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In vivo and *in vitro* modulation of the Eph/ephrin System as a key element in intestinal inflammation

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CONTENTS

INTRODUCTION

1 Eph/ephrin System

1.1 Discovery and structure

Eph receptors represent the largest family of receptor tyrosine kinases (RTKs) and are classified in A and B subtypes, based on their binding affinity for two different subclasses of membrane-tethered ligands, the ephrins (Eph family receptor interacting proteins).
The acronym EPH stands for erythropoietin-producing hepatocellular carcinoma, since they were identified by molecular cloning in this carcinoma cell line by Hirai and colleagues in 1987; as a result of this discovery Eph gene overexpression has been originally correlated to possible neoplastic processes of some tumors (Hirai et al. 1987).
The increasing attention on Eph/ephrin proteins triggered a large series of works aimed at exploring their structures and functions in human pathophysiology and, to date, it is well described that the bond between Eph receptors and their membrane-linked ligands mediate a short distance cell-cell communication affecting cellular motility, morphology, survival and proliferation (Taylor, Campbell, e Nobes 2017). Sequence homology studies identified EphA (1-8,10) and EphB (1-4,6) and their respective glycosylphosphatidylinositol (GPI)-linked ephrin-A (1-5) or transmembrane ephrin-B (1-3) ligands.

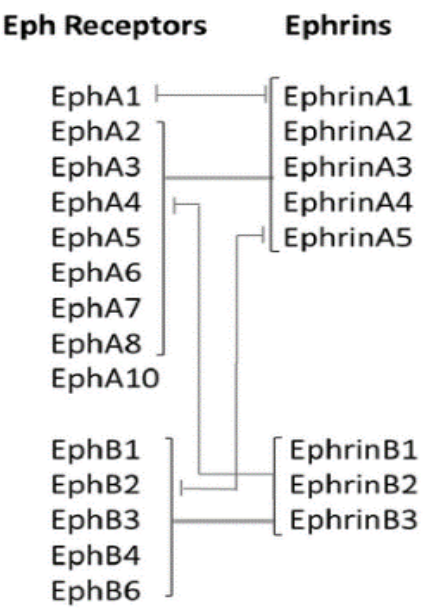


Figure 1: Schematic picture of preferential Eph/ephrin interactions showing cross-type linking (Charmsaz, Sara & Scott, 2017)

There is a matching of linking between receptors and ligands of the same subclass with differences in binding affinity (Figure 1); moreover, there is a case of specificity as for EphB4/ephrin-B2. Even so, Eph/ephrin interactions are also promiscuous considering A and B types because EphA4 binds to ephrin-B2 and ephrin-B3 (Blits-Huizinga et al. 2004)

and EphB2 binds for the most to A-type ephrins, in particular ephrin-A5 (J.-P. Himanen et al. 2004).

The conserved multi-domain Eph structure corresponds to an N-terminal ligand binding domain (LBD), followed by an epidermal growth factor-like motif marked by a cysteine-rich domain (CRD) and two fibronectine-type III repeats (FN III1 and FN III2). A transmembrane domain (TM) allows the link with cell membranes and brings to the C-terminal region. The last intracellular part counts of a juxtamembrane region (JM), followed by a tyrosine kinase domain (TKD), a sterile α motif (SAM) and a postsynaptic density protein 95, discs large 1, and zonula occludens-1 (PDZ) binding motif.

The ligands ephrins show a conserved extra-cellular receptor binding domain (RBD). Ephrins A are linked to the membrane through a GPI portion, while the B class has a C-terminal tail that extends into the cytoplasm of the ligand-exposing cell through a TM element and a PDZ domain which loads a Tyr residue (P) (Figure 2A and 2B).

Biological responses are induced following the phosphorylation of Tyr residues (P) found in the juxtamembrane domain between the TK and TM domains, as well as in the TK and SAM domains (Perez White et al. 2014).

1.2 Eph/ephrin signalling

1.2.1 Forward and reverse signalling

The canonical Ephs/ephrins activation starts by cell-cell contact, because they are membrane tethered proteins. In particular the ligand-binding domain of the Eph receptor interacts with the receptor binding domain of the ephrin expressed on a neighbouring cell. The result is a bidirectional signalling: eph receptor bearing cell brings the so called *forward* signalling, while ephrin ligand propagates the *reverse* signalling (Figure 2D).

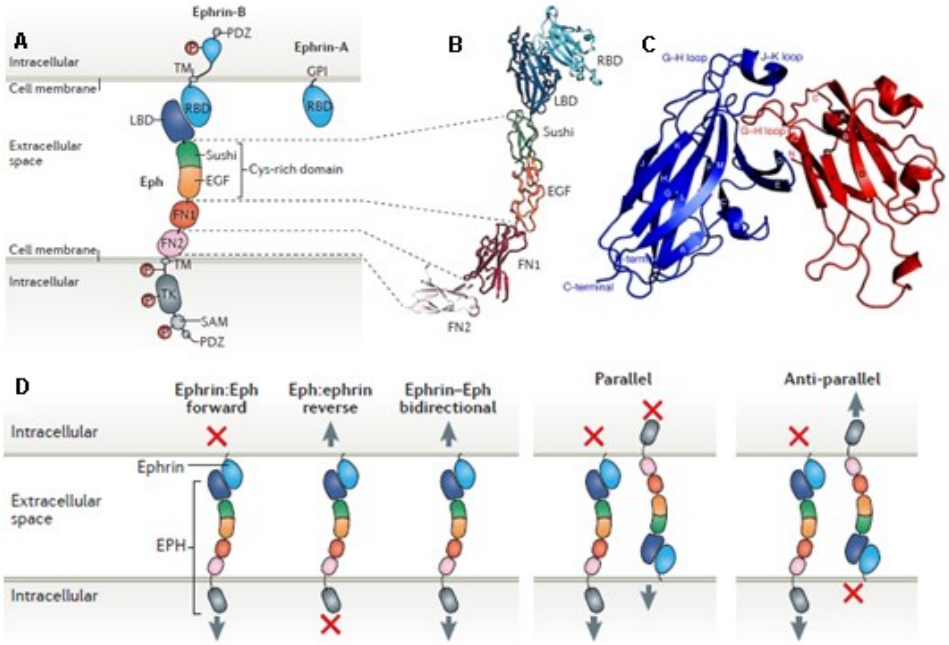


Figure 2: Representative display of Eph/ephrin structures (A) and interaction modes (D); crystal complexed EphA2/ephrin-A5 and specific RBD/LBD bond (B) (Kania et al. 2016); crystal structure of the complex of EphA2 (blue) and ephrin-A1 (red) with labelled secondary structures (C) (J. P. Himanen et al. 2009)

The first ephrin:Eph *forward* signal involves the kinase feature of receptors until the activation of mechanisms of autophosphorylation of juxtamembrane Tyr residues.

In the second Eph:ephrin *reverse* B signalling, SRC kinase mediated phosphorylation is crucial for downstream signalling. As regard *reverse* A signal, lacking Tyr domains, it is based on supposed triggering interaction with other membrane proteins, like p75 low-affinity nerve growth factor receptor (T.R. McLaughlin, 2007).

This kind of interaction between opposite cells is named *trans* activation and it can spread in a parallel or antiparallel way. In fact, the parallel one happens when Eph and ephrins located on the same cell respond to the counterparts presented by neighbouring cell. In contrast, anti-parallel pathway brings to a simultaneous activation of *forward* signals between related cells (Figure 2D) (Kania et al. 2016).

A second type of signalling named *cis* activation is possible when Eph receptors and the ephrin ligands interact on the same cell, resulting in the reduction of the *forward* signalling (Falivelli et al. 2013).

Despite *cis* or *trans* activation requires in both cases the autophosphorylation of Tyr residues, recent works showed that beyond this catalytic action, Eph receptors might

exhert non-catalytic signalling. This is the case of EphA10 and EphB6 which are also called pseudokinases because they do not expose catalytic sites. The signalling is allowed by other B receptors such as EphB1 and EphB4 which transphosphorilate EphA10 and EphB6. Moreover, the formation of stable hetero-complexes can initiate cytoplasmic signalling events. (Freywald et al. 2002).

Besides this well-known direct cell-cell communication, evidence is provided of a paracrine manner of Eph activation by a soluble and active form of ephrin-A1 ligand released from glioblastoma multiforme cells and breast cancer cells. In this case the involvement of metalloproteases is crucial to cleave ligands from the plasma membranes (Wykosky et al. 2008).

Regardless of the presence of soluble forms, it has been established that EphA interacts with ephrins in different manner compared to EphB. In particular B-types interact through an *induced-fit* mechanism, while A-class types show a *lock and key* one.

This concept came since the discovery of the complementarity between the interaction surfaces of EphA2 and ephrin-A1: G-H loop of ephrin-A1 is inserted in a pocket of EphA2 constituted by its D, E and J strands on one hand and G and M on the other side (Figure 2C). This binding is dominated by Van der Waals interactions between hydrophobic portions. Moreover, hydrogen bonds between ephrin-A1 β-sandwich strands C, G and F interact with B-C loop of EphA2 strengthening the binding, and no conformational changes are observed, resulting in firm and complementary structures.

In contrast, following the *induced-fit* mechanism, EphB/ephrin-B interactions require energy and conformational adjustment to put in action proper interaction surfaces (J. P. Himanen et al. 2009).

1.2.2 Clustering modes

In order to trigger a signal, Eph receptors need to form dimers like other RTK's as first step. But the typical feature of this system is that it requires pre-clustered ligands and multimeric structures to ensure down-stream responses. Before activation, initial distribution of receptors on cell surfaces is poor with no or low basal kinase activity. After first Eph/ephrin binding, receptors start to homodimerize without any biological effect in most cases. By the way there are examples of a heterodimer inducing responses

such as the complex EphA2/ErbB2 which promotes metastatic progression in human and murine breast adenocarcinoma cells (Brantley-Sieders et al. 2008).

Then receptors and ligands start to colocalize and concentrate in microdomain or rafts and they start to build higher order complexes which amplify the signal (Figure 3A).

In fact, the extension of clustering determines the strength of the signal such as demonstrated on HeLa cells expressing EphB2. Here EphB2 dimers produced very minor cell contractions compared to the collapsed response obtained after EphB2 clustering (Schaupp et al. 2014).

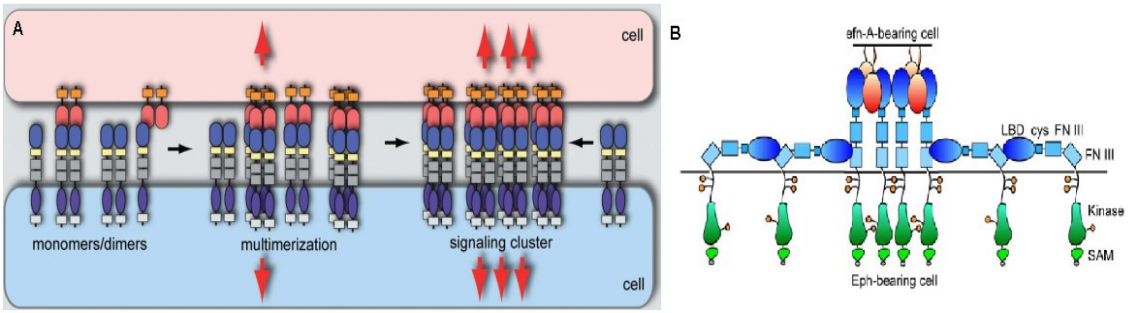


Figure 3: Schematic Eph/ephrin monomer/dimer and cluster representation (A) (Pitulescu et al. 2014); EphA4 cluster oligomerization far away from the area of ephrin contact (B) (Nikolov et al.2013)

Since clustering started, higher order aggregate might be formed in a ligand independent manner too. As shown in figure 3B, EphA4 extracellular domain binds the fibronectin III moiety of neighbouring receptors thus implementing EphA clusters and signal without any interaction with ligands (Nikolov et al. 2013).

1.2.3 Attenuation of Eph/ephrin signalling

Like others RTKs, general mechanisms of Eph receptor pathway attenuation are phosphatase-dependent dephosphorylation, proteolytic cleavage, or endocytosis.

Negative regulation of Eph receptors signalling can be exerted by different protein tyrosine phosphatases (PTP). For instance, ephrin-A1/EphA2 interaction can induce repulsive response, cell proliferation, cell adhesion and spreading on PC3 cells, through MAPK pathway attenuation, while low molecular weight PTPs operate EphA2 dephosphorylation, thus dampening proliferative and adhesive outcomes (Parri et al. 2005).

Under ephrin binding, Eph receptors could be internalized and degraded by trans-endocytosis, with a combined internalization of Eph/ephrin complexes through a clathrin and Rac-1 dependent manner. So, the recruitment of Rac-1 regulators can influence the destiny of Eph receptors on cell surface (Boissier et al.2013).

Internalization could be also mediated by receptor proteolytic cleavage as for EphA3/ephrin-A5 binding. EphA3 cleavage is disintegrin- and metalloprotease 10 (ADAM10) -dependent in kidney epithelial cells (Janes et al. 2009).

Ubiquitin-mediated degradation is another mechanism of attenuation of Eph receptor signalling. Following EphA/ephrin-A interaction, C-CBL (***C****asitas* ***B****-lineage* ***L****ymphoma*) protein operates ubiquitin ligation of EphA class thus resulting in receptor degradation (Boissier et al.2013). Furthermore it has been demonstrated that Src-like adaptor protein (SLAP) negatively regulates EphA2 exposure on cell surface with suppressive action on colorectal cancer cells (Naudin et al. 2014).

Taken together, these studies represent a panel of attenuation modes of Eph/ephrin signalling.

1.3 Pharmacological tools for Eph/ephrin system modulation

Ephs and ephrins are involved in many pathophysiological functions. Their altered expression in pathology offers the possibility to apply different pharmacological strategies in order to restore correct Eph/ephrin activation signalling. There are multiple tools that can be adopted to have an agonist or an antagonistic effect on the Eph/ephrin pathway but its typical feature of bidirectional or ligand-independent signalling complicates the pharmacological modulation. In fact, **ephrins** or **Ephs fused with Fc**, besides having a longer in vivo half-life than native proteins, can act as both ectopic activators or as competitors of endogenous Eph–ephrin interactions. Indeed Fc-fused proteins activate in a specific way *forward* or *reverse* signalling. For example, ephrin-Fc-conjugated (Eph agonist) can activate exclusively *forward* signals by linking the respective receptor and competes with endogenous ephrins thus preventing *reverse* signalling. In the same way, Eph-Fc-conjugated (ephrin agonist) can activate exclusively the *reverse* signal, by linking endogenous ephrin ligands and blocking the *forward* one.

Recombinant proteins have a long in vivo half-life, especially when they are Fc-conjugated (Barquilla e Pasquale 2015).

For this reason Eph/ephrin modulators cannot be strictly classified from a classical pharmacological point of view (Giorgio et al. 2020).

The main strategies for the blockade of this system are the use of **kinase inhibitors** and **protein-protein interactions inhibitors** (Figure 7). The first strategy brings to selectively inhibit Eph *forward* signalling while the second prevents the receptor-ligand binding thus blocking the bidirectional signalling (Tognolini et al. 2014).

Compensatory redundancy remains the first challenge that needs to be overcome, also considering off-target effects of a potential unselective Eph/ephrin modulator.

1.3.1 Kinase Inhibitors

This class is constituted by small molecules with high affinity to the ATP binding pocket of Eph receptors. For instance the selective molecule **NVP-BHG712**, synthesized by Novartis, was able to inhibit EphB4 autophosphorylation and vessel formation in vivo after oral administration, in mice (Martiny-Baron et al. 2010). Some of these compounds also reached clinical Phase II in non-small-cell lung cancer management, which is the case of compound **XL647**, developed by Exelixis, which is able to inhibit EphB4 activity in vivo (Pietanza et al. 2012).

1.3.2 Protein-protein interaction inhibitors (PPI)

PPIs can belong to Eph or ephrin recombinant proteins, peptides, antibodies and small molecules.

Recombinant extra-cellular domains (ECDs) of **Eph receptors** or **ligands** in soluble form are employed as surrogates of their counterparts to produce downstream stimulation or inhibition. **Eph ECDs** can block both *forward* and *reverse* signalling interacting with ephrin ligands and preventing classical clustering. This was the case of monomeric EphB4 that displayed promising anticancer and antiangiogenic effect in animal models (Pasquale et al. 2010).

Small peptides are more specific than soluble proteins because they are thought to selectively bind the binding pocket of Eph receptors. Several dodecapeptides have been identified having antagonist properties versus EphA2, EphA4, EphB2 and EphB4

(Noberini et al. 2012). Some EphA2 targeting peptides displayed both inhibition of ephrin binding and phosphorylation of EphA2 thus modulating EphA2 signalling (Mitra et al. 2010). Conversely to what happens with endogenous ephrins, these peptides do not provoke receptor internalization and degradation (Koolpe, et al. 2002). Among them the EphA4 targeting **KYL peptide** showed the capacity to interfere with integrin-mediated T cell adhesion to endothelial cells (Sharfe et al. 2008; Noberini et al. 2012).

Antibodies can take advantage of their elevated binding affinity, specificity and of their long in vivo half-life. Monoclonal antibodies having both activating and inhibitory effects have been developed. Through the recognition of Eph/ephrin ECDs they were used in anticancer and antiangiogenesis applications (Pasquale et al. 2010).

Lastly, **small molecules** can interpose the Eph/ephrin system simultaneously linking specific regions of the receptors and the ligands. The identification of compounds able to do that is challenging because they should interfere with a large surface of protein-protein contact. In this class it is possible to count lithocholic acid derivatives. **UniPR1331** belongs to this class and it has been defined as a potent ephrin-Eph pan-antagonist endowed with good oral bioavailability (Castelli et al. 2015), and in vivo anti-angiogenic and anti-vasculogenic properties (Festuccia et al. 2018).

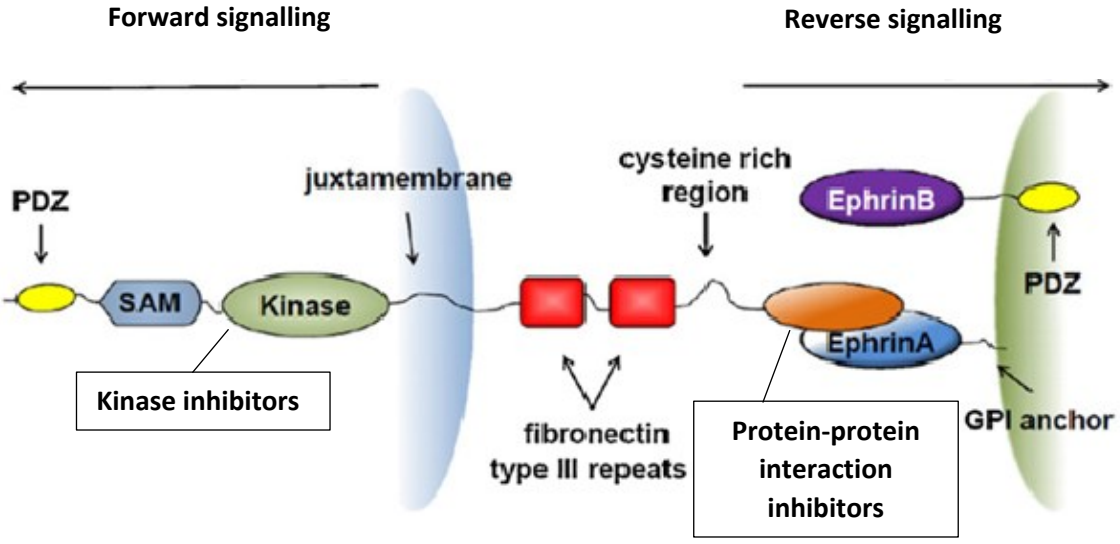


Figure 7: Pharmacological blockade of Eph/ephrin System (adapted from Funk et al. 2013)

2 Pathophysiology of Eph/ephrin system

2.1 Eph/ephrin system between attraction and repulsion in tissue development, maintenance and function

The discovery of modes of Eph/ephrin interaction helped to elucidate the physiological processes governing the developmental and adult organ homeostasis. Indeed this system is extensively expressed during embryogenesis, playing a pivotal role in compartmentalization and boundary formation especially in cardiovascular and central nervous systems (Pasquale E.B., 2008). Furthermore, Ephs/ephrin system affects cytoskeleton organization, cellular junctions, cell shape, cell-substrate adhesion and finally cell movement (Egea e Klein, 2007). Different Eph/ephrin-mediated functions are present even in adult life as regard bone and intestinal homeostasis (Kosinski et al. 2007; Davy et al. 2004) and immune system (Juan Jose Muñoz et al. 2011).

Central nervous system: during embryogenesis Ephs and ephrins are expressed in the leading moiety of growing neurons in order to keep axon guidance and help to produce the complex network of the nervous system. In fact, one of the most important functions of Ephs and ephrins is the repulsive effect after cell-cell interactions during the embryonic life. One example is the repulsion of trunk neural crest cells from motor axons of the caudal half of somites due to EphB3-ephrin-B1 interaction. In fact, neural crest cells expressing EphB3 bind caudal sclerome cells expressing ephrin-B1, avoiding the extension of neural crest into the caudal region. This suggests an important role in the definition of axon direction (Krull et al. 1997). In parallel with axon guidance, Eph/ephrins seem to be involved in cell compartmentalization. In fact pharmacological Eph/ephrin modulation provoked disruption of CNS structures in zebrafish embryos (Durbin et al. 1998).

By the way Eph/ephrin system is still expressed after complete CNS development and both A and B classes have been found in different neural cells, including glial cells, synaptic terminals of hippocampal and cortical neurons and perisynaptic astrocytes (Liebl et al. 2003). In particular, neuron-glia interaction is essential in CNS maintenance and plasticity; in adult hippocampus astrocytes expressing ephrin-A3 communicate with

EphA4 expressed by dendritic spines, resulting in the retraction of dendritic spines and the reduction of their number and size. This spine retraction is the result of β1-integrin disruption mediated by EphA4 *forward* signal activation following astrocyte interaction (Murai et al. 2011).

The importance of Eph/ephrin system in the adult CNS has been confirmed in a mouse model of Alzheimer's disease in which downregulation of EphA4 and EphB2 has been detected in the hippocampus and put in relation with object recognition and spatial memory failure (Simón et al. 2009).

Bone tissue: Eph/ephrin pathway plays a central role also in bone development and cell segregation as indicated by the defects in chondrocytes, that form skeletal elements, detected in ephrin-B1 knockout mice. In fact, the complementary expression of ephrin-B1 in chondrocytes and of EphB receptors in surrounding tissues reminds to their potential action on chondrotissues segregation. Moreover in the same mouse model the uprising of skeletal malformation and palatal clefts bringing to cranio-frontonasal syndrome have been detected (Davy et al. 2004).

Despite this clear embryonic function Ephs and ephrins modulate osteoblast and osteoclast balance also in the adult life thus controlling bone remodelling. It has been demonstrated that osteoblasts produce cytokines which lead to osteoclast activation, differentiation and ephrin-B2 overexpression. In turn, this osteoclast ligand interacts with EphB receptors on osteoblasts avoiding osteoclast bone resorption and promoting osteoblast bone formation (Pasquale EB, 2008).

Pancreas: Since the discovery of Eph/ephrin expression on pancreatic islet β-cells, its potential function in glucose metabolism has been postulated. It seems that bidirectional signalling can control insulin secretion. In fact, β-cells communicate through EphA/ephrin-A system in response to glucose. In particular, under basal condition, β-cells activate EphA *forward* pathway to suppress insulin secretion; in contrast the activation of ephrin-A *reverse* signalling promotes insulin production when the plasmatic concentration of glucose is high. This scenario opens to new pharmacological tools for type 2 diabetes treatments. It was further showed that EphA-ephrin-A-mediated β cell communication is bidirectional: EphA *forward* signaling inhibits

insulin secretion, whereas ephrin-A *reverse* signaling stimulates insulin secretion. EphA *forward* signaling is downregulated in response to glucose, which indicates that, under basal conditions, β cells use EphA forward signaling to suppress insulin secretion and that, under stimulatory conditions, they shift to ephrin-A *reverse* signaling to enhance insulin secretion (Konstantinova et al. 2007).

Cardiovascular system: EphB/ephrin-B system is involved in the development of the vasculature and the mechanisms repulsion-based lead to arterial and the venous compartment separation. In particular ephrin-B2 is expressed mostly on arterial path while its receptor, EphB4 on venous one. In fact, at a capillary level, endothelial precursors are a mixed population expressing the ligand or the receptor, and repulsive interactions bring to the two different vascular compartments (Adams et al. 1999). Ephrin-B2/EphB4 pathway also induces sprouting angiogenesis via clathrin-mediated VEGF (vascular endothelial growth factor) receptors internalization (Wