephrin system in acute DSS were investigated: to this end, besides the treatments already tested in TNBS colitis model and focussed on B-type proteins, the responses evoked by equimolar doses of agonists, EphA2-Fc and ephrinA1-Fc, activating respectively A-*reverse* and -*forward* signalling, and of the monomeric protein EphA2, presumably acting as EphA-ephrinA antagonist, were assessed. Finally, given the positive results obtained through modulation of B-type proteins in TNBS-induced colitis, the same treatments were tested in chronic colitis evoked by DSS administration.

The same clinical parameters were considered in the three different models: disease activity index, indicating the severity of the colitis induced, colonic macroscopic score, length and thickness as marker of local damage were evaluated. Moreover, myeloperoxidase levels within the colon and the lungs were used as index of neutrophils infiltration and thus as a sign of local and systemic damage respectively. To characterise the immune response in the three different models, flow cytometric analysis of lymphocytes subpopulations, obtained from spleen and mesenteric lymph nodes, was performed.

METHODS

ANIMALS

Male and female C57BL/6 mice (8-12 weeks old) (Charles River Laboratories, Calco, Italy), weighing 20-24g, were housed, five per cage, and maintained under standard conditions at our animal facility (12:12 h light–dark cycle, 22-24°C, food and water available *ad libitum*). Experimental procedures were conducted between 9:00 a.m and 12:00 a.m and all efforts were made to minimize animals' suffering. Animal experiments were performed according to the guidelines for the use and care of laboratory animals and they were authorized by the local Animal Care Committee "Organismo Preposto al Benessere degli Animali" and by Italian Ministry of Health "Ministero della Salute" (DL 26/2014).

INDUCTION AND ASSESSMENT OF COLITIS

TNBS COLITIS INDUCTION

Female mice, fasted for 20 hours with free access to water containing 5% glucose, were anaesthetised (isoflurane 2%) and a 10 cm long PE-50 tubing attached to a syringe was inserted 4 cm into the anus. Colitis was induced by enema administration of 50 µL of a 10% (w/V) 2,4,6-TrinitroBenzene Sulfonic acid (TNBS) solution (5mg/mouse) in 50% ethanol in mice kept in a head-down position for 3 minutes to avoid leakage of the intracolonic instillate.

Subcutaneous (s.c.) pharmacological treatments started 8 hours after the induction of colitis and were applied daily or twice daily until euthanasia, 3 days later *[Table 2]*.

DSS COLITIS INDUCTION

Dextran Sulphate Sodium (DSS) colitis was induced giving female or male animals free access to a 3% or 4% w/V DSS solution in drinking water for 5 or 7 days (1 cycle) -***acute DSS***- or for 3 cycles, of 5 days each, interrupted by 9 days of drinking water, wash out period, -***chronic DSS***-. Sham mice received drinking water. Animals were euthanized by CO_2 inhalation on day 8, acute DSS, or on day 33, chronic colitis *[Table 2]*.

COLITIS ASSESSMENT

Disease Activity Index (DAI), measured daily throughout the experiment, was assessed by unaware investigators. Body weight, stools consistency and rectal bleeding were

considered. Immediately after euthanasia, the macroscopic damage of colonic mucosa was assessed as macroscopic score (MS). The weight and the length of each colon were measured, and the weight/length ratio was evaluated as parameter of intestinal wall thickening (Bischoff et al., 2009). Colon, lungs, spleen and mesenteric lymph nodes were collected for subsequent biochemical or flow cytometry analyses. Spleen weight was measured and normalized with respect to body weight (BW): the ratio was expressed as spleen(mg)*1000/BW(g).

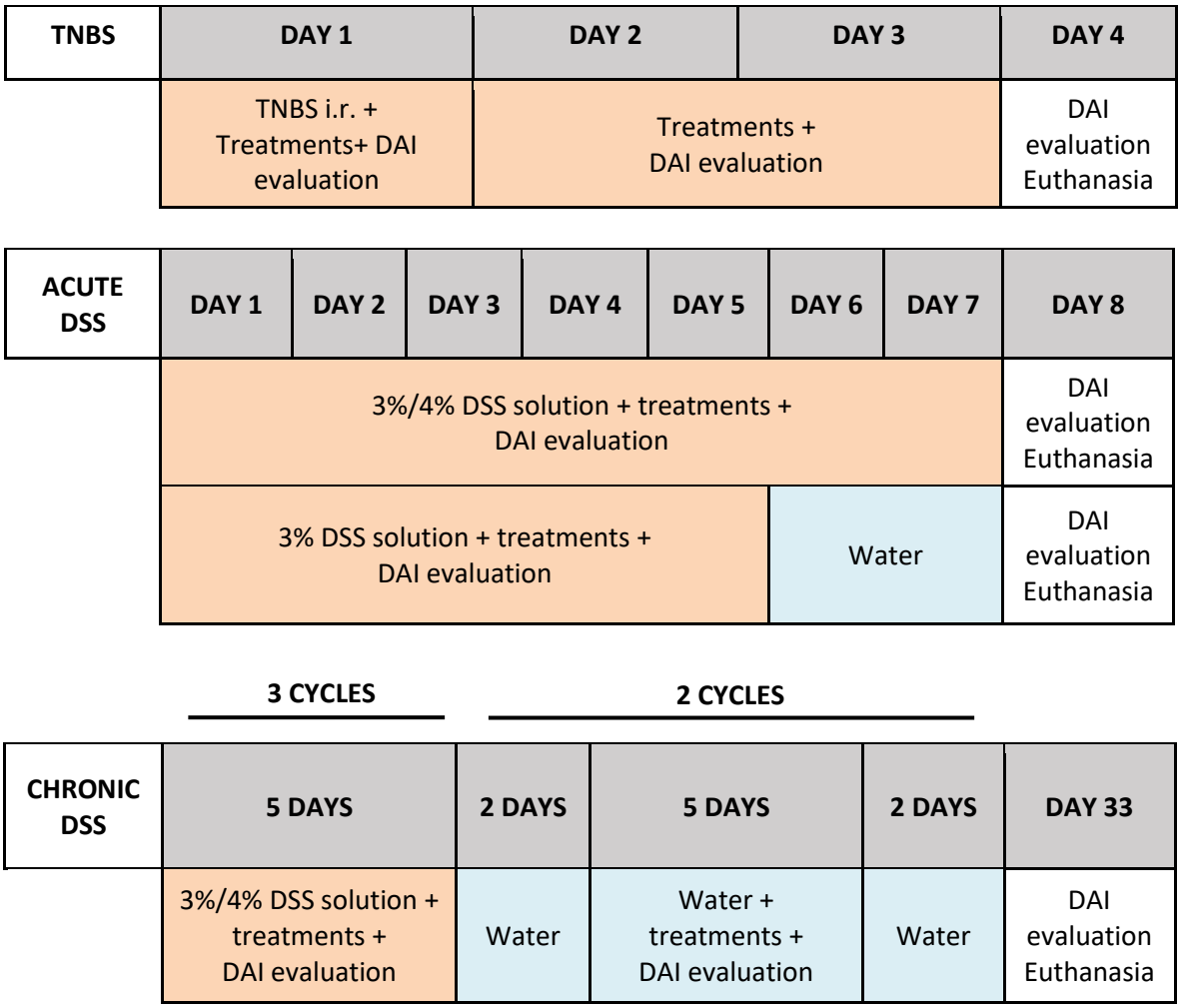


Figure 12. Schematic representation of TNBS and DSS experimental protocols.

PHARMACOLOGICAL TREATMENTS

Pharmacological treatments started 8 hours after TNBS colitis induction, on day 1 for acute DSS colitis, and on day 8 for chronic DSS colitis and were applied once daily by subcutaneous (s.c.), intraperitoneal injection (i.p.) or oral administration (os). Animals were randomly divided in the following different experimental groups (n=6-10):

- *Sham or S:* 0.9% NaCl (saline) i.r. or drinking water + saline 10 ml/kg/die s.c.
- *TNBS:* TNBS i.r.+ saline s.c.
- *3% 7 d F:* 7 days 3% DSS + saline s.c., female mice
- *4% 7 d F:* 7 days 4% DSS + saline s.c., female mice
- *3% 7 d M:* 7 days *3*% DSS + saline s.c., male mice
- *4% 7 d M:* 7 days 4% DSS + saline s.c., male mice
- *3% 5+2 d F or DSS A:* 5 days 3% DSS + 2 days of wash out + saline s.c., female mice, selected acute protocol
- *3% 5+2 d M:* 5 days 3% DSS + 2 days of wash out + saline s.c., male mice
- *3% C or DSS C:* 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out + saline s.c., female mice, selected chronic protocol
- *4% C:* 3 cycles of 5 days 4% DSS + 2 cycles of 9 days water + saline s.c., female mice
- *EphrinB1-Fc:* TNBS i.r. or acute DSS/chronic DSS + ephrinB1-Fc 17 µg/kg/die s.c.
- *EphB1-Fc:* TNBS i.r. or acute DSS/chronic DSS + EphB1-Fc 30 µg/kg/die s.c.
- *EphB4:* TNBS i.r. or acute DSS/chronic DSS + EphB4 20 µg/kg/die s.c.
- *CsA:* DSS (acute protocol) + cyclosporine A 25 mg/kg/die ip
- *CsA os*: DSS (acute protocol) + cyclosporine A 25 mg/kg/die os
- *Sulfa*: DSS (acute protocol) + Sulfasalazine 50 mg/kg/die s.c.
- *EphrinA1-Fc:* DSS (acute protocol) + ephrinA1-Fc 16 µg/kg/die s.c.
- *EphA2-Fc:* DSS (acute protocol) + EphA2-Fc 30 µg/kg/die s.c.
- *EphA2:* DSS (acute protocol) + EphA2-Fc 20 µg/kg/die s.c.

EVALUATION OF INFLAMMATORY PARAMETERS

DISEASE ACTIVITY INDEX (DAI)

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

The scores were quantified as follows:

- Stool consistency: 0 (normal), 1 (soft), 2 (liquid);
- Rectal bleeding: 0 (no bleeding), 1 (light bleeding), 2 (heavy bleeding)
- Body weight loss: 0 (<5%), 1 (5–10%), 2 (10–15%), 3 (15–20%), 4 (20–25%), 5 (>25%).

The maximum reachable score was 9.

MACROSCOPIC SCORE (MS)

After euthanasia, the colon was explanted, opened longitudinally, cleaned and the macroscopic score was immediately assigned 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 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 haemorrhage (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).

The maximum reachable score was 12.

COLONIC LENGTH AND THICKNESS

To evaluate the deposition of fibrotic material induced by the inflammatory state, the length of colon and its weight were measured; moreover weight (mg)/length (cm) ratio was calculated to estimate colon thickness (Bischoff et al., 2009).

COLONIC AND PULMONARY MYELOPEROXIDASE ACTIVITY (MPO)

Myeloperoxidase (MPO) is a heme-containing peroxidase expressed mainly in neutrophils and monocytes able to catalyze the formation of reactive oxygen intermediates, as hypochlorous acid (HOCl): the MPO/HOCl axis plays an important role in microbial killing. Furthermore, MPO is a local mediator of tissue damage and it is abundantly released in inflamed tissues. Myeloperoxidase activity becomes therefore a marker of tissue neutrophil infiltration; in this work it was determined according to Krawisz's modified method (Krawisz et al., 1984). After being weighed, colon and lung samples were homogenized in ice-cold potassium phosphate buffer (100 mM, pH 7.4) containing aprotinin 1 µg/mL and centrifuged for 20 min at 10000 rpm at 4°C. The obtained pellets were re-homogenized in five volumes of ice-cold potassium phosphate buffer (50 mM, pH 6) containing 0.5% hexadecylthrimethyl-ammonium bromide (HTAB) and aprotinin 1 µg/mL. The samples were frozen (10 minutes in liquid nitrogen) and thawed (15 minutes in 37°C water bath) for three cycles, and then centrifuged for 30 min at 12000 rpm at 4°C. 100 µL of the supernatant was then made to react with 900 µL of a buffer solution containing o-Dianisidine (0.167 mg/mL) and 0.0005% H_2O_2.

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, one unit of MPO was defined as the quantity of enzyme able to degrade 1 µmol of peroxide per minute at 25°C. Data were normalized with respective colon or lungs oedema values [(wet weight-dry weight)/dry weight] and expressed as U/g of dry weight tissue.

SPLEEN/BODY WEIGHT RATIO (SP/BW)

Immediately after euthanasia the spleen (SP) was weighed and the weight was normalized with respect to animal body weight (BW): the ratio was expressed as spleen(mg)*1000/BW(g). This parameter was used as a marker of systemic inflammation.

ISOLATION OF SPLENOCYTES AND MESENTERIC LYMPH NODES

Spleen and mesenteric lymph nodes were removed immediately after euthanasia, cleaned from adipose tissue and mechanically broken up through a 100 µm cell-strainer, and washed respectively with PBS containing 0.6 mM EDTA (PBS-EDTA) or with *Hank's Balanced Salt Solution* (HBSS). The suspensions were centrifuged at 1000 rpm for 10 minutes at 4°C, and the pellet re-suspended in PBS-EDTA and HBSS respectively. Spleen suspensions were 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 dark, to get rid of erythrocytes; while nothing was added in mLNs suspensions. Samples were then centrifuged at 1000 rpm for 10 minutes at 4°C, the spleen pellet was washed with PBS-EDTA and re-suspended in 5 mL of cell staining buffer (PBS containing 0.5% fetal calf serum (FCS) and 0.1% sodium azide), while mLNs were re-suspended in 3 mL of cell staining buffer. The obtained single cell suspensions were stained with fluorescent antibodies.

IMMUNOFLUORESCENT STAINING AND FLOW CYTOMETRY ANALYSIS

In order to avoid non-specific binding, before the incubation with fluorescent antibodies, 200 µL of spleen or mLNs suspension was incubated with IgG1-Fc (1µg/10^6 cells) for 10 minutes at 4°C in the dark.

We used the subsequent antibodies: Phycoerythrin-Cyanine 5 (PE-Cy5) conjugated anti-mouse CD3ε (0.25 µg/106 cells) emitting red fluorescence, Fluorescein Isothiocyanate (FITC) anti-mouse CD4 (0.25 µg/106 cells) emitting green fluorescence and PE anti-mouse CD8a (0.25 µg/106 cells) emitting yellow fluorescence. Cells were incubated for 1 hour at 4°C in the dark, washed with PBS to remove excessive antibodies, centrifuged and resuspended in cell staining buffer to perform flow cytometry (FACS) analysis.

Cells viability was assessed using propidium iodide (PI): PI is a red fluorescent dye not permeant to live cells, thus able to bind DNA only in apoptotic or dead cells. Cells were incubated with 10 µg/mL PI for 1 minute in the dark, at room temperature, immediately prior to FACS analysis.

Samples were analysed using Guava *easyCyte*^{TM} and *InCyte*^{TM} software (Merck Millipore, Darmstadt, Germany). Lymphocytes were identified on the basis of their size in the *Forward Scatter* (FSC)-*Side Scatter* (SSC) plot (FSC low, SSC low), and T cells' number was determined by selecting $CD3^+$ T cells. T-cells subpopulations were determined by measuring the number of $CD4^+$ and $CD8^+$ cells within $CD3^+$ lymphocytes. Cells viability was determined by assessing PI^- cells; all PI^+ lymphocytes were excluded from the analysis.

STATISTICS

All data are presented as means ± SEM. Comparisons among experimental groups were made using the analysis of variance (one-way or two-way ANOVA) followed by Bonferroni's post-test or using unpaired Student's t-test. $P<0.05$, $P<0.01$, and $P< 0.001$ were considered, respectively, statistically significant, highly significant, or extremely highly significant. Statistical analysis was performed using Prism 4 software (GraphPad Software Inc. San Diego, CA, USA).

DRUGS, ANTIBODIES AND REAGENTS

TNBS, ethanol, HTAB and 30% hydrogen peroxide were purchased from Sigma Aldrich® (St. Louis, MO) while DSS was bought from MP Biomedicals® (Germany). Fc-recombinant proteins (ephrinA1/B1 and EphA2/B1) were purchased from R&D system® (Minneapolis, MN); whereas EphA2 and EphB4 from Life Technologies® (Carlsbad, CA).

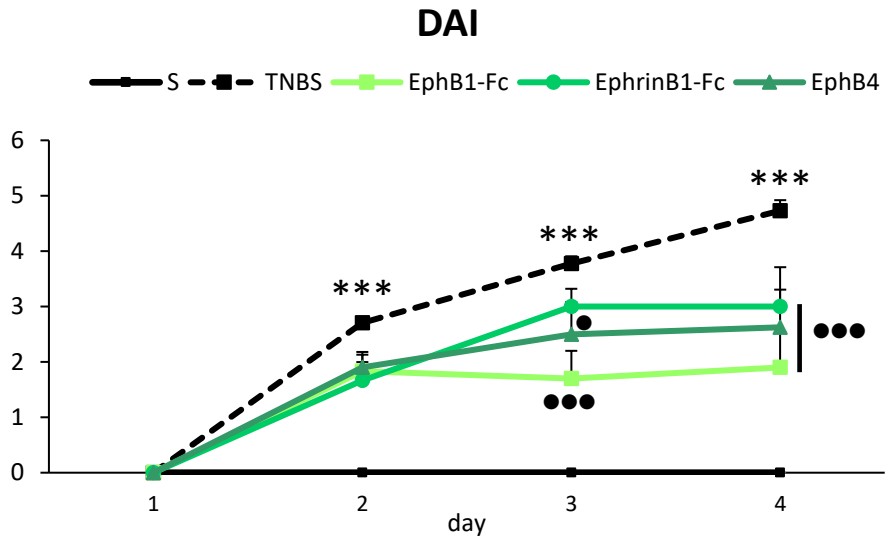
Fluorescent antibodies used for flow cytometry (FITC anti-mouse CD4, PE anti-mouse CD8) and Propidium Iodide were purchased from BioLegend® (San Diego, CA), while PE-Cy5 anti-mouse CD3ε from affymetrix eBioscience® (San Diego, CA).

RESULTS

TNBS COLITIS: Eph/ephrin system involvement

In the first part of the project the TNBS model of colitis was used to assess whether the pharmacological modulation of EphB-ephrinB signalling could ameliorate the haptenizing agent-induced inflammation. Female C57BL/6 mice were challenged with TNBS administration and treated with saline (control group-TNBS) or with EphB1-Fc, activating *reverse* signalling, ephrinB1-Fc, activating *forward* signalling, and the monomeric receptor EphB4, purportedly blocking the bidirectional signalling. Local and systemic inflammatory parameters were analysed; in addition, flow cytometric analysis of spleen and mesenteric lymph nodes was performed in case of effective treatments.

DISEASE ACTIVITY INDEX



Graph 1: Disease activity index -DAI

Disease activity index assessed in vehicle-treated normal mice (S) and in TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group).

**** P<0.001 vs S; ●●● P<0.001, ● P <0.05 vs TNBS.*

Two-way ANOVA + Bonferroni's post test

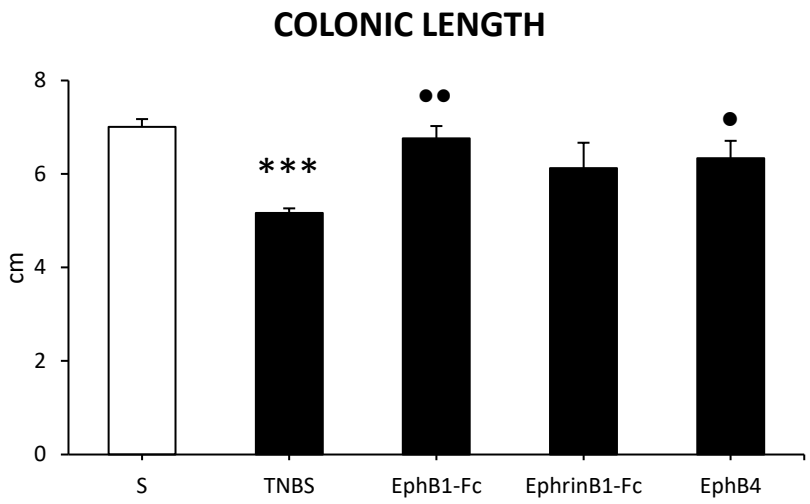
Disease activity index (DAI) was evaluated daily starting from the day of TNBS instillation as index of the severity of the colitis induced. For each animal, weight loss and the presence of soft stools or diarrhoea was scored and added up to obtain DAI

score. In *Graph 1* DAI score for each group is reported day by day until the day of euthanasia. As shown, in sham animals DAI remained 0 throughout the whole period, whilst TNBS group marked the highest score on days 2-3-4, sign of the extremely significant worsening of animals' condition with respect to S mice ($P<0.001$). All the treatments were effective in strongly reducing DAI score at day 4 compared to TNBS group ($P<0.001$ vs TNBS); notably EphB1-Fc ($P<0.05$) and EphB4 ($P<0.001$) lowered this parameter already at day 3.

MACROSCOPIC SCORE

Macroscopic score was determined right after colon removal. The presence of ulcers, erythema and fibrosis contributed to grade the severity of the damage induced by TNBS administration. Sham animals scored 0, while TNBS group score was highly significantly increased to 3.49±0.28 ($P<0.001$ vs S). All the treatments were able to prevent mucosal injury: besides ephrinB1-Fc, which reduced the macroscopic damage score to 2.75±0.63 ($P<0.01$ vs S), especially EphB1-Fc and EphB4 exerted a clear mucosal protection, as shown by the respective score values of 1.30±0.63 and 1.20±0.73 ($P<0.05$ and $P<0.01$ vs TNBS).

COLONIC LENGTH

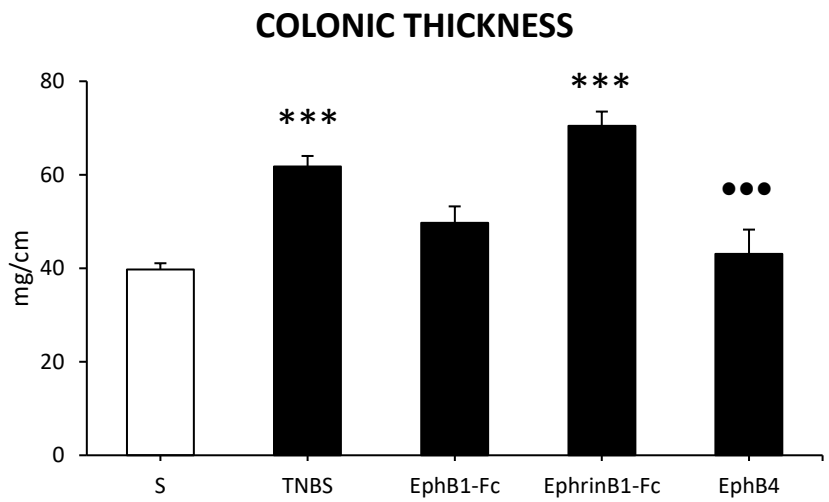


Graph 2: Colonic length

*Colonic length assessed in vehicle-treated normal mice (S) and in TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001 vs S; ●● P<0.01, ● P <0.05 vs TNBS. One-way ANOVA + Bonferroni's post test*

Colitis induced a strong reduction of colonic length in TNBS group compared to S animals (P<0.001 vs S) *(Graph 2)*. Colonic shortening was prevented by EphB1-Fc and EphB4 administration in a significant way (P<0.01, P<0.05 vs S respectively), whilst ephrinB1-Fc protection did not reach statistical significance.

COLONIC THICKNESS

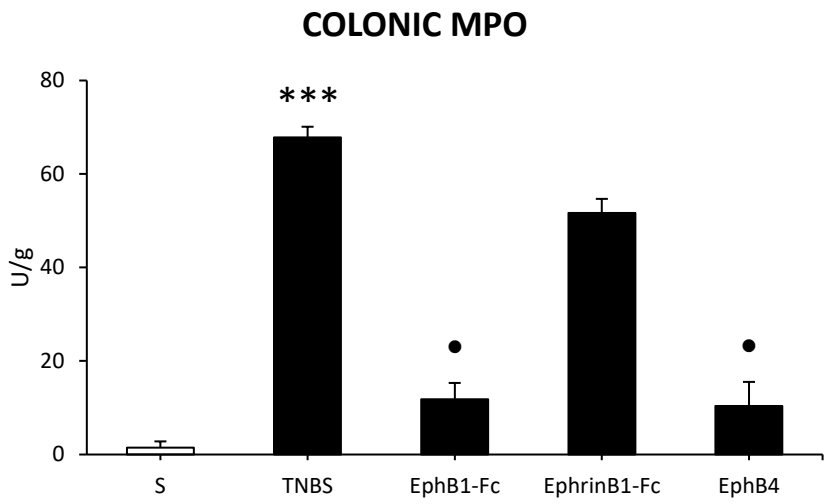


Graph 3: Colonic thickness

*Colonic thickness assessed in vehicle-treated normal mice (S) and in TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001 vs S; ●●●P<0.001 vs TNBS. One-way ANOVA + Bonferroni's post test*

A striking thickening of colonic wall was observed in control animals (P<0.001) *(Graph 3)*. EphB4 administration strongly counteracted colonic thickness increase (P<0.001), that was only moderately controlled by EphB1-Fc administration. By opposite, ephrinB1-Fc was completely ineffective and reached thickening values similar to those of TNBS group.

COLONIC MYELOPEROXIDASE ACTIVITY



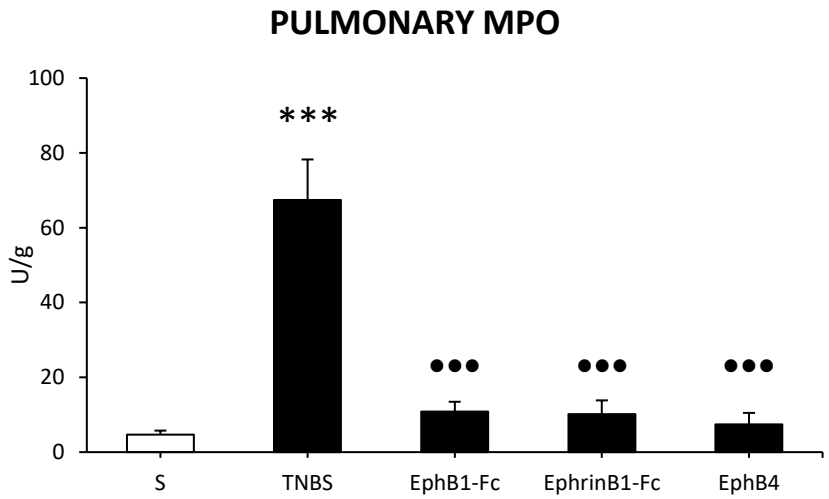
Graph 4: Colonic MPO

*Myeloperoxidase (MPO) activity assessed in the colon of vehicle-treated normal mice (S) and of TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001 vs S; ● P <0.05 vs TNBS.*

One-way ANOVA + Bonferroni's post test

Myeloperoxidase (MPO) activity is a parameter used to estimate the infiltration of neutrophils within the inflamed tissue (*Graph 4)*. Colitis induction triggered a massive recruitment of neutrophils within the colon in TNBS group (P<0.001 vs S). This infiltration was strongly curtailed by EphB1-Fc and EphB4 in a similar way (P<0.05 vs TNBS), whilst ephrinB1-Fc administration only weakly reduced neutrophils recruitment.

PULMONARY MYELOPEROXIDASE ACTIVITY



Graph 5: Pulmonary MPO

*Myeloperoxidase activity assessed in the lungs of vehicle-treated normal mice (S) and of TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001 vs S; ●●● P <0.001 vs TNBS*

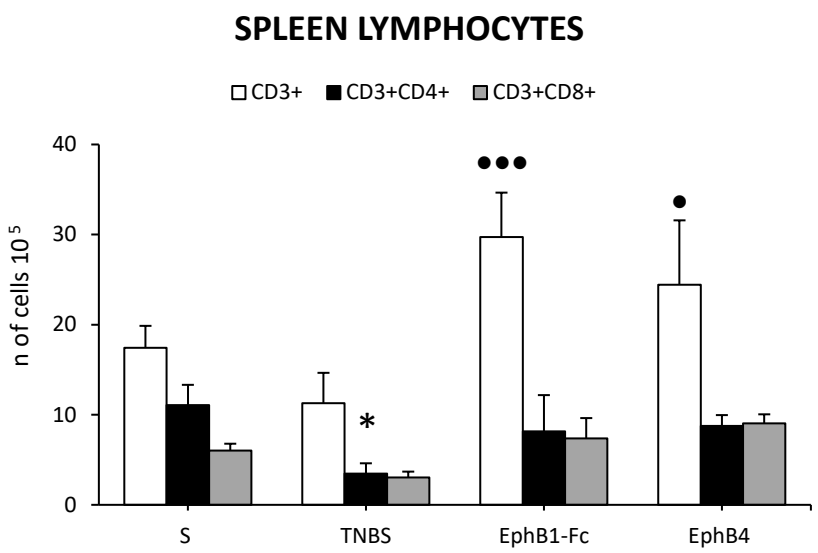
One-way ANOVA + Bonferroni's post test

The evaluation of MPO levels within the lungs can give information about the systemic inflammatory state consequent to colitis induction. Looking at *Graph 5,* it is clear that inflammation-induced neutrophils recruitment was exacerbated in TNBS group (P<0.001 vs S) and effectively limited by all the treatments (P<0.001 vs TNBS).

SPLEEN/BODY WEIGHT RATIO

Similarly to pulmonary MPO levels, this parameter is useful to assess the systemic level of inflammation in mice. TNBS mice showed a slight increase in SP/BW ratio (P<0.05 vs S), while in the other groups no clear differences were found, either compared to S or to TNBS mice (data not shown).

SPLENIC LYMPHOCYTES



Graph 6: Spleen lymphocytes

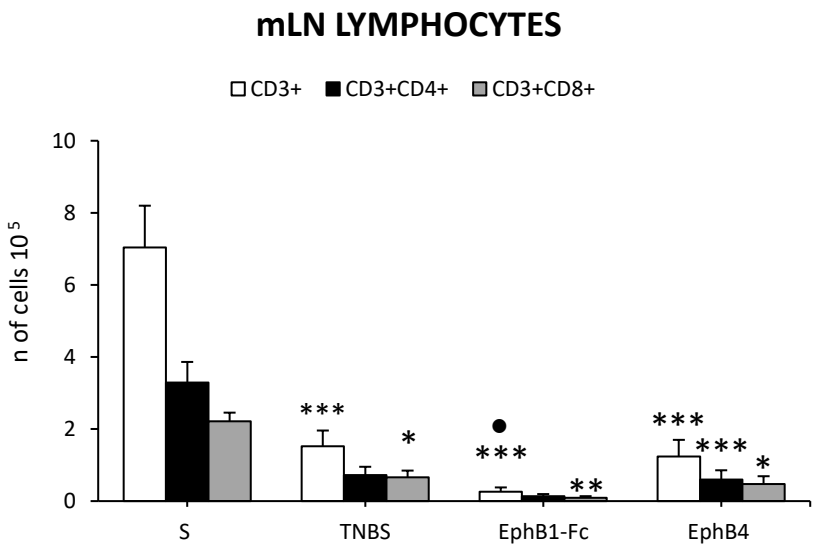
Number of splenic T cells assessed in vehicle-treated normal mice (S) and in TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc or EphB4 (n=6-10 values per group).

** P<0.05 vs S; ●●● P<0.001, ● P<0.05 vs TNBS. One-way ANOVA + Bonferroni's post test*

Flow cytometry is a technique that allows the identification of different cells subtypes in a complex population. In *Graph 6* $CD3^+$ (T lymphocytes), $CD3^+CD4^+$ (T helper cells) and $CD3^+CD8^+$ (T cytotoxic cells) subpopulations, obtained from spleens of mice belonging to the different experimental groups, are presented.

Colitis was responsible for a decrease in the number of T cells subpopulations compared to sham animals, particularly evident as regards T helper cells ($P<0.05$ vs S). In EphB1-Fc and EphB4 groups, T cells reduction was counteracted by the treatment with recombinant proteins: the number of $CD3^+$ T cells was increased compared to S animals in both groups ($P<0.001$ and $P<0.05$ vs S respectively), while the number of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was restored to levels comparable to those of Sham animals.

MESENTERIC LYMPH NODES T CELLS



Graph 7: mLN lymphocytes

*Number of mesenteric lymph nodes (mLN) T cells assessed in vehicle-treated normal mice (S) and in TNBS-treated mice administered with vehicle (TNBS), EphB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001, **P<0.01, * P<0.05 vs S; ● P<0.05 vs TNBS*

One-way ANOVA + Bonferroni's post test

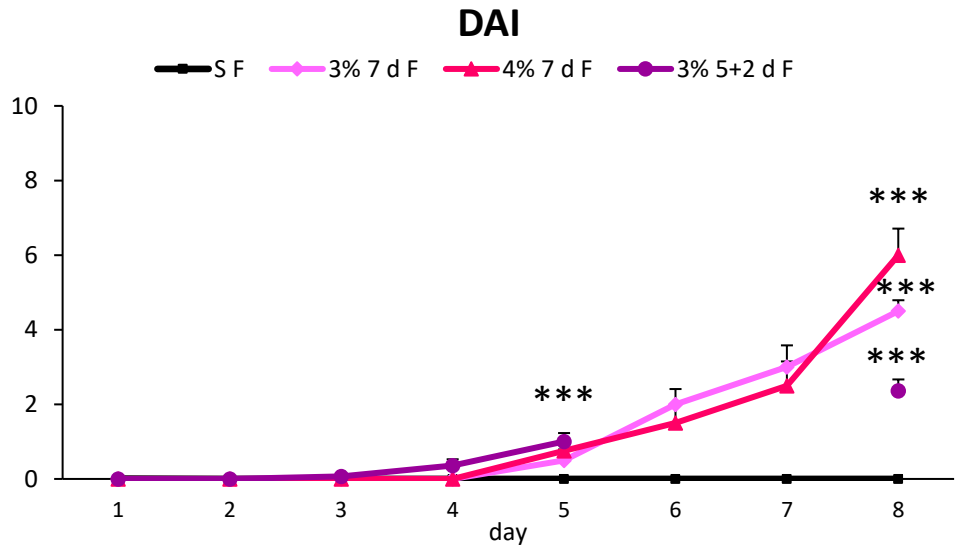
In *Graph 7*, the different subpopulations of lymphocytes determined in mesenteric lymph nodes (mLNs) are represented. Similarly to spleen results, in TNBS group the number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was strongly reduced following colitis ($P<0.001$) but none of the treatments was able to rescue them to Sham values. The number of $CD3^+$ cells detected in EphB1-Fc was even lower than those of TNBS mice ($P<0.05$).

DSS MODELS DEVELOPMENT

In the second part of the project, suitable models for acute and chronic DSS colitis were developed. Firstly, the concentration of DSS solution, the timing of administration and the sex of mice to be used in the acute protocol were investigated. Secondly, drugs, already in use for the treatment of humans IBDs, were tested as positive control. Thirdly, the chronic DSS protocol was developed in female animals.

ACUTE DSS MODEL -female mice

DISEASE ACTIVITY INDEX



Graph 8: Disease activity index-DAI

*Disease activity index assessed in female water-treated normal mice (S F) and in DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group). *** P<0.001 vs S*

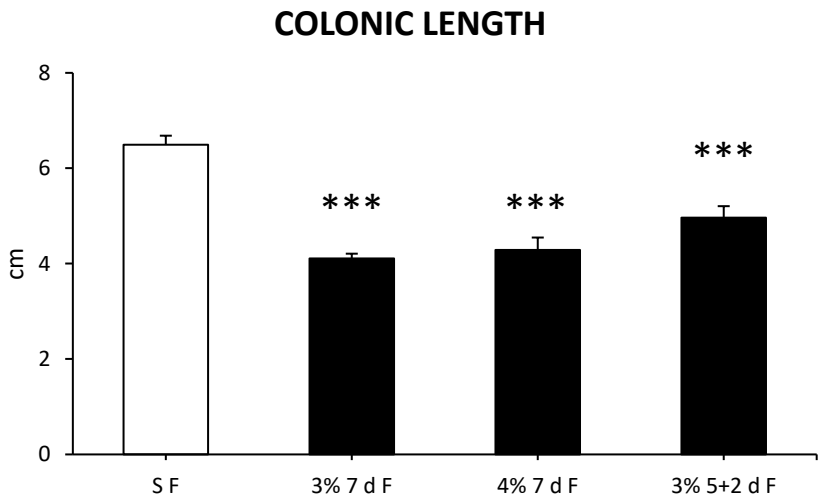
Two-way ANOVA + Bonferroni's post test

Graph 8 shows the DAI score from day 1 to day 8 of DSS exposure.

Administration of DSS to female mice for 7 days induced a similar profile of severity at 3% (3% 7d F) and 4% (4% 7d F): it peaked at day 5 and culminated at day 8 showing a highly significant increase (P<0.001) of DAI value. 3% 5+2d group received 5 days of 3% DSS solution followed by 2 days of wash out (water administration). At day 5, DAI score

of this group overlapped that of the others two groups, while it was lower at day 8, but still significantly higher than S value.

COLONIC LENGTH



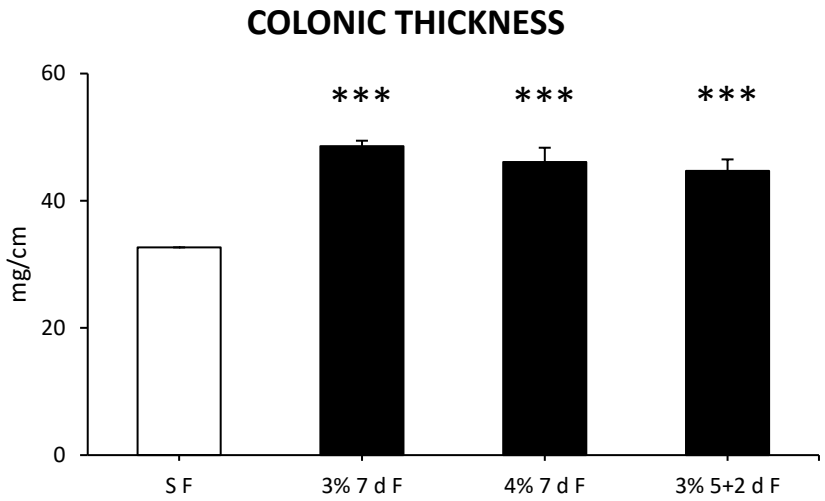
Graph 9: Colonic length

*Colonic length assessed in female water-treated normal mice (S F) and in DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group). *** P<0.001 vs S*

One-way ANOVA + Bonferroni's post test

Colon length was strongly reduced by the administration of 3% DSS solution for 7 days with respect to sham mice (P<0.001). Increasing the concentration of DSS to 4% or abbreviating the period of administration to 5 days did not further worsen colon shortening with respect to 3% 7d group (P<0.001 vs. S).

COLONIC THICKNESS



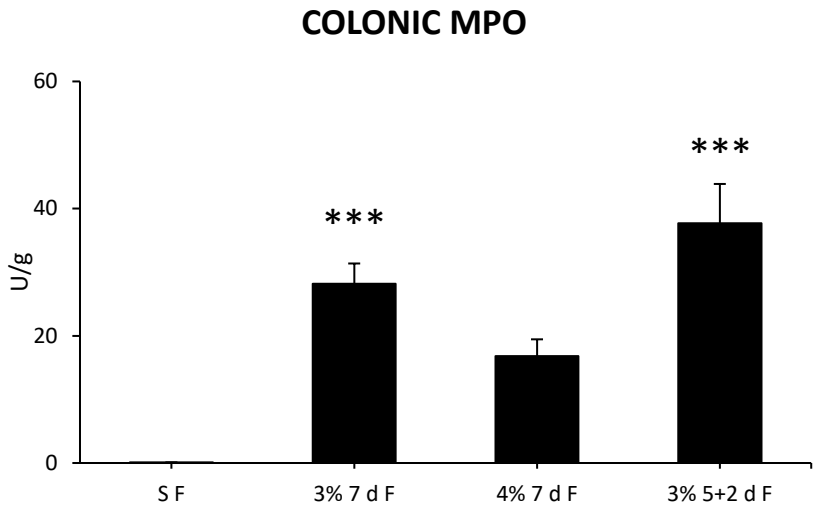
Graph 10: Colonic thickness

*Colonic thickness assessed in female water-treated normal mice (S F) and in DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group). *** P<0.001 vs S*

One-way ANOVA + Bonferroni's post test

As seen in *Graph 9* for colonic length, colonic thickness was strongly increased upon DSS administration in a similar way in the three groups regardless of whether the concentration of DSS was increased or the period of administration reduced (P<0.001 vs S) (*Graph 10*).

COLONIC MYELOPEROXIDASE ACTIVITY



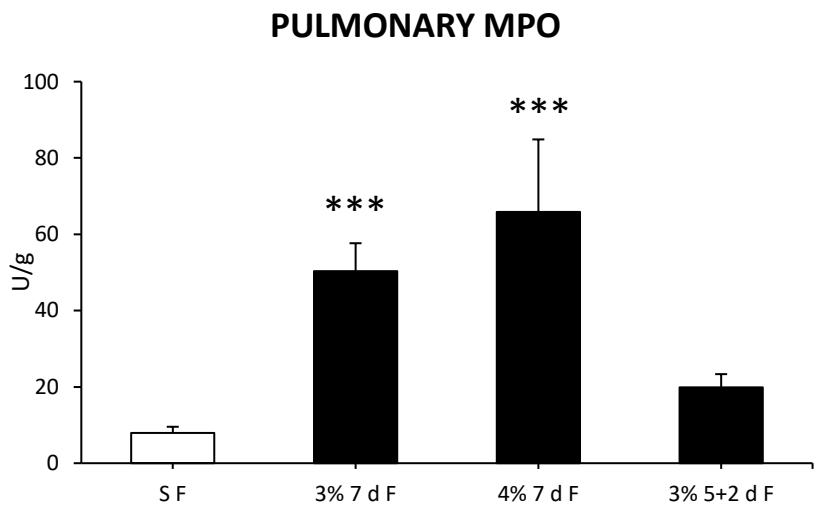
Graph 11: Colonic MPO

Myeloperoxidase (MPO) activity assessed in the colon of female water-treated normal mice (S F) and of DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group).

**** P<0.001 vs S. One-way ANOVA + Bonferroni's post test*

As in TNBS colitis model, DSS administration led to a firm increase of neutrophils recruitment (*Graph 11*). Surprisingly the highest levels of MPO activity were obtained for 3% 5+2 d group (P<0.001 vs S), while the lowest for 4% 7d F group, a value that was not statistically different from S animals.

PULMONARY MYELOPEROXIDASE ACTIVITY



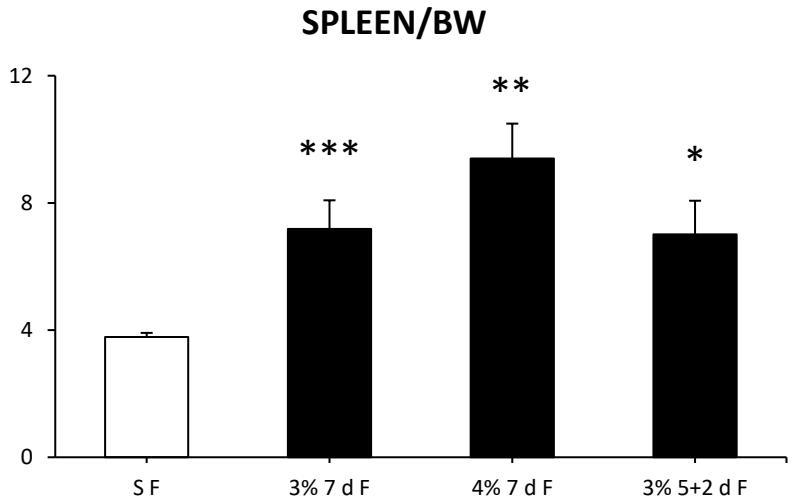
Graph 12: Pulmonary MPO

Myeloperoxidase (MPO) activity assessed in the lungs of female water-treated normal mice (S F) and of DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group).

**** P<0.001 vs S. One-way ANOVA + Bonferroni's post test*

DSS administration increased MPO levels in the lungs, suggesting the presence of systemic inflammation also for this acute model (*Graph 12*). 7 days of DSS administration led to values of neutrophils infiltration higher than those obtained in the shorter period of 5 days and remarkably increased compared to S group (P<0.001).

SPLEEN/BODY WEIGHT RATIO



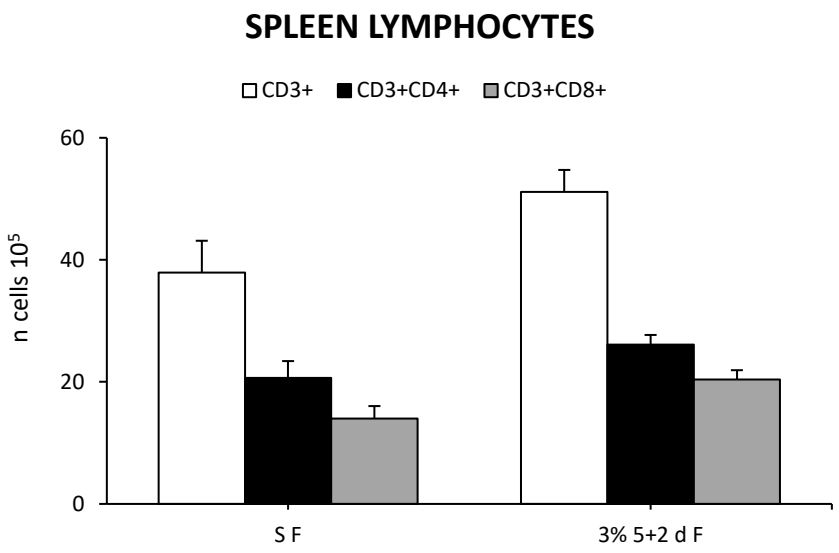
Graph 13: Spleen/BW

Spleen/body weight ratio assessed in female water-treated normal mice (S F) and in DSS-treated mice: 3% DSS for 7 days (3% 7 d F), 4% DSS for 7 days (4% 7 d F) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group).

**** P<0.001, ** P<0.01, * P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

Spleen dimension and weight were increased upon DSS administration at day 8 compared with water-treated mice but no clear differences appeared among the three groups of mice exposed to the colitogenic agent.

SPLENIC LYMPHOCYTES

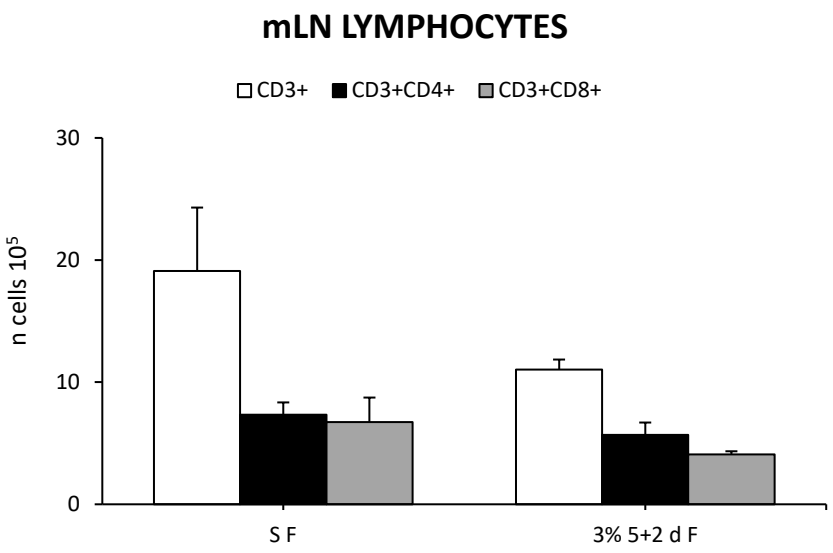


Graph 14: Spleen lymphocytes

Number of splenic T cells assessed in female water-treated normal mice (S F) and in 3% DSS-treated mice for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group).

Lymphocytes subpopulations are reported in *Graph 14* only for S animals and for 3% 5+2d group, that was selected as acute DSS protocol. Changes in T cells subpopulations are representative of the involvement of adaptive immune system. In our case, no significant differences in the number of $CD3^+$ T cells and their T helper and T cytotoxic subtypes were observed between the two groups.

MESENTERIC LYMPH NODES T CELLS



Graph 15: mLN lymphocytes

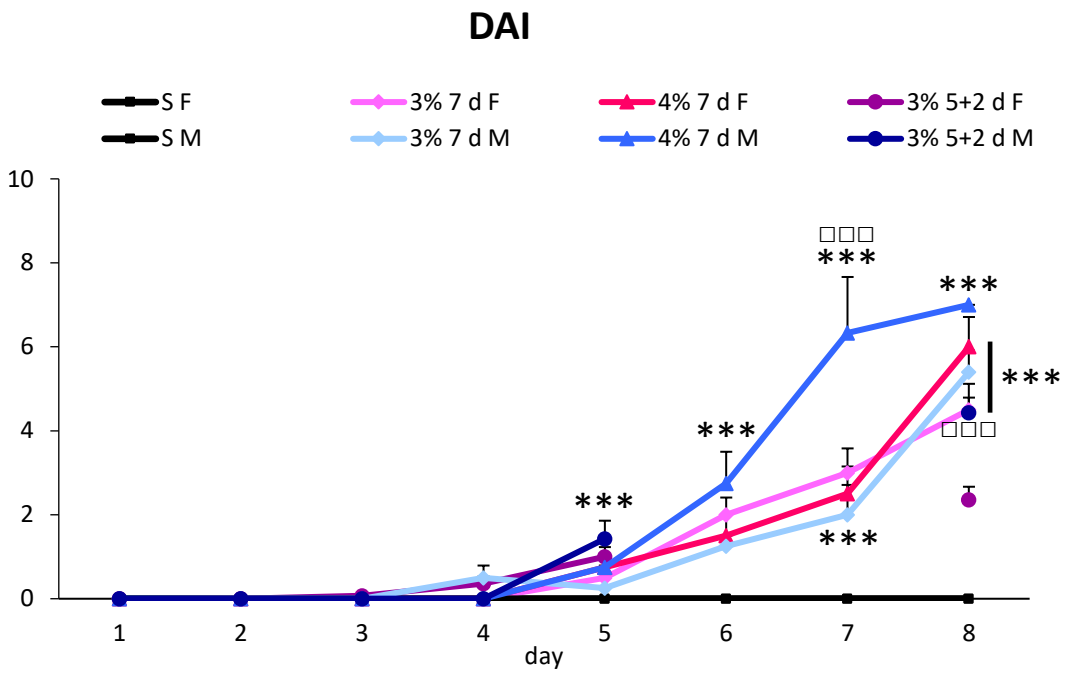
Number of mesenteric lymph nodes (mLN) T cells assessed in female water-treated normal mice (S F) and in 3% DSS-treated mice for 5 days followed by 2 days of water (3% 5+2 F) (n=5-7 values per group).

Similarly to spleen T cells subpopulations, also in mesenteric lymph nodes, no differences were noticed between Sham and colitic animals as regards the different adaptive cell types.

ACUTE DSS MODEL-male mice

To assess whether there was a different susceptibility to DSS colitis between male and female animals, the same acute DSS protocols tested in females were tested in male mice.

DISEASE ACTIVITY INDEX



Graph 16: Disease activity index-DAI

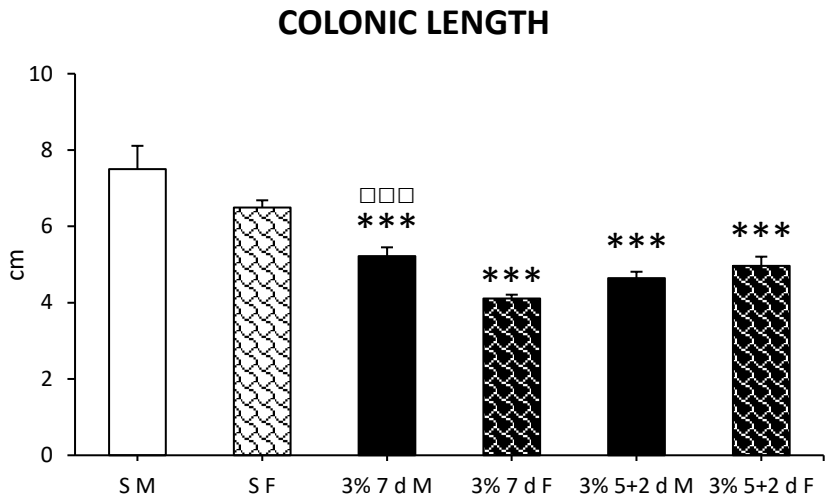
*Disease activity index assessed in male water-treated normal mice (S M) and in DSS-treated mice, 3% DSS for 7 days (3% 7 d M), 4% DSS for 7 days (4% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M), compared to respective female groups (n=5-7 values per group). *** P<0.001vs S; □□□ P<0.001 vs respective F group*

Two-way ANOVA + Bonferroni's post test or Student's unpaired t test

Starting from day 5, DAI score was extremely increased in the three groups receiving DSS with respect to vehicle-treated mice (P<0.001 vs S) (*Graph 16*). Notably, 90% of the mice assigned to group 4% 7d M died before the end of experimental period, whilst in the female group no death was reported. Moreover, DAI values were significantly higher than the respective female group on day 7 (P<0.001 vs respective F). Interestingly, also male mice of 3% 5+2 d group resulted more susceptible to DSS

colitis compared to corresponding female group: DAI score at day 8, in fact, was extremely higher than that of corresponding F animals (P<0.001 vs respective F).

COLONIC LENGTH



Graph 17: Colonic length

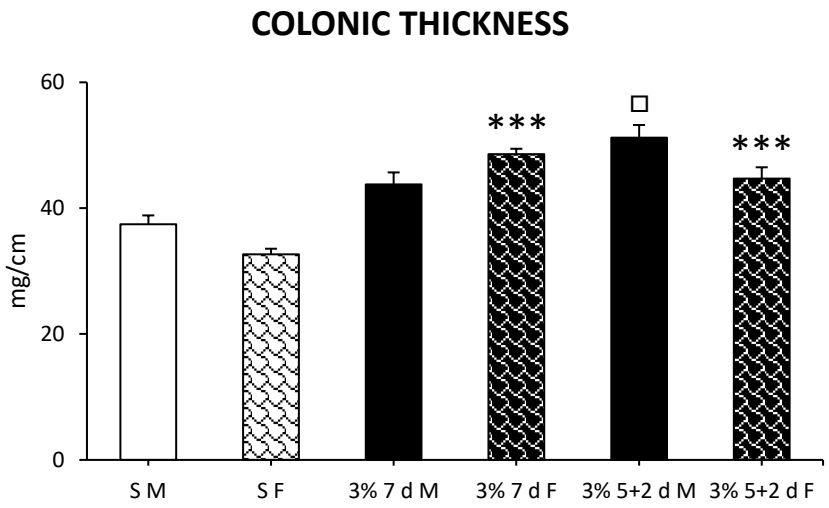
Disease activity index assessed in male water-treated normal mice (S M) and in DSS-treated mice, 3% DSS for 7 days (3% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M), compared to respective female groups (n=5-7 values per group).

**** P<0.001vs S; □□□ P<0.001 vs respective F group*

One-way ANOVA + Bonferroni's post test or Student's unpaired t test

Colonic length was reduced by DSS administration in a similar way for the two experimental conditions (*Graph 17*). The 3% 7 days protocol caused a strong colon shortening in female mice that was significantly lower in male animals (P<0.001). No differences occurred between males and females of 3% 5+2 d groups.

COLONIC THICKNESS



Graph 18: Colonic thickness

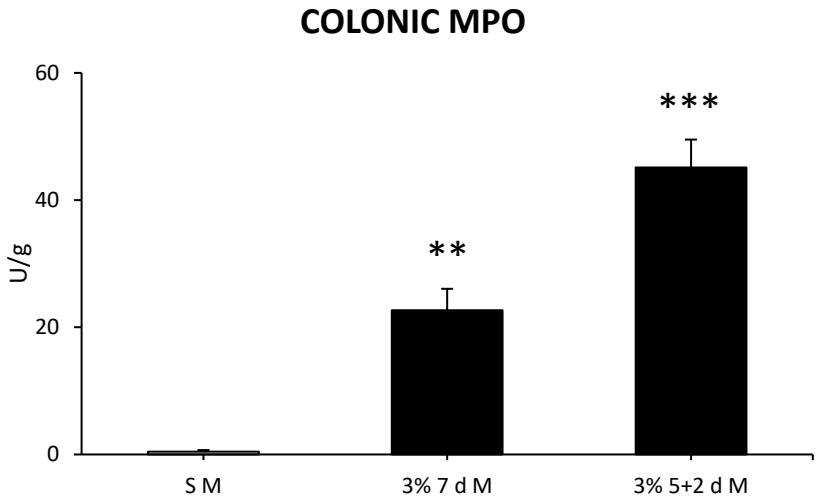
Disease activity index assessed in male water-treated normal mice (S M) and in DSS-treated mice, 3% DSS for 7 days (3% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M), compared to respective female groups (n=5-7 values per group).

**** P<0.001 vs S; □ P<0.05 vs respective F group*

One-way ANOVA + Bonferroni's post test or Student's unpaired t test

In *Graph 18* changes of colonic thickness are reported for male mice. In 3% 7d group a small increase of this parameter was demonstrated, whilst in 3% 5+2d group a huge increment was seen. This value was significantly higher than that of S animals (P<0.001 vs S), and of respective female animals (P<0.05 vs respective F).

COLONIC MYELOPEROXIDASE ACTIVITY



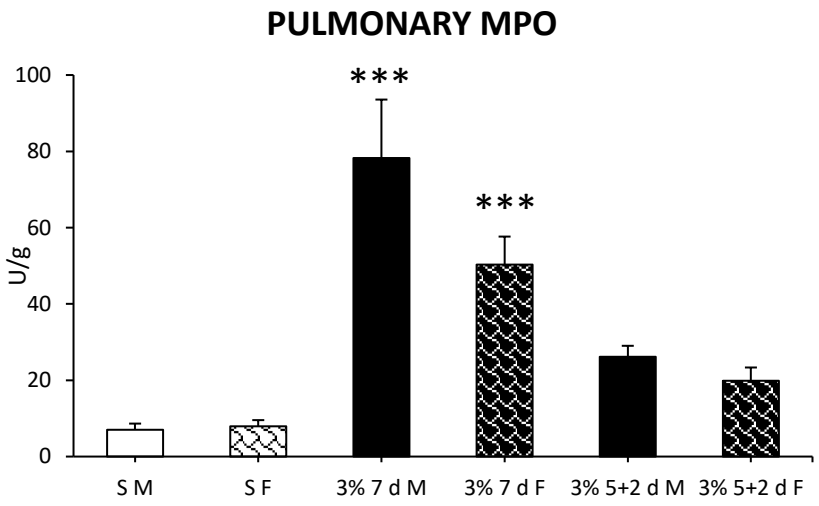
Graph 19: Colonic MPO

*Myeloperoxidase (MPO) activity assessed in the colon of male water-treated normal mice (S M) and of DSS-treated mice: 3% DSS for 7 days (3% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M) (n=5-7 values per group). *** P<0.001, ** P<0.01 vs S*

One-way ANOVA + Bonferroni's post test

Neutrophils infiltration within the colon is reported in *Graph 19*. DSS administration for 5 days caused a massive activation of leukocytes recruitment (P<0.001) analogous to that of corresponding female mice. Neutrophils infiltration consequent to 7 days DSS administration was twenty-fold higher than sham animals (P<0.01), but moderately lower than 3% 5+2 d group.

PULMONARY MYELOPEROXIDASE ACTIVITY



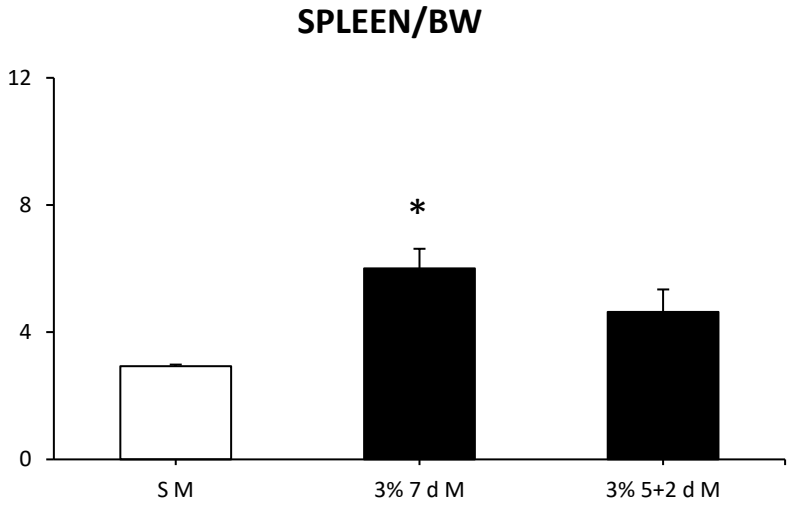
Graph 20: Pulmonary MPO

*Myeloperoxidase (MPO) activity assessed in the lungs of male water-treated normal mice (S M) and of DSS-treated mice: 3% DSS for 7 days (3% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M) (n=5-7 values per group). *** P<0.001 vs S*

One-way ANOVA + Bonferroni's post test

According to the results obtained in female mice, MPO levels were higher for animals treated with 3% DSS solution for 7 days with respect to 5 days treatment. Moreover, the MPO increment observed for male mice was higher than the one in females, even if there was no statistical significance.

SPLEEN/BODY WEIGHT RATIO

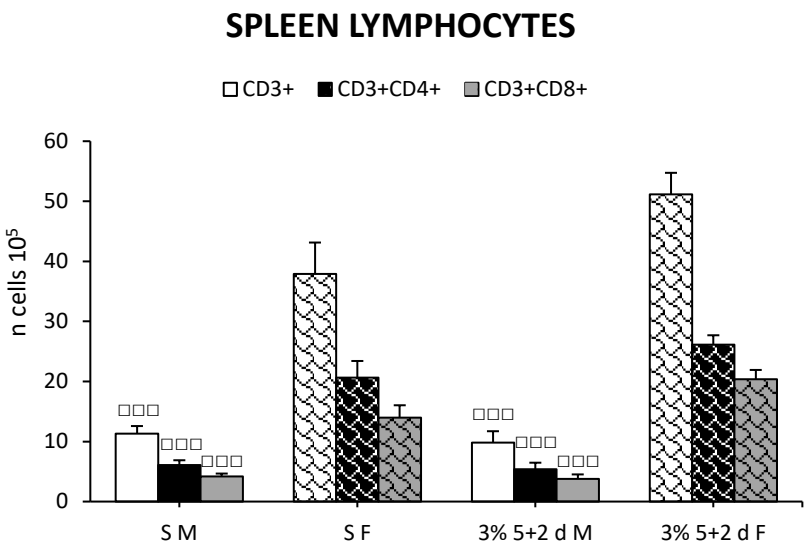


Graph 21: Spleen/BW

Spleen/body weight ratio assessed in male water-treated normal mice (S M) and in DSS-treated mice: 3% DSS for 7 days (3% 7 d M) and 3% DSS for 5 days followed by 2 days of water (3% 5+2 M) (n=5-7 values per group). P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

Similarly to female mice, SP/BW ratio was increased by DSS administration in the two groups, but a statistically significant difference with respect to sham mice was reached only in 3%7 d group (P<0.05), while in 3% 5+2d M splenomegaly was slightly lower than in 3% 7d M group and in female mice.

SPLENIC LYMPHOCYTES



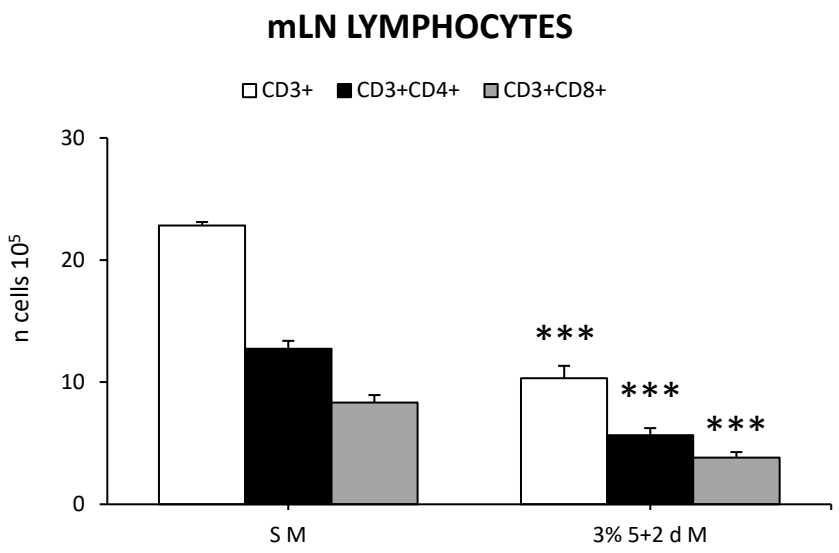
Graph 22: Spleen lymphocytes

Number of splenic T cells assessed in male water-treated normal mice (S M) and in 3% DSS-treated mice for 5 days followed by 2 days of water (3% 5+2 M), compared to respective female groups (n=5-7 values per group). □□□ P<0.001 vs respective F group

One-way ANOVA + Bonferroni's post test

As for female groups, only animals assigned to sham and 3% 5+2 group were analysed and compared. From the data reported in *Graph 22*, a striking difference appeared: the number of T cells obtained for both sham and colitic male animals was strongly lower than corresponding females (P<0.001). No further differences were spotted between the two male groups.

MESENTERIC LYMPH NODES T CELLS



Graph 23: mLN lymphocytes

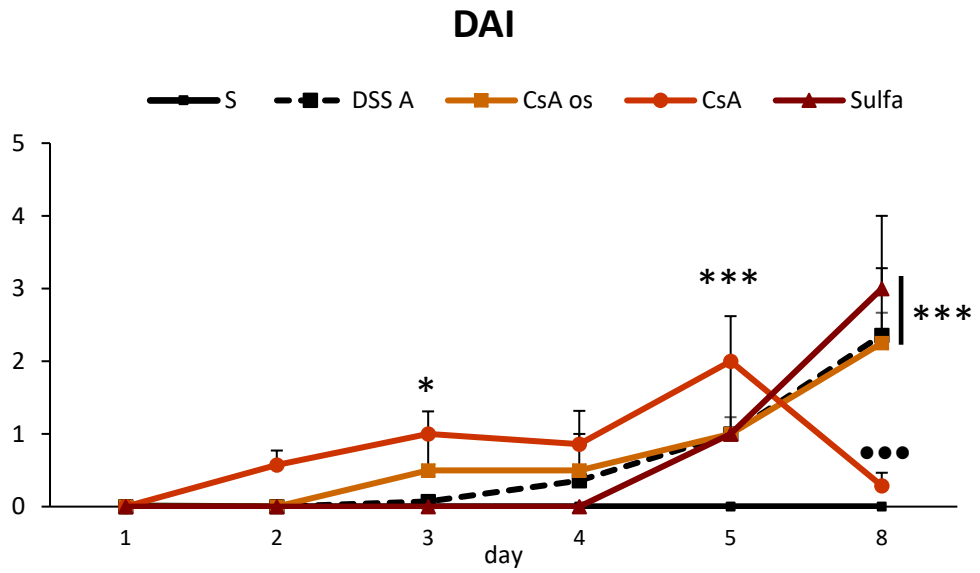
*Number of mesenteric lymph nodes (mLN) T cells assessed in male water-treated normal mice (S M) and in 3% DSS-treated mice for 5 days followed by 2 days of water (3% 5+2 M) (n=5-7 values per group). *** P<0.001vs S. One-way ANOVA + Bonferroni's post test*

Within mesenteric lymph nodes, the number of T cells was clearly reduced upon DSS administration (P<0.001) with respect to sham mice (*Graph 23*). Levels of lymphocytes subpopulations for male animals were comparable to those of females.

ACUTE DSS MODEL-positive control selection

In order to select a positive control drug, sulfasalazine and cyclosporine A, drugs already used in clinics, were tested on female animals exposed to 3% DSS 5+2 days, the administration protocol chosen as acute DSS model. The control group, represented by vehicle-treated colitic animals, has been subsequently re-named DSS-A. Since no differences in T cells subpopulations of spleen and lymph nodes were detected in control animals with respect to sham mice, FACS analysis was not performed on animals treated with sulfasalazine and cyclosporine A.

DISEASE ACTIVITY INDEX



Graph 24: Disease activity index-DAI

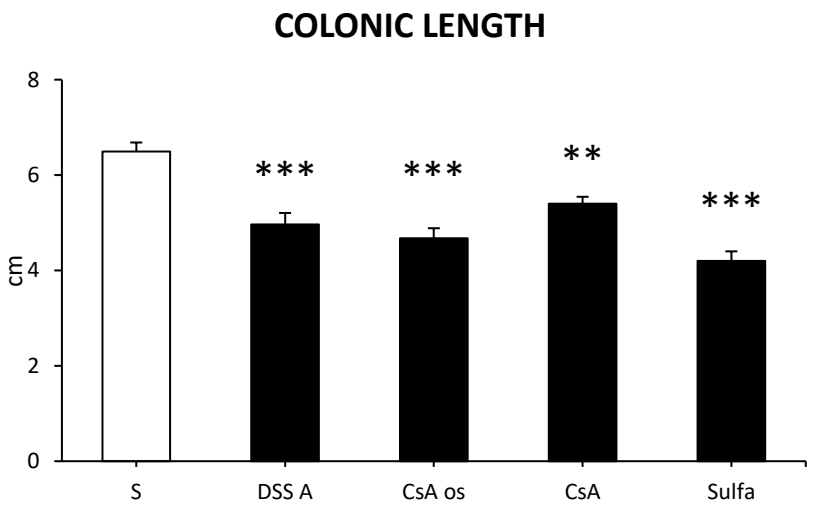
Disease activity index assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group).

**** P<0.001, * P<0.05 vs S; ●●● P <0.001 vs DSS A. Two-way ANOVA + Bonferroni's post test*

Sulfasalazine, subcutaneously injected, and cyclosporine A, orally administered, were not able to counteract DSS-induced disease severity: at day 8 DAI scores were similar to those of DSS A group. Cyclosporine A intraperitoneally injected worsened DAI score at day 3 (P<0.05 vs S) and at day 5 (P<0.001) with respect to S animals, but was the

only treatment able to strongly reduce DAI at day 8 compared to control animals (P<0.001 vs DSS A). Remarkable body weight loss initially contributed to high DAI score of CsA group, while the absence of rectal bleeding throughout the whole experiment and full recovery of body weight at day 8 accounted for the beneficial effects of this treatment.

COLONIC LENGTH



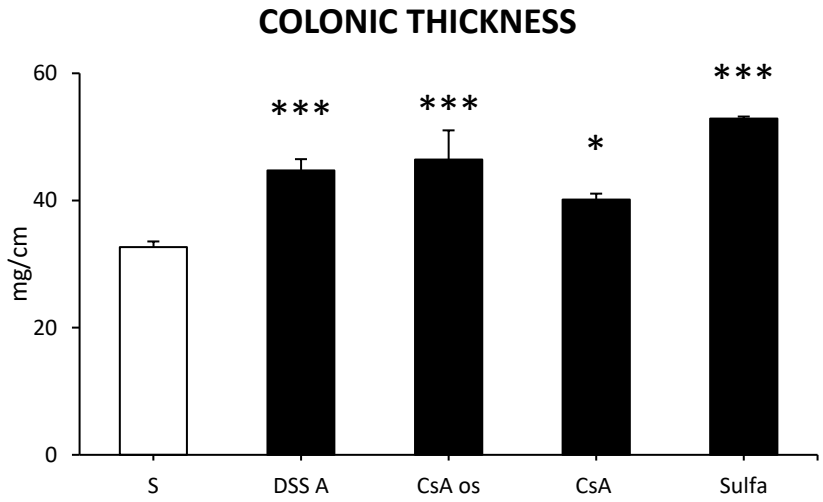
Graph 25: Colonic length

*Colonic length assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group). *** P<0.001, ** P<0.01 vs S*

One-way ANOVA + Bonferroni's post test

DSS-induced colonic shortening was not counteracted by sulfasalazine administration (P<0.001 vs S), that in turn led to an increased reduction of colonic length, even if not significant, with respect to DSS A animals. Similarly, cyclosporine A administration, either per os or intraperitoneally injected, was not effective in preventing colonic length shortening.

COLONIC THICKNESS



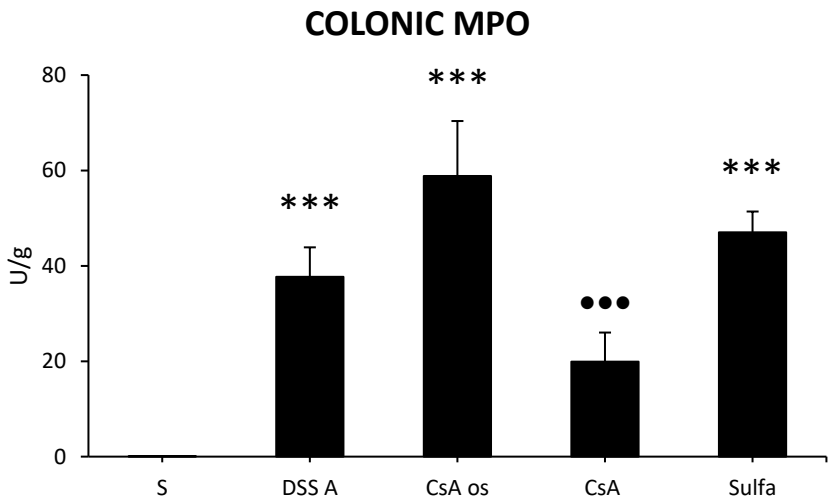
Graph 26: Colonic thickness

*Colonic thickness assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group). *** P<0.001, * P<0.05 vs S*

One-way ANOVA + Bonferroni's post test

In *Graph 26*, values corresponding to colonic weight/length ratio are represented. Clearly DSS administration triggered an increase of colonic thickness that was not controlled by sulfasalazine and by cyclosporine A orally administered (P<0.001 vs S), whilst in mice injected with intraperitoneal cyclosporine A the thickening of the colon appeared slightly reduced compared to DSS A group (P<0.05 vs S).

COLONIC MYELOPEROXIDASE ACTIVITY



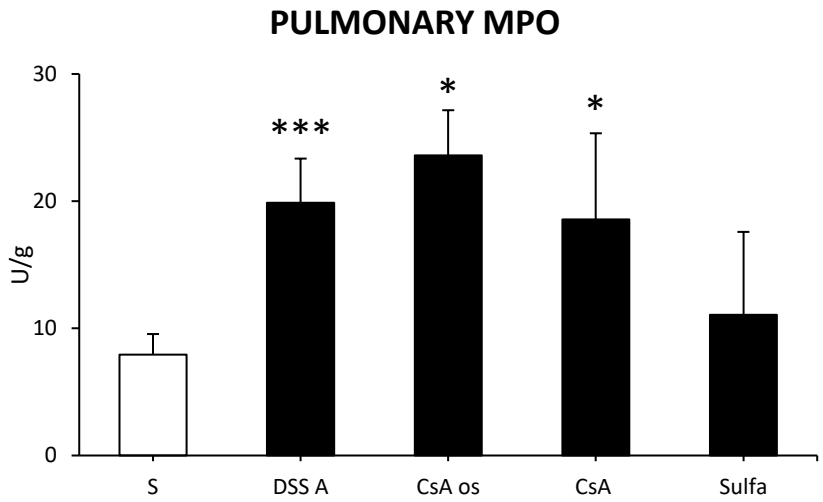
Graph 27: Colonic MPO

Myeloperoxidase (MPO) activity assessed in the colon of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group).

**** P<0.001vs S, ●●● P <0.001vs DSS A. One-way ANOVA + Bonferroni's post test*

The reduction of neutrophils infiltration in the colon is the primary endpoint considered for the identification of an effective drug in our model. MPO levels were strongly increased by DSS administration (P<0.001 vs S), while they were significantly reduced by cyclosporine A ip injection (P<0.001 vs DSS A). The protective effect exerted by CsA ip was lost when the same drug was administered by oral route. Sulfasalazine could not control leukocytes recruitment in the colon.

PULMONARY MYELOPEROXIDASE ACTIVITY



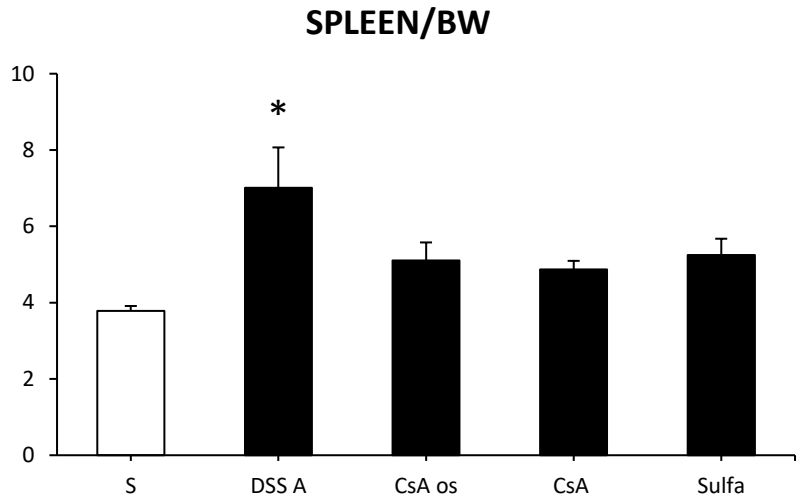
Graph 28: Pulmonary MPO.

Myeloperoxidase (MPO) activity assessed in the lungs of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group).

**** P<0.001, *P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

Neutrophils infiltration in the lung district was not as massive as in the colon. However, in DSS A group a significant increase of this parameter was recorded (P<0.001 vs S). Neither Cyclosporine A, per os or ip injected, or sulfasalazine could effectively lower MPO levels (P<0.05 vs S).

SPLEEN/BODY WEIGHT RATIO



Graph 29: Spleen/BW

*Spleen/body weight ratio assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), cyclosporine A os (CsA os), cyclosporine A s.c. (CsA) or sulfasalazine s.c. (Sulfa) (n=5-7 values per group). *P<0.05 vs S*

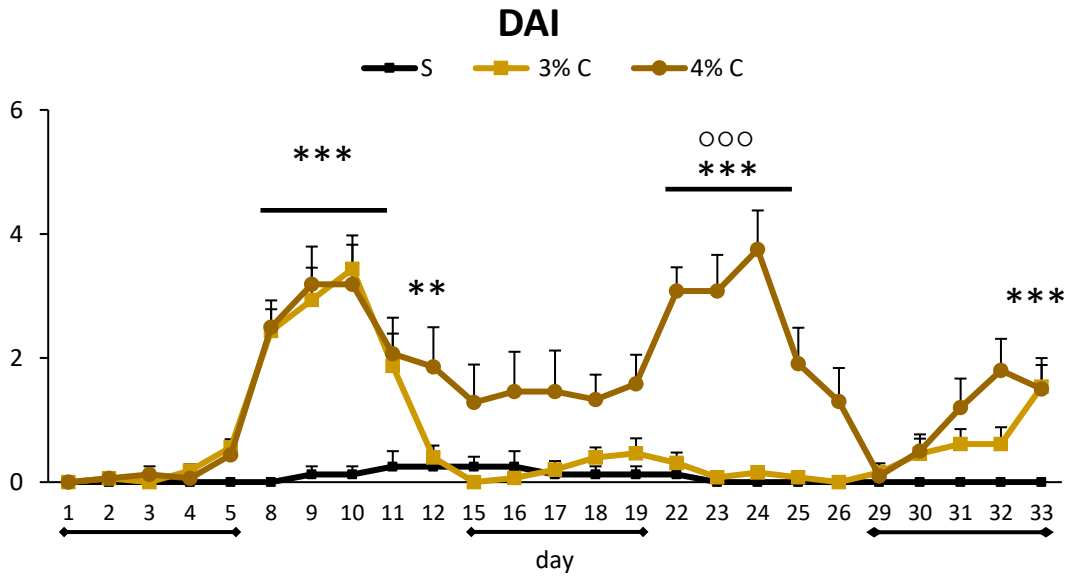
One-way ANOVA + Bonferroni's post test

As shown in *Graph 29*, a moderate increase in SP/BW ratio was observed for DSS A group (P<0.05 vs S). Such a small difference was lost in the other groups, suggesting a partial control of splenomegaly exerted by the tested drugs.

CHRONIC DSS MODEL DEVELOPMENT

Based on the sex-dependent susceptibility emerged in the acute model and on the DSS concentrations tested, only female mice were used for the development of chronic DSS model and 3% and 4% concentrations of DSS were tested: DSS solution was administered to mice for 3 cycles of 5 days each (day 1-5; day 15-19; day 30-33), alternated by 9 days of water. Animals were euthanized at the end of the last cycle of DSS administration (day 33).

DISEASE ACTIVITY INDEX



Graph 30: Disease activity index-DAI

Disease activity index assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group).

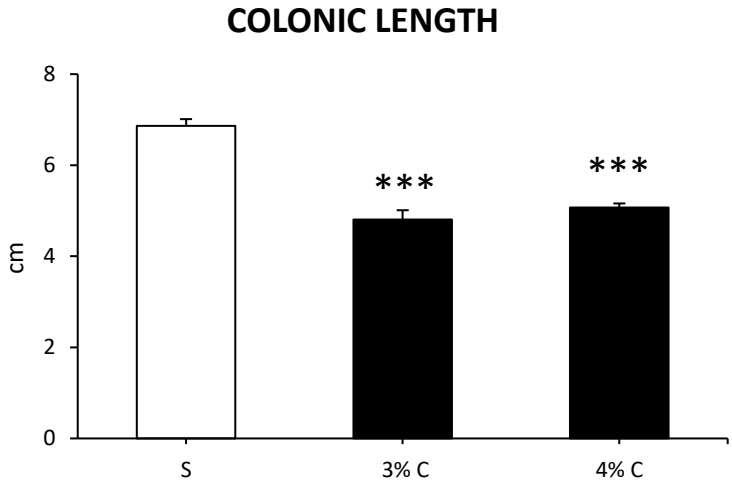
Arrows under X-axis indicate DSS administration period

**** P<0.001, **P<0.01 vs S; ○○○ P<0.001 vs 3% C. Two-way ANOVA + Bonferroni's post test*

The first cycle of DSS administration, lasting 5 days, increased highly significantly the DAI score between day 8 and 11 with respect to sham animals (P<0.001 vs S), irrespective of the different DSS concentrations. While in 3% C group DAI score remained low until the last cycle of DSS administration (day 30-33), 4% C group curve

presented a second peak of high DAI score right after the end of the second cycle of DSS exposure. In particular, DAI values reached significantly high levels from day 22 to day 25 for 4% C group compared to S and 3% C groups (P<0.001 vs S and 3% C). At day 33 both 3% C and 4% C groups' scores were significantly higher than that of S mice (P<0.001 vs S).

COLONIC LENGTH



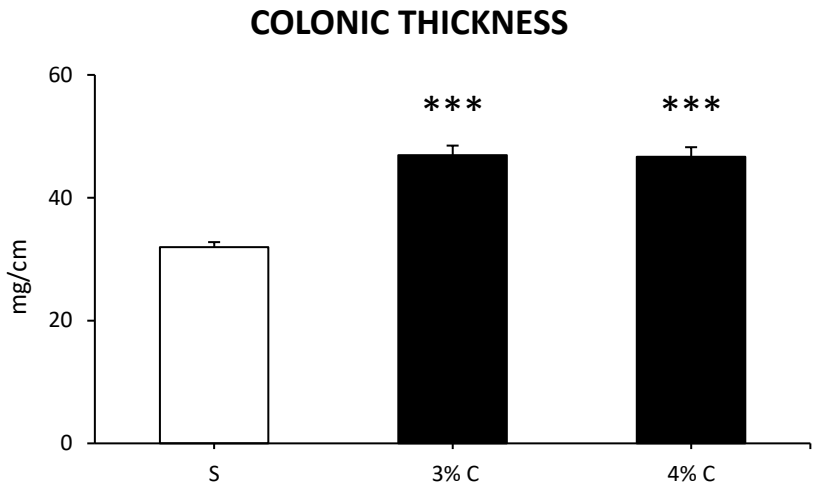
Graph 31: Colonic length

Colonic length assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group).

**** P<0.001 vs S. One-way ANOVA + Bonferroni's post test*

In a way similar to acute colitis, also in chronic colitis DSS administration was followed by a strong reduction of colonic length; however, the highest DSS concentration (4%) did not worsen colonic shortening in comparison to the lowest one (3%).

COLONIC THICKNESS



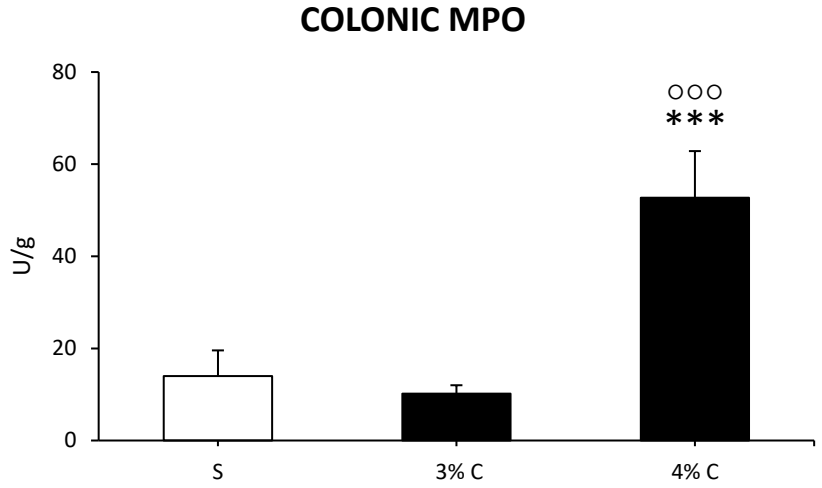
Graph 32: Colonic thickness

Colonic thickness assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group).

**** P<0.001 vs S. One-way ANOVA + Bonferroni's post test*

Weight/length ratio of the colon was increased by chronic DSS both at 3% and 4% concentration (P<0.001), as shown in *Graph 32*.

COLONIC MYELOPEROXIDASE ACTIVITY

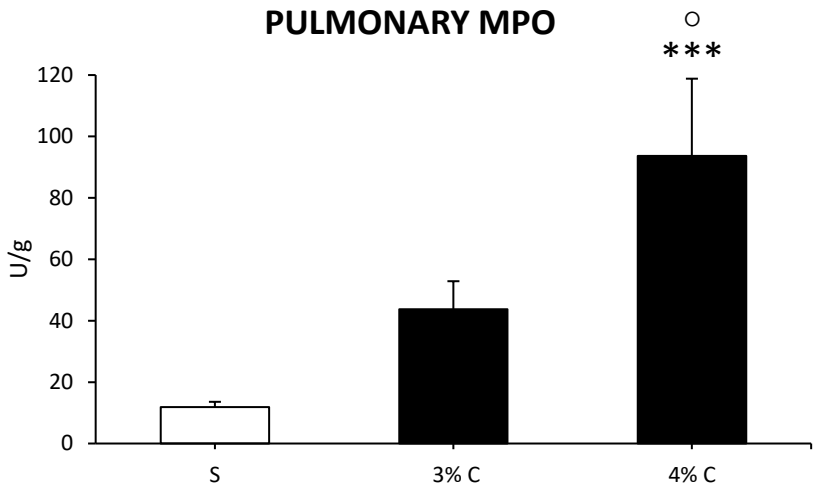


Graph 33: Colonic MPO

*Myeloperoxidase (MPO) activity assessed in the colon of water-treated normal mice (S) and of chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group). *** P<0.001 vs S; ○○○ P<0.001 vs 3% C. One-way ANOVA + Bonferroni's post test*

Following chronic 3% DSS administration no differences in MPO levels could be detected with respect to S group. By opposite 4% DSS administration led to a massive recruitment of leukocytes from the periphery (P<0.001 vs S), more resembling the typical features of acute DSS colitis. The difference between the 3% C and 4% C group was significant (P<0.001 vs 3% C).

PULMONARY MYELOPEROXIDASE ACTIVITY

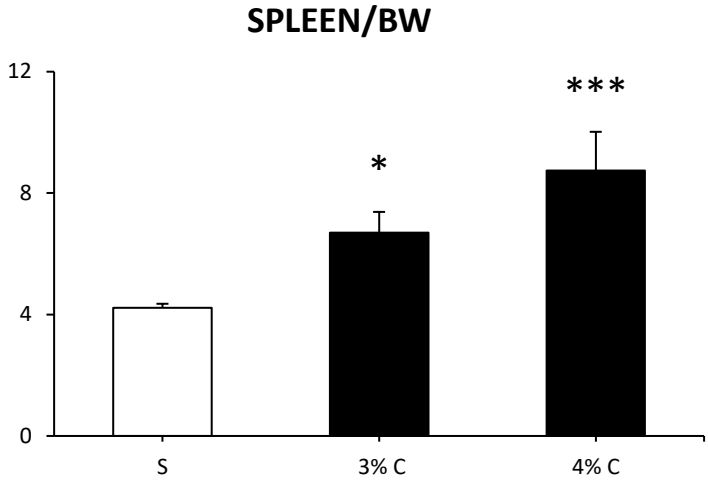


Graph 34: Pulmonary MPO

*Myeloperoxidase (MPO) activity assessed in the lungs of water-treated normal mice (S) and of chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group). *** P<0.001 vs S; ○ P<0.05 vs 3% C. One-way ANOVA + Bonferroni's post test*

In the chronic model a strong systemic inflammation developed consequently to the repeated challenge of mice with DSS. Similarly to what observed in the colon, 4% DSS led to a massive infiltration of neutrophils within the lungs compared to sham mice ($P<0.001$). The difference in MPO levels was also significant ($P<0.05$ vs 3% C) with respect to 3% C group, whose levels were four-fold increased compared to sham mice.

SPLEEN/BODY WEIGHT RATIO



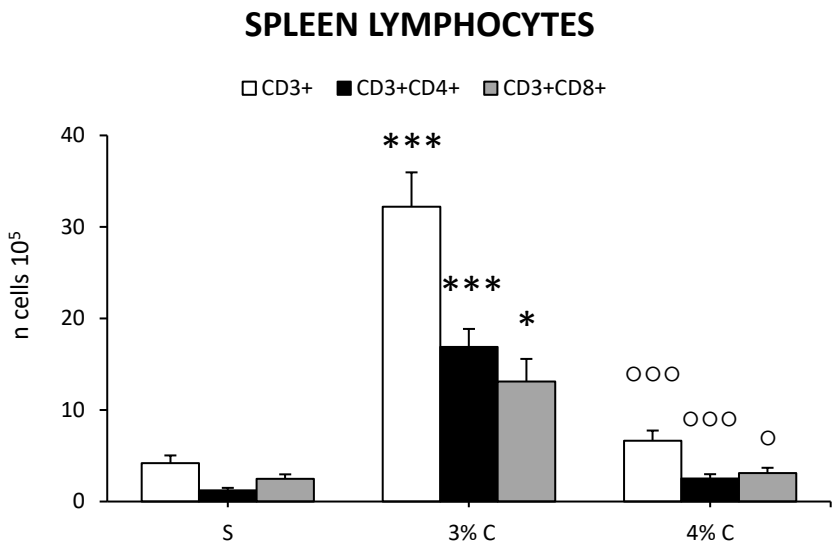
Graph 35: Spleen/BW

Spleen/body weight ratio assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group).

**** P<0.001, * P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

The presence of systemic inflammation was confirmed by the increase in spleen/body weight ratio after DSS administration. The highest concentration of DSS demonstrated once more to be responsible for major colitis severity. A two-fold increase was in fact observed for SP/BW ratio in 4% C group in comparison to S ($P<0.001$ vs S), whilst 3% C group ratio was lower but still significantly augmented with respect to sham mice ($P<0.05$ vs S).

SPLENIC LYMPHOCYTES



Graph 36: Spleen lymphocytes

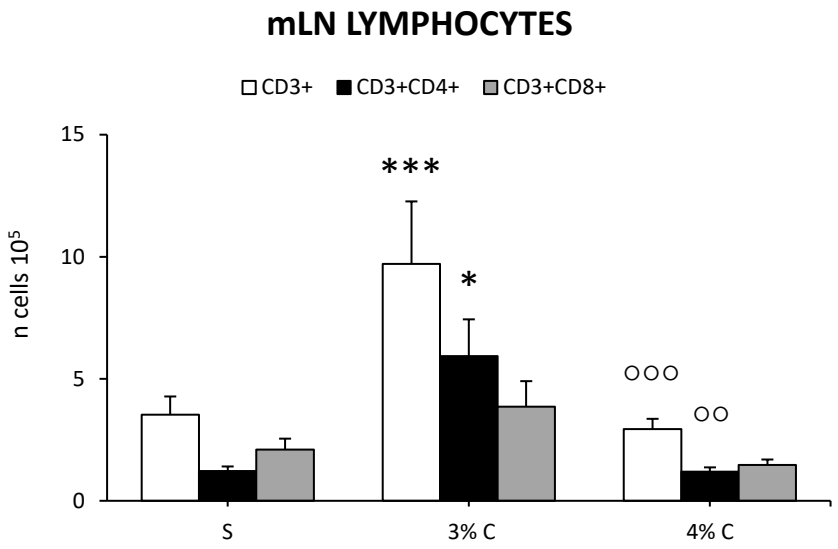
Number of splenic T cells assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group).

**** P<0.001, * P<0.05 vs S; ooo P<0.001, o P<0.05 vs 3% C*

One-way ANOVA + Bonferroni's post test

Chronic DSS colitis model relies on the activation of adaptive immune responses and on T cells increased number in secondary lymphoid organs and migration to the sites of inflammation. In *Graph 36* the different T cells subsets analysed in the spleen are represented. 3% C group showed a clear increase in the three different subpopulations $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ with respect to S group ($P<0.001$ for $CD3^+$, $CD3^+CD4^+$ and $P<0.05$ for $CD3^+CD8^+$ subset vs S). By opposite, in 4% C, the number of cells in these three subpopulations was significantly lower when compared to that of 3% C group ($P<0.001$ for $CD3^+$, $CD3^+CD4^+$ and $P<0.05$ for $CD3^+CD8^+$ subset vs 3%C), while no differences were spotted in T cells numbers compared to sham mice.

MESENTERIC LYMPH NODES T CELLS



Graph 37: mLN T cells

*Number of mesenteric lymph nodes (mLN) T cells assessed in water-treated normal mice (S) and in chronically DSS-treated mice: 3 cycles of 5 days 3% DSS + 2 cycles of 9 days of wash out (3% C) or 3 cycles of 5 days 4% DSS + 2 cycles of 9 days of wash out (4% C) (n=5-7 values per group). *** P<0.001, * P<0.05 vs S; ○○○ P<0.001, ○○ P<0.01 vs 3% C*

One-way ANOVA + Bonferroni's post test

FACS analysis of mesenteric lymph nodes mirrored the situation obtained in the spleen. 3% DSS treated mice presented higher levels of $CD3^+$ lymphocytes, T helper and T cytotoxic cells compared to both sham mice ($P<0.001$ for $CD3^+$ and $P<0.05$ for $CD3^+CD8^+$) and 4% DSS treated mice ($P<0.001$ for $CD3^+$ and $P<0.01$ $CD3^+CD8^+$), where no differences were spotted with respect to S mice.

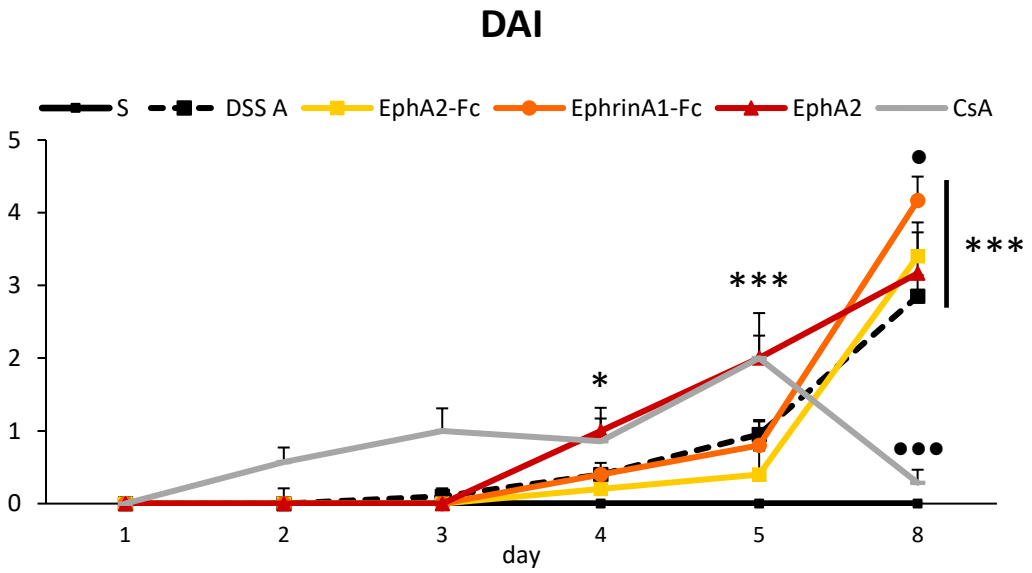
ACUTE DSS COLITIS: Eph/ephrin system involvement

In the third part of this work, the involvement of the Eph/ephrin system in the previously developed acute and chronic models of colitis was assessed. To this end, female mice were first challenged with 3% 5+2 days protocol and received saline (control group-DSS A), cyclosporine A 25mg/kg/die ip injected, selected as positive control drug (CsA) or recombinant receptors and ligands as to modulate class A and B signalling. Local and systemic inflammatory parameters were analysed.

EphA-ephrinA SIGNALLING MODULATION

In order to modulate EphA-ephrinA signalling, mice, administered with 3% DSS solution, were assigned to three different experimental groups and treated with EphA2-Fc, activating *reverse* signalling, ephrin-A1-Fc, activating *forward* signalling and monomeric EphA2, blocking the bidirectional signalling.

DISEASE ACTIVITY INDEX

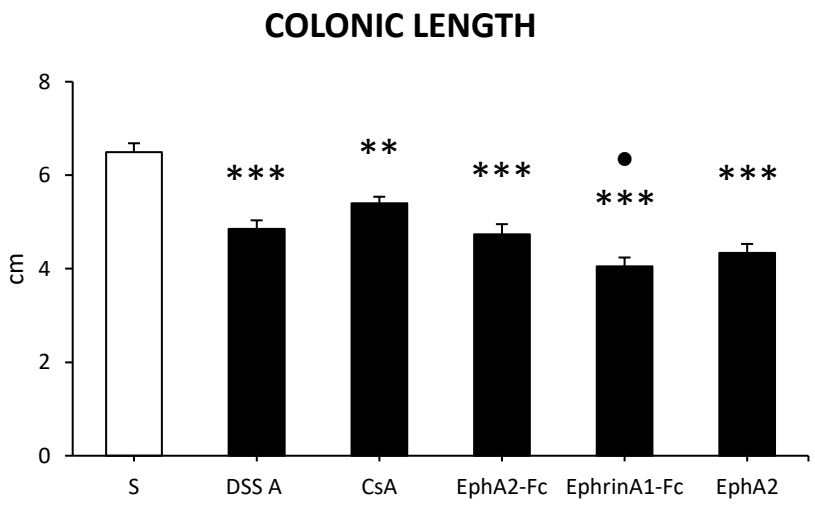


Graph 38: Disease activity index-DAI

*Disease activity index assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, * P<0.05 vs S, ●●● P <0.001, ● P<0.05 vs DSS A Two-way ANOVA + Bonferroni's post test*

As shown in *Graph 38*, none of the treatments could reduce colitis severity, except for CsA: EphA2-Fc and ephrinA1-Fc DAI score curves were perfectly overlapped to the one of DSS A group, whilst EphA2 group score was significantly higher than sham mice already at day 4 (P<0.05 vs S) and at day 5 (P<0.001 vs S). At day 8 all the groups, apart from the positive control group CsA, marked similar scores ranging between 3-4 (P<0.001 vs S), and, moreover, ephrinA1-Fc score was significantly higher than control group (P<0.05). Remarkable rectal bleeding was the parameter contributing the most to total DAI score.

COLONIC LENGTH



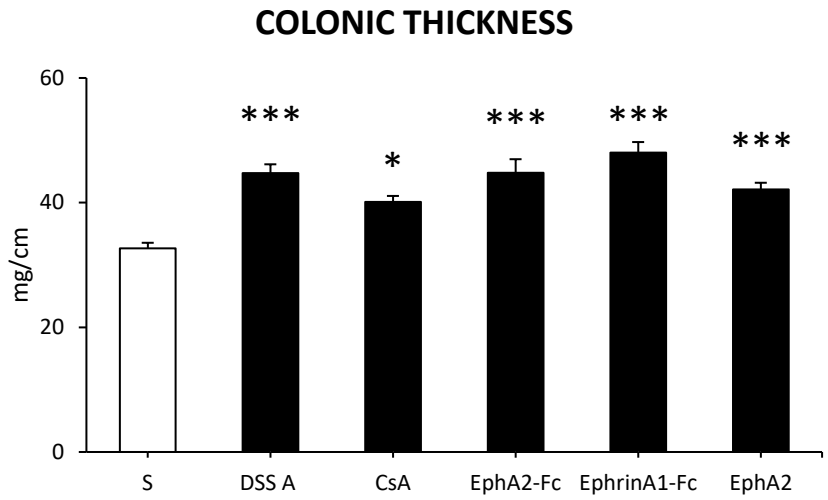
Graph 39: Colonic length

*Colonic length assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, ** P<0.01 vs S; ● P<0.05 vs DSS A*

One-way ANOVA + Bonferroni's post test

EphA2-Fc and EphA2 administration could not control colon shortening (P<0.001 vs S) induced by DSS administration, while ephrinA1-Fc even worsened colon length reduction with respect to control group (P<0.05 vs DSS A).

COLONIC THICKNESS



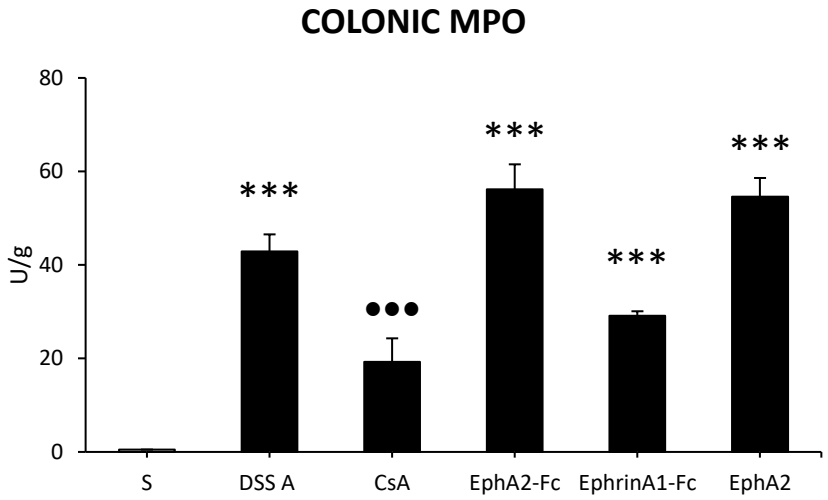
Graph 40: Colonic thickness

*Colonic thickness assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, * P<0.05 vs S*

One-way ANOVA + Bonferroni's post test

Cyclosporine A partially controlled colon thickening induced by DSS, while EphA2-Fc, ephrinA1-Fc and EphA2 failed in reducing colon weight/length ratio with respect to control animals.

COLONIC MYELOPEROXIDASE ACTIVITY

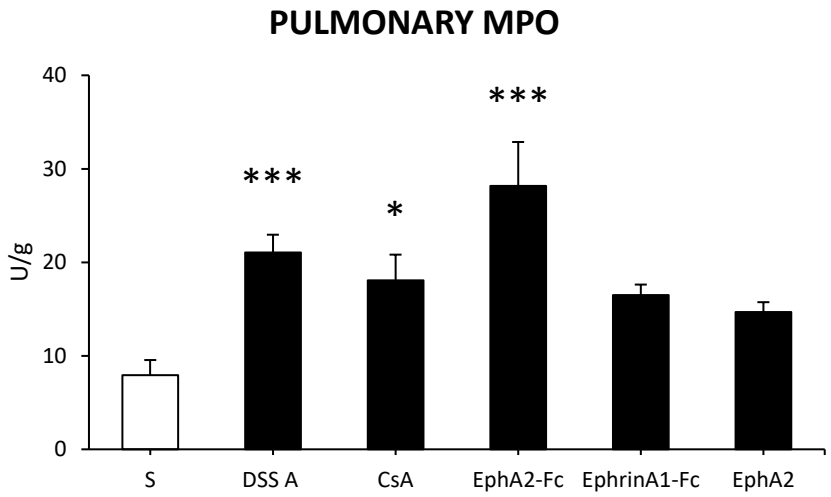


Graph 41: Colonic MPO

*Myeloperoxidase (MPO) activity assessed in the colon of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001 vs S; ●●● P<0.001 vs DSS A One-way ANOVA + Bonferroni's post test*

As already reported in *Graph 11*, neutrophils infiltration was massively triggered by DSS administration (P<0.001 vs S). Both monomeric and IgG-conjugated EphA2 receptors were unable to limit leukocytes recruitment in the colon, while ephrinA1-Fc administration partially reduced MPO levels, even if not significantly, when compared to DSS A group. As a result, only CsA administration could control this local inflammatory parameter with respect to control mice (P<0.001 vs DSS A) (*Graph 41*).

PULMONARY MYELOPEROXIDASE ACTIVITY

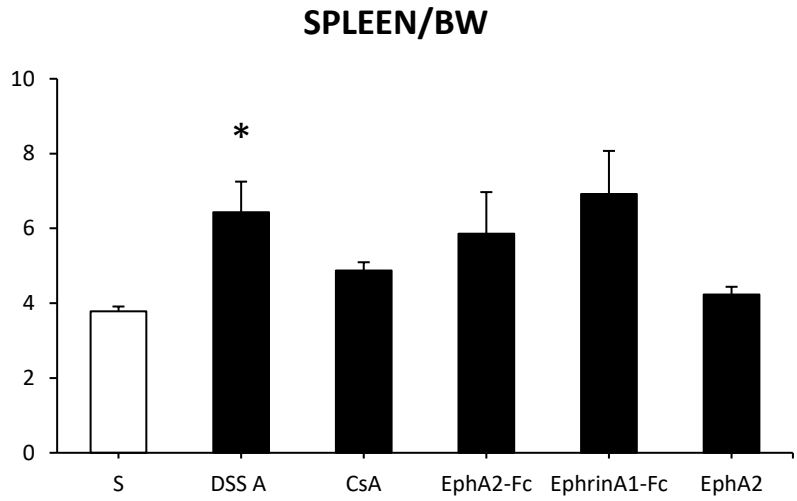


Graph 42: Pulmonary MPO

Myeloperoxidase (MPO) activity assessed in the lungs of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). **** P<0.001, * P<0.05 vs S*
One-way ANOVA + Bonferroni's post test

As represented in *Graph 42*, EphA2-Fc treatment slightly increased MPO activity levels compared to control group, whilst ephrinA1-Fc and EphA2 managed to control neutrophils infiltration in a way comparable to cyclosporine A.

SPLEEN/BODY WEIGHT RATIO



Graph 43: Spleen/BW

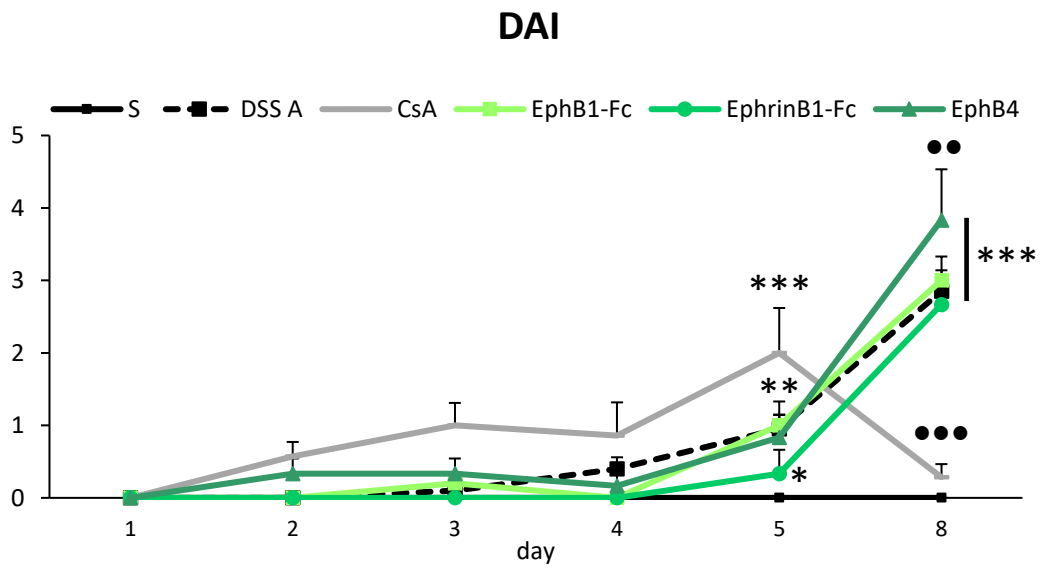
*Spleen/body weight ratio assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphA2-Fc, ephrinA1-Fc, EphA2 or cyclosporine A (CsA) (n=6-10 values per group). * P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

As regards spleen-body weight ratio, EphA2, similarly to CsA, weakly attenuated splenomegaly induced by DSS A, while no effects were evident for EphA2-Fc and ephrinA1-Fc groups.

EphB-ephrinB SIGNALLING MODULATION

EphB-ephrinB signalling involvement in acute DSS colitis was assessed by administering mice the same treatments already tested in TNBS colitis: id est, EphB1-Fc, able to activate *reverse* signalling pathway, ephrinB1-Fc, selectively activating *forward* signalling and EphB4 monomeric receptor as inhibitor of the bidirectional signalling.

DISEASE ACTIVITY INDEX

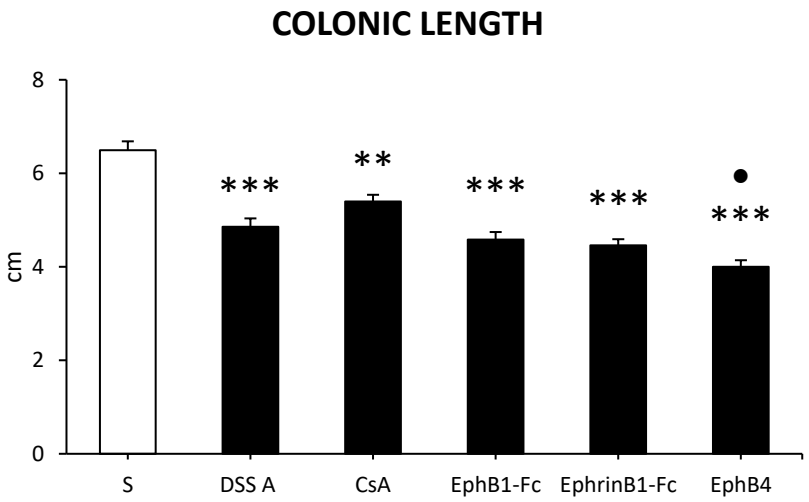


Graph 44: Disease activity index-DAI

*Disease activity index assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, ** P<0.01, * P<0.05 vs S; ●●● P<0.001, ●● P<0.01 vs DSS A Two-way ANOVA + Bonferroni's post test*

Disease activity index curve increased for EphB1-Fc and for ephrinB1-Fc group perfectly overlapping that of DSS A group, the score at day 8 being almost equal (P<0.001 vs S). EphB4 administration, instead, mirrored DSS A score profile until day 5 but was significantly higher at the end of the protocol with respect to the control group (P<0.001 vs S; P<0.01 vs DSS A).

COLONIC LENGTH



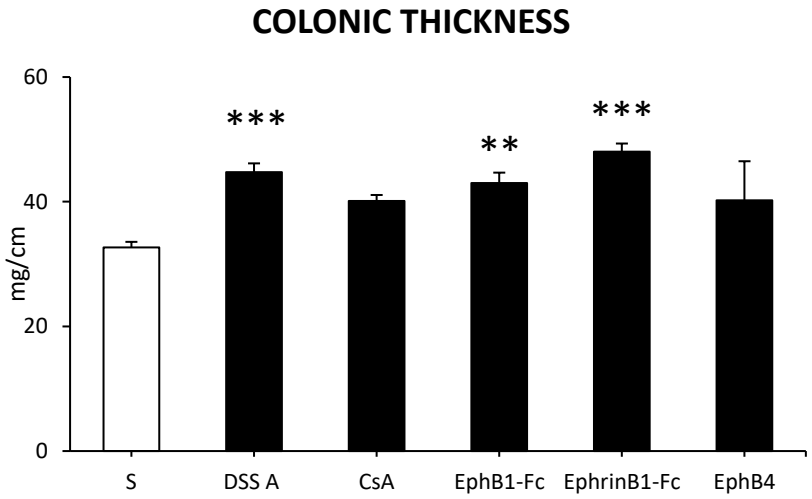
Graph 45: Colonic length

*Colonic length assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, ** P<0.01 vs S; ● P<0.05 vs DSS A*

One-way ANOVA + Bonferroni's post test

Activation of the *reverse* or *forward* signalling, mediated respectively by EphB1-Fc and ephrinB1-Fc, did not counteract colonic length reduction with respect to DSS A group (P<0.001 vs S), while the blockade of both signalling pathways by EphB4 led to a worsening of colonic shortening compared to control mice (P<0.001 vs S, P<0.05 vs DSS A).

COLONIC THICKNESS



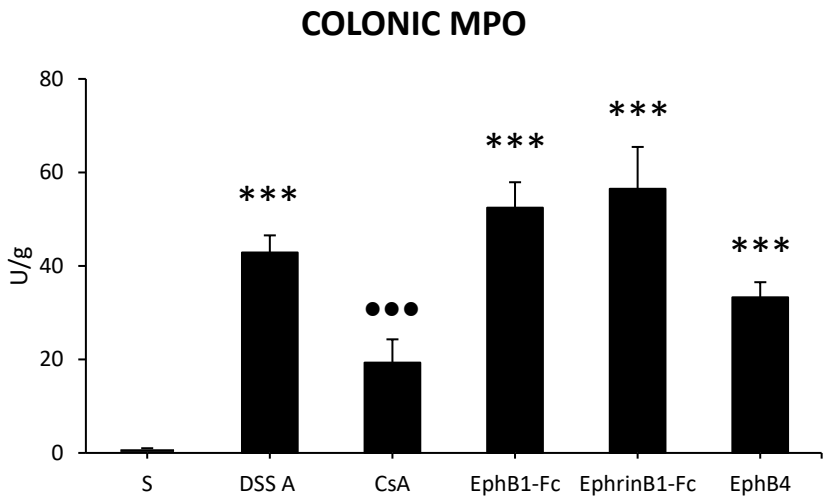
Graph 46: Colonic thickness

*Colonic thickness assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, ** P<0.01 vs S*

One-way ANOVA + Bonferroni's post test

None of the treatments modulating EphB-ephrinB signalling could ameliorate DSS-induced increase of colon weight/length ratio, the values of thickness recorded in the different experimental groups being comparable to that of DSS A group.

COLONIC MYELOPEROXIDASE ACTIVITY

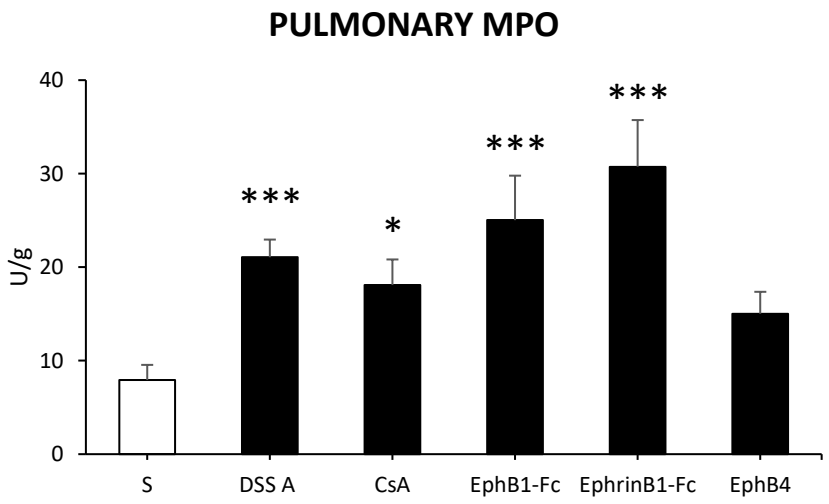


Graph 47: Colonic MPO

*Myeloperoxidase (MPO) activity assessed in the colon of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001 vs S; ●●● P<0.001 vs DSS A One-way ANOVA + Bonferroni's post test*

In *Graph 47* colon neutrophils infiltration is shown. It is evident that none of the treatments modulating EphB-ephrinB system had a beneficial effect comparable to that produced by CsA, MPO levels obtained in EphB1-Fc and ephrinB1-Fc groups being even higher than those of control mice.

PULMONARY MYELOPEROXIDASE ACTIVITY



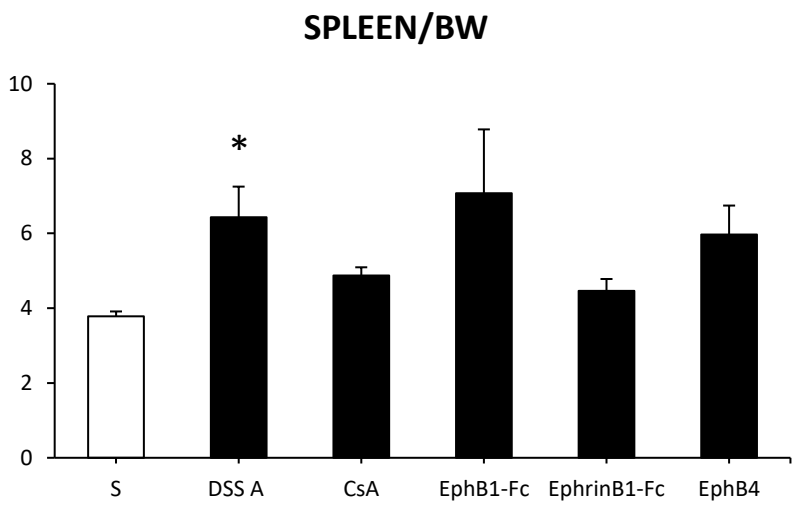
Graph 48: Pulmonary MPO

*Myeloperoxidase (MPO) activity assessed in the lungs of water-treated normal mice (S) and of DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). *** P<0.001, * P<0.05 vs S*

One-way ANOVA + Bonferroni's post test

As regards neutrophils infiltration in the lung, only EphB4 treatment could moderately suppress granulocytes recruitment induced by DSS, whilst none of the other tested treatments, CsA included, were able to control this parameter.

SPLEEN/BODY WEIGHT RATIO



Graph 49: Spleen/BW

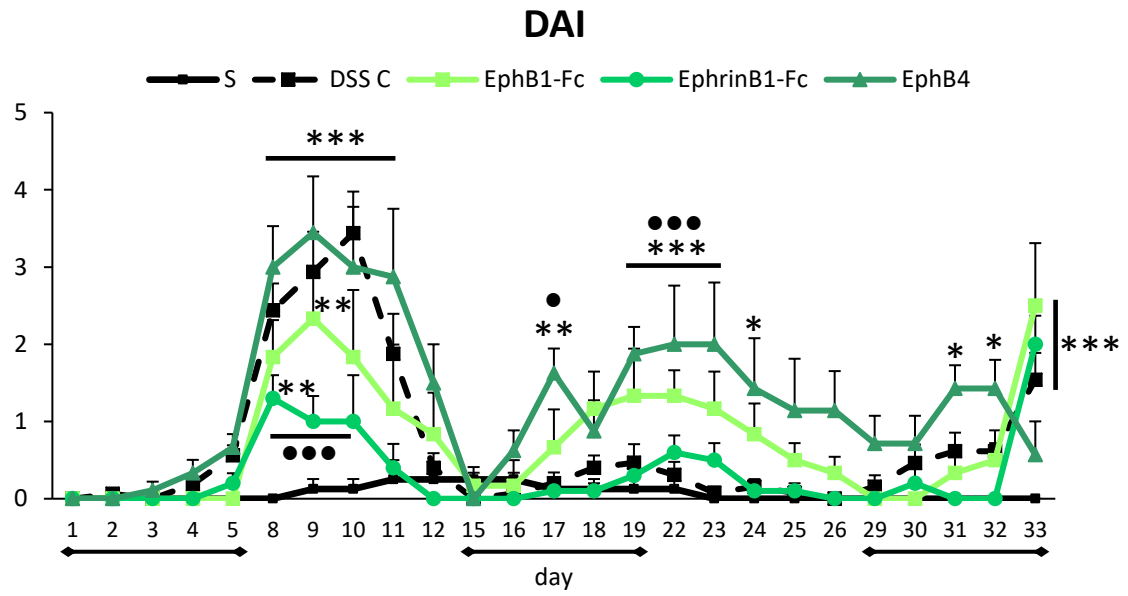
*Spleen/body weight ratio assessed in water-treated normal mice (S) and in DSS-treated mice administered with vehicle (DSS A), EphB1-Fc, ephrinB1-Fc, EphB4 or cyclosporine A (CsA) (n=6-10 values per group). * P<0.05 vs S. One-way ANOVA + Bonferroni's post test*

EphB4 and EphB1-Fc did not reduce SP/BW ratio in comparison to DSS A group, while ephrinB1-Fc partially controlled splenomegaly as cyclosporine A did.

CHRONIC DSS COLITIS: Eph/ephrin system involvement

Considered that, similarly to TNBS model, also DSS chronic model relies on activation of adaptive immune responses, in the last part of the project the effects of EphB-ephrinB signalling modulation were assessed. According to the chronic developed protocol, female mice were administered with 3% DSS solution and randomly assigned to control group, DSS C, or to EphB1-Fc, ephrinB1-Fc and EphB4 groups respectively. Local and systemic inflammatory parameters were analysed; in addition, for the most effective treatment, flow cytometric analysis of spleen and mesenteric lymph nodes was performed.

DISEASE ACTIVITY INDEX



Graph 50: Disease activity index-DAI

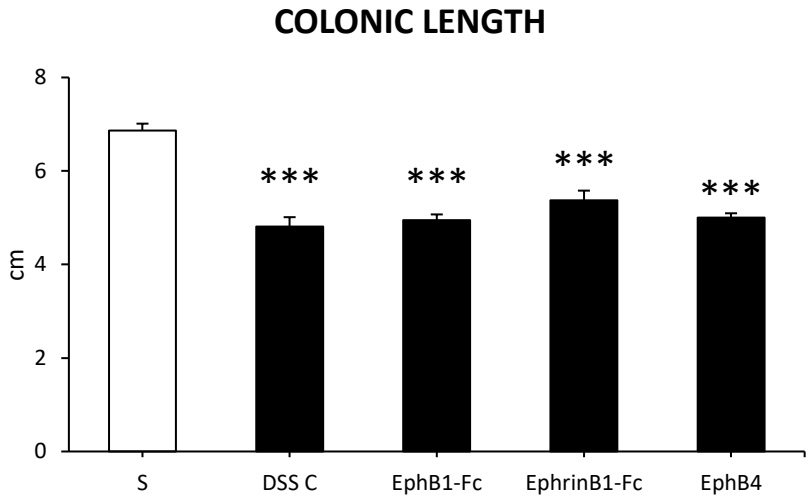
Disease activity index assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). Arrows under X-axis indicate DSS administration period

**** P<0.001, ** P<0.01, * P<0.05 vs S; ●●● P<0.001, ● P<0.05 vs DSS C*

Two-way ANOVA + Bonferroni's post test

DSS administration induced a first peak in DAI score curve lasting from day 8 to day 11 (P<0.001 vs S). Till day 15, EphB4 curve was perfectly comparable to the one obtained in DSS C group, whilst DAI score of EphB1-Fc and ephrinB1-Fc was lower. In particular, ephrinB1-Fc administration significantly reduced DAI score from day 8 to 10 with respect to DSS C group (P<0.001). After the second cycle of DSS administration, DAI score peaked again for EphB1-Fc and for EphB4 group, whose score was significantly higher compared to both S and DSS C groups from day 19 to 23 (P<0.001 vs S and DSS C). At the end of the experimental procedure on day 33, DAI score was significantly higher for ephrinB1-Fc and EphB1-Fc groups compared to sham mice (P<0.001 vs S).

COLONIC LENGTH

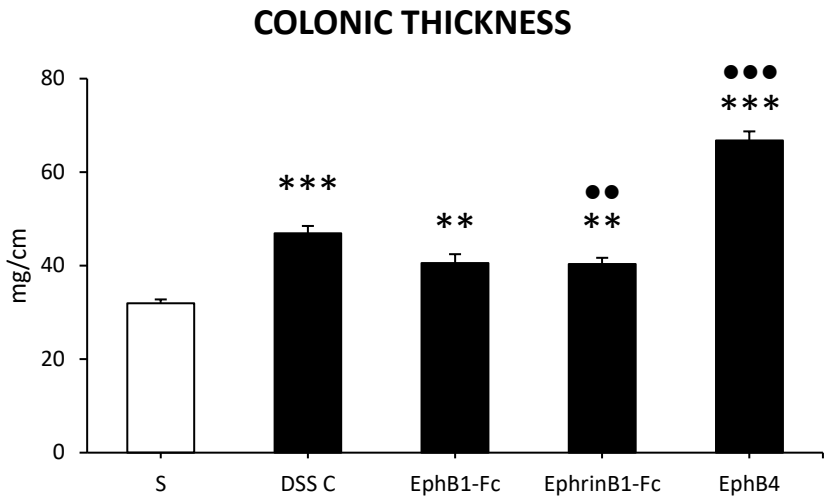


Graph 51: Colonic length

*Colonic length assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001 vs S. One-way ANOVA + Bonferroni's post test*

None of the treatments was able to counteract colonic shortening with respect to S mice (P<0.001 vs S): in fact, no changes were observed after EphB1-Fc, ephrinB1-Fc or EphB4 administration compared to DSS C mice (*Graph 51*).

COLONIC THICKNESS



Graph 52: Colonic thickness

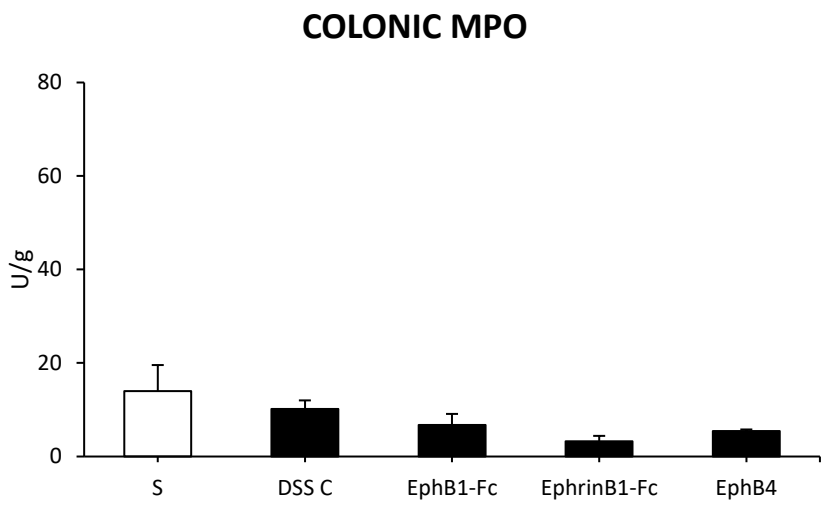
Colonic thickness assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group).

**** P<0.001, ** P<0.01 vs S; ●●● P<0.001, ●● P<0.01 vs DSS C*

One-way ANOVA + Bonferroni's post test

Changes in colonic thickness are reported in *Graph 52*. EphB4 administration worsened colonic thickening compared to control mice (P<0.001 vs S and vs DSS C). By opposite, ephrinB1-Fc managed to reduce colonic thickness increase with respect to DSS C group (P<0.01 vs S and DSS C). Similarly, EphB1-Fc controlled this local inflammatory parameter, even if not significantly compared to DSS C mice (P<0.01 vs S).

COLONIC MYELOPEROXIDASE ACTIVITY

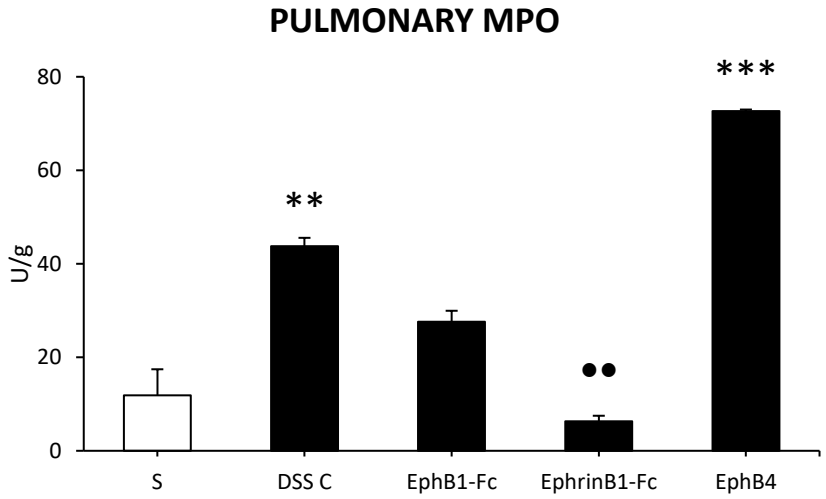


Graph 53: Colonic MPO

Myeloperoxidase (MPO)activity assessed in the colon of water-treated normal mice (S) and of chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group).

Contrarily to what observed in DSS acute colitis (*Graph 47*), colonic MPO activity levels were not increased at the end of chronic DSS exposure, either in vehicle-treated or in drug-treated mice (*Graph 53*).

PULMONARY MYELOPEROXIDASE ACTIVITY



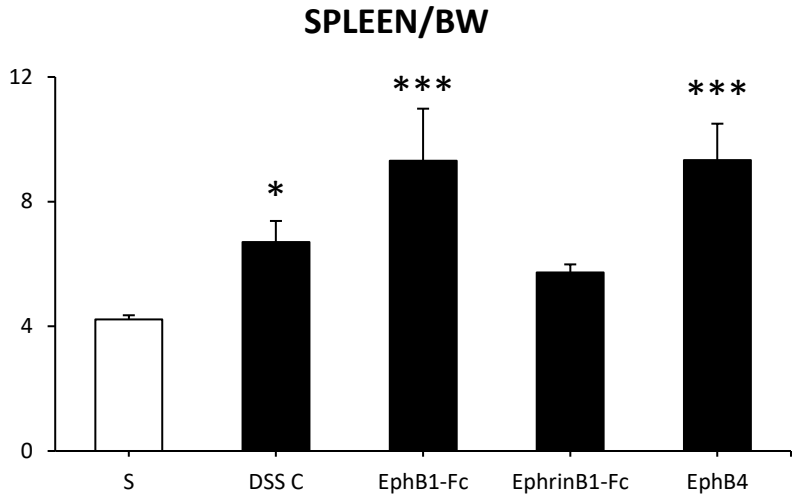
Graph 54: Pulmonary MPO

*Myeloperoxidase (MPO) activity assessed in the lungs of water-treated normal mice (S) and of chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001, ** P<0.01 vs S; ●● P<0.01 vs DSS C*

One-way ANOVA + Bonferroni's post test

Conversely to what observed in the colon, in the lungs MPO activity levels were remarkably increased by chronic DSS assumption (*Graph 54*). According to the chronic model of colitis, the prolonged DSS administration is responsible for the development of systemic inflammation and thus for high levels of neutrophils infiltration in the lungs. As a result, in the control group, MPO activity levels were significantly increased compared to S animals (P<0.01 vs S). EphB4 administration increased neutrophils infiltration in the lungs with respect to S group (P<0.001 vs S) and to control mice. By opposite, EphB1-Fc could partially control MPO levels, while EphrinB1-Fc drastically reduced leukocytes recruitment compared to DSS C group (P<0.01).

SPLEEN/BODY WEIGHT RATIO



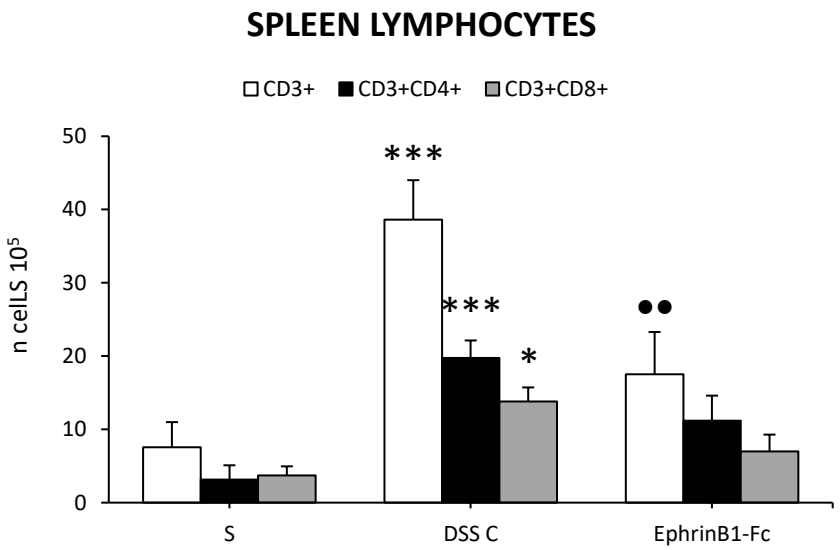
Graph 55: Spleen/BW

*Spleen/body weight ratio assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C), EphB1-Fc, ephrinB1-Fc or EphB4 (n=6-10 values per group). *** P<0.001, * P<0.05 vs S*

One-way ANOVA + Bonferroni's post test

Splenomegaly was increased in control group by DSS administration (*Graph 55*). EphB4 and EphB1-Fc treatment worsened systemic inflammation as demonstrated by the increase in SP/BW ratio in the respective groups. As observed before for lung MPO levels, also in this case, EphrinB1-Fc treatment seemed to partially reduce systemic inflammation.

SPLENIC T CELLS



Graph 56: Spleen lymphocytes

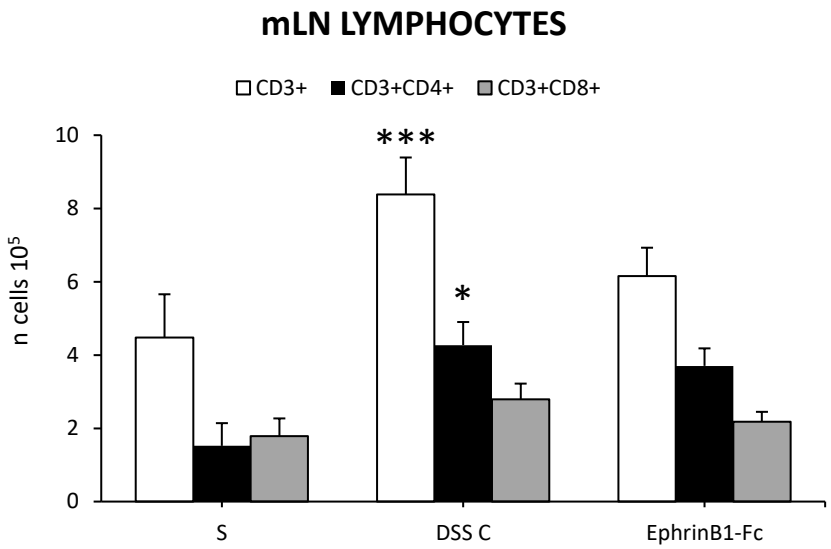
Number of splenic T cells assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C) or ephrinB1-Fc (n=6-10 values per group).

**** P<0.001, * P<0.05 vs S; ●● P<0.01 vs DSS C*

Two-way ANOVA + Bonferroni's post test

DSS administration led to a huge increase in the number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells subpopulations. Since ephrinB1-Fc was the only treatment effective in controlling some of the inflammatory responses induced by the chemical agent, the changes of T cells subpopulations were analysed only for it. In ephrinB1-Fc group a strong reduction of all the three subsets of T cells was observed: $CD3^+$ lymphocytes were strongly reduced with respect to DSS C mice ($P<0.01$), while T helper and T cytotoxic cells reduction did not reach statistical significance.

MESENTERIC LYMPH NODES T CELLS



Graph 57: mLN lymphocytes

*Number of mesenteric lymph nodes T cells assessed in water-treated normal mice (S) and in chronically DSS-treated mice administered with vehicle (DSS C) or ephrinB1-Fc (n=6-10 values per group). *** P<0.001, * P<0.05 vs S. Two-way ANOVA + Bonferroni's post test*

As in the spleen, also in mesenteric lymph nodes the number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was increased in DSS C group ($P<0.001$ for $CD3^+$ and $P<0.05$ for $CD3^+CD4^+$ vs S). EphrinB1-Fc administration partially lowered the number of $CD3^+$ T cells, while it failed in controlling T helper and T cytotoxic subpopulations increase.

DISCUSSION AND CONCLUSIONS

In this project the involvement of Eph/ephrin system in intestinal inflammation was investigated. As to better reproduce the complexity of human disease and to elucidate the mechanisms responsible for Eph-ephrin mediated effects, three different models of colitis were used. TNBS colitis is based on the activation and recruitment of T cells and is characterised by Th1-Th17-mediated immune responses (Antoniou et al., 2016); acute DSS colitis relies primarily on the activation of innate immune mechanisms (Bento et al., 2012); chronic DSS colitis model, instead, should mimic better the relapsing-remitting features of human condition and is characterised by Th1-Th2 mixed inflammatory responses (Dieleman et al., 1998).

In the first part of the project, the local and systemic effects produced by modulation of EphB-ephrinB signalling was tested in TNBS colitis: to this end, equimolar doses of recombinant agonists EphB1-Fc and ephrinB1-Fc, selectively activating *reverse* and *forward* signalling but, conversely, blocking *forward* and *reverse* signalling, respectively, and the monomeric EphB4 receptor, proposed as inhibitor of bidirectional signalling (Kertesz et al., 2006), were administered to mice intra-rectally injected with the haptenating agent.

In fact, several evidences point out a possible role of EphB-ephrinB pathway in intestinal inflammation: some receptors and ligands of this class are expressed in intestinal epithelial cells as active regulators of their maturation and positioning along the crypt-villus axis (Genander et al., 2010); Eph receptors on monocytes and ephrin ligands on endothelial cells are involved in monocytes adhesion (Funk et al., 2013); EphBs and ephrinBs localised on T cells facilitate the differentiation from naïve T cells to effector T cells in lymphoid organs and act as TCR co-receptors (Wu et Luo, 2005). Furthermore, Kitamura has recently shown that the levels of expression of ephrin-B1 are high in $CD3^+$ T cells present in the synovial tissue, in fibroblasts and peripheral blood mononuclear cells (PBMCs) of rheumatoid arthritis patients, and that the exogenous ephrinB1-Fc administration was responsible for high PBMCs migratory ability and for the increased production of TNF-α (Kitamura et al., 2008). Moreover, Hafner observed mRNA changes of EphBs and ephrinBs in mucosal lesions of IBD

patients compared to healthy subjects and demonstrated that ephrin-B2 *reverse* signalling is implicated in increased wound healing competence of intestinal epithelial cells due to activation of downstream genes involved in tissue repair (Hafner et al., 2005).

TNBS model, characterised by immune-mediated responses of Th1-Th17 type and by a dense colon tissue infiltration of T cells, was used as a mice model of human CD. In our model, TNBS intrarectally administered to mice triggered a severe colitis characterised by high DAI and macroscopic score, strong reduction of colon length and increase of colon thickness, as well as by a massive infiltration of neutrophils within the colon. Systemic inflammation followed TNBS colitis induction: spleen/body weight ratio was slightly increased in TNBS mice, while pulmonary MPO levels were extremely high. Among the treatments, EphB1-Fc 30 μg/kg and EphB4 20 μg/kg were able to counteract disease onset and severity: they reduced macroscopic and DAI score, controlled colon length reduction and thickening, strongly counteracted neutrophils infiltration within colon and lungs. By opposite, the protection mediated by ephrinB1-Fc 17 μg/kg administration was weak: only DAI score and lung MPO levels were reduced compared to TNBS mice. Accordingly, in the attempt to shed some light on the mechanisms underlying the beneficial effects of EphB-ephrinB blockade, FACS analysis of splenocytes and mesenteric lymph nodes was performed. As a result, a reduction in the number of splenic $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in the control group was reported, reduction that was reverted to levels similar to that of sham animals by EphB1-Fc and EphB4 administration.

Overall, in TNBS-induced colitis, given the beneficial effects of both EphB1-Fc and EphB4 recombinant proteins, the protection seems to derive from blockade of EphB-*forward* signalling; ephrinB1-Fc administration, in fact, able to activate *forward* signalling, did not lead to the same positive outcomes in mice.

The targets and the mechanisms responsible for EphB1-Fc and EphB4 beneficial results are still far from our understanding. Nevertheless, considered the changes in spleen T cells subpopulations and the central role displayed by T cells in this model, we can hypothesize that EphB1-Fc and EphB4 administration either blocked T cells migration

from secondary lymphoid organs towards the colon, reducing the T cells mediated perpetuation of inflammation, or reduced the proliferation and differentiation of these cells in the spleen. Other studies have already reported that B signalling might play a pivotal role in T cells proliferation, maturation and migration. Hu and colleagues, for example, demonstrated that in double KO mice for ephrin-B1/B2 on T cells, the clinical score for collagen-induced arthritis was strongly reduced compared to wild type mice. This beneficial effect was linked to CD4 cells defective development into Th17 cells and, consequently, to the reduced ability of T cells to undergo chemotaxis toward CXCL12 in vitro and to migrate in vivo to the inflamed paw (Hu et al., 2015). The positive effects mediated by EphB1-Fc and by monomeric EphB4 both able to block *forward* signalling activated by ephrin-B in our model, might therefore be explained according to the reduced migration of T cells from secondary lymphoid organs to the inflamed colon and/or altered maturation of $CD4^+$ T cells. Moreover, as regards T cells proliferation, Yu reported that ephrinB2-Fc, used as a co-stimulator in the presence of a sub-optimal concentration of anti-CD3 monoclonal antibody, was able to promote T cells proliferation, cytotoxic activity and interferon-γ production in vitro. Notably, soluble monomeric EphB4 inhibited ephrinB2-Fc co-stimulation (Yu et al., 2003), prompting us to hypothesize similar effects in our TNBS model. Direct effects on tissue repair due to activation of EphB1-Fc-mediated *reverse* signalling, according to Hafner's observations (Hafner et al., 2005), can be proposed in addition: to investigate this possible mechanism of action, we therefore evaluated the effects of Eph/ephrin ligands also in acute DSS colitis, a model of chemical colitis characterized by loss of barrier integrity where the beneficial action of molecules promoting mucosal healing could be better evidenced. Finally, in order to verify whether the protective action of Eph/ephrin ligands is maintained also in a chronic setting, the same agents tested in acute TNBS colitis were investigated in a chronic model of DSS colitis, Th1-Th2 dependent.

Accordingly, suitable acute and chronic DSS protocols were established.

Both female and male mice were employed for the development of acute DSS colitis model. Male mice showed greater susceptibility than females: 90% of the animals challenged with 4% DSS solution administered for 7 days died in the male group while no deaths were detected in the female one. Moreover, also upon administration of DSS 3% for 7 days, a higher number of male mice died compared to females (data not shown). Finally, DAI score at day 8, colonic thickening and colonic MPO levels presented higher values in some male groups than in corresponding females. Male greater sensitivity to DSS induced colitis was reported also by other authors: Elderman and colleagues investigated sex impact in intestinal and systemic immunity highlighting a lower number of adaptive T cells and a higher proportion of innate immune cells in male mice compared to females (Elderman et a., 2016); furthermore, Bábíčková and colleagues suggested that, in experimentally-induced colitis, female protection might be ascribed to estradiol, the predominant female sex hormone (Bábíčková et al., 2015). In order to adopt a model of colitis of moderate but not excessive severity, we decided to work with female mice. Among the different tested protocols, 3% 5+2d protocol was selected: this model is characterised by clear increase of DAI score, colonic shortening and thickening, and by a huge recruitment of neutrophils within the colon. Moreover, there was a limited infiltration of leukocytes in the lungs and a reasonable increment of spleen/body weight ratio, but no changes in splenic and mLN T cells subpopulations and no macroscopical differences in the colon of DSS animals compared to sham mice. All those features are in accordance with an acute model of colitis where most of the damage is localised at the first site of inflammation, the colon, and native immune players are recruited (Bento et al., 2012).

Once selected the protocol, we looked for a positive control, a drug already in use for humans IBD, able to ameliorate colitis severity in mice and specifically to reduce neutrophils infiltration. Initially, sulfasalazine 50 mg/kg was orally administered to mice for five days: no benefits, on both local and systemic inflammatory parameters, were appreciated upon its administration. The use of sulfasalazine or other 5-ASA family compounds, like olsalazine and mesalazine, has been already tested by other groups and conflicting results have been reported: their lack of efficacy has been

accounted for by low 5-ASA concentration able to reach the mouse colon or by its short retention at the colonic site (Sann et al., 2013).

The same negative results were obtained for cyclosporine A orally delivered: the reason could be its low oral bioavailability due to poor water solubility, that prevents an adequate absorption through the intestinal mucosa (Muller et al., 2006), or a rapid metabolism by mouse enterocytes that leads to diminished plasma or local concentrations of biologically active cyclosporine, as previously reported by Murthy (Murthy et al., 1993). In humans, as well, bioavailability of oral cyclosporine A is subjected to high inter and intra-individual variations (Sandborn et al., 1991; Webber et al., 1992). We therefore decided to administer cyclosporine A by intraperitoneal injection: animals assigned to this group had lower DAI at day 8, lower reduction of colonic length and lower increase of colonic thickness as well as remarkably lower infiltration of neutrophils within the colon. Despite the presence of side effects like the consistent loss of body weight at day 3 and 5, but thanks to the overall protection exerted, CsA ip was selected as positive control drug for the acute model of colitis. Cyclosporine A is a potent immunosuppressive drug, whose main mechanism of action is the blockade of the transcription of cytokines gene, id est IL-2, in activated T cells by inhibiting the phosphatase activity of calcineurin. The question “how can CsA be effective in DSS colitis model, a model in which T cells are apparently not involved?” spontaneously arises. However, besides the main immunosuppressive mechanism, other mechanisms of action have been proposed for cyclosporine A: for example, Soriano-Izquerdo demonstrated that CsA is directly involved in the reduced expression of adhesion molecules’, like ICAM1 and VCAM1, in a model of DSS colitis, thus leading to a reduction in leukocytes recruitment by endothelial cells (Soriano-Izquierdo et al., 2004). Moreover, other studies showed that cyclosporine A can abrogate the activation of macrophages and the recruitment of neutrophils, major mediators of tissue damage during acute inflammation (Wagner et al., 1983; Kubes et al., 1991). Finally, more recently, cyclosporine A demonstrated to be able to reduce epithelial cells apoptosis by up-regulating TGF-β expression and by inhibiting Caspase-8 in the colon (Satoh et al., 2009), a very advantageous action if we consider that DSS colitis is

associated with an increment in the epithelial apoptotic ratio, due to increased apoptosis and reduced proliferation of epithelial cells (Perse et Cerar, 2012).

Subsequently, the chronic DSS model was optimised. Using female animals, two different concentrations of DSS, 3% and 4%, were administered for 3 cycles of 5 days each alternated by 2 cycles of 9 days of water. While the highest concentration led to a more severe disease with features still resembling the acute DSS model (high MPO levels in colon and lungs and no T cells changes in spleen and mLN), the lowest concentration (3% w/V) was responsible for a condition characterised by low neutrophils infiltration in the colon but increased systemic inflammation (high MPO levels in the lungs and SP/BW ratio) and augmented number of T cells in spleen and mLNs. The latter situation reflected the pathophysiology of chronic DSS colitis as described by other authors (Bento et al., 2012), thus 3% chronic protocol was selected for subsequent tests aimed at evaluating the effects of EphB-ephrinB pharmacological modulation. Cyclosporine A was tested as positive control for the chronic model, but due to the sustained side effects, mainly represented by heavy body weight loss, it was rapidly abandoned (data not shown). Indeed, the use of a positive control drug to demonstrate the predictivity of a chronic DSS colitis protocol can be found rarely in literature. Recently an article has been published in which cyclosporine A was tested and selected as control drug for chronic DSS: however, in this investigation, BALB/C mice, instead of C57BL/6, and different DSS concentrations and timing were used (Hoffmann et al., 2018), possibly accounting for the different results we collected in our model.

In the third part of the project, the effects produced by Eph-ephrin signalling modulation were therefore investigated in the acute and chronic DSS colitis models.

As already mentioned, acute DSS colitis model is considered a well-established experimental method to study drugs aimed at epithelial barrier repair and at modulating innate immune responses in human intestinal inflammatory disorders. The rationale for studying EphA/ephrinA class in DSS colitis is based on several observations: some of the genes encoding for A receptors and ligands are TNF-α

responsive (Ivanov et al., 2006); EphA2 receptors are involved in vascular permeability regulation by re-organising actin in the cytoskeleton (Ivanov et al., 2006); EphA2 is expressed on both monocytes and lymphocytes (Funk et al., 2013; Sharfe et al., 2008) and it is implicated in their migration or adhesion to the endothelium; EphA2 signalling can activate NF-kB leading to increased expression of adhesion molecules like ICAM1-VCAM1 (Carpenter et al., 2012). Some members of this class, like EphA2 and ephrin-A1, are also described as possible mediators of tissue repair in in vivo models of hypoxic skin injury or spinal cord injury due to their re-vascularisation and axon repair abilities (Vihanto et al., 2005; Coulthard et al., 2012). Moreover, EphA2 is up-regulated in several acute inflammatory conditions like renal (Baldwin et al., 2006), myocardial (Dries et al., 2011), or mesenteric ischemia/reperfusion injury as we recently demonstrated (Vivo et al., 2017). Changes in EphAs/ephrinAs expression were found also in chronic inflammatory diseases like in biopsies from the carotid of subjects with atherosclerosis (Sakamoto et al., 20008), or from mucosal lesions of IBD patients (Hafner et al., 2005).

In our project mice were assigned to three different groups: EphA2-Fc 30 µg/kg was injected daily to activate selectively *reverse* signalling; ephrinA1-Fc 16 µg/kg was administered in order to selectively activate *forward* signalling, while monomeric EphA2 20 µg/kg was used as inhibitor of bidirectional signalling.

Unfortunately, despite the encouraging background, none of the treatments could produce beneficial effects to colitic mice. In particular, the blockade of *forward* signalling by EphA2 or EphA2-Fc administration was not effective in controlling colonic MPO levels, DSS-induced colon shortening and thickening as well as DAI score at day 8, while its exogenous stimulation by ephrinA1-Fc worsened some of the parameters associated to increased colitis severity: DAI score, as well as colon shortening, were significantly increased at day 8 with respect to control mice. On the whole, the findings collected in our model of colitis indicated that the contribution of EphA-ephrinA signalling to acute intestinal inflammation appears weak and that exogenous activation of EphA *forward* signalling appeared detrimental, highlighting the importance of a

perfectly balanced EphA-ephrinA signalling for the maintenance of epithelial barrier integrity.

The same proteins and dosages used for the pharmacological modulation of EphB-ephrinB signalling in TNBS colitis were applied to acute DSS colitis.
Unexpectedly, none of the treatments was beneficial in counteracting the inflammatory responses induced by acute DSS colitis. In fact, EphB1-Fc and ephrin-B1-Fc results were perfectly overlapping those of the control group, whilst EphB4 administration worsened only DAI score at day 8 and colonic shortening with respect to control group. On the whole, as for family A, the pharmacological manipulation of EphB-ephrinB signalling seems to have a minor impact in a condition where intestinal homeostasis is perturbed by epithelial barrier disruption and T cells are only marginally involved.

In the last part of the project, to assess if the beneficial effects produced by blockade of EphB-ephrinB *forward* signalling could be maintained in a model of intestinal inflammation able to better resemble the cycles of flares and remission of human IBD, the chronic DSS colitis model was applied. Surprisingly, the protective effects expected for EphB1-Fc and EphB4 were not achieved, while, on the contrary, ephrinB1-Fc demonstrated to be the most beneficial treatment. In fact, ephrinB1-Fc led to a reduction of DAI score along the whole experimental period, a decreased colonic thickening and neutrophils infiltration within the lungs with respect to control mice. Moreover, taking into consideration the data obtained after FACS analysis, the chimeric ligand was able to prevent the increase in the number of splenic T cells induced by repeated DSS administration. By opposite, while EphB1-Fc did not ameliorate any of the parameters evaluated, EphB4 administration increased DAI score significantly with respect to the control group, between days 19 and 23, and worsened colonic thickening and leukocytes infiltration in the lungs.
Interestingly, in vitro low concentrations of ephrinB1/ephrinB2-Fc can increase EphB-mediated production of IFN-γ while blocking IL-4 production in T cells, key cytokines

for Th1 and Th2 skewing, respectively (Yu et al., 2003; Kawano et al., 2012). Thus, we can argue that in chronic DSS colitis, a disease more associated with immune responses of Th2 type, the activation of the *forward* signalling by ephrinB1-Fc might re-balance the equilibrium between Th1 and Th2 T cells subpopulations, by increasing IFN-γ production, therefore attenuating colitis severity.

On the basis of the lack of protection of exogenous Eph/ephrin stimulation in acute DSS colitis, a model based primarily on the involvement of innate immune responses, and of the protective effects mediated by *forward* signalling manipulation in TNBS and chronic DSS colitis, we can conclude that EphB-ephrinB signalling is involved in intestinal inflammation and his major target cells are T lymphocytes. Indeed, according to our results, TNBS and chronic DSS colitis were associated to opposite effects on splenic T cells counts: interestingly, similar beneficial responses were produced when these changes were reverted by blockade or exogenous activation of *forward* signalling, respectively. Moreover, we can hypothesize that, according to the purportedly different Th1- or Th2-inflammatory profiles elicited in TNBS and chronic DSS models, a distinct pharmacological approach should be considered for the treatment of inflammatory diseases driven by dys-regulation of different subsets of adaptive immune cells.

At the end of this study, the exact mechanism through which the EphB/ephrinB system can modulate T cells and whether their proliferation, maturation, differentiation or migration can be affected by pharmacological targeting of type A or B proteins remain unanswered questions: already ongoing and future studies are trying to shed light on those aspects fundamental to fully understand and characterise the Eph/ephrin role in intestinal inflammation.

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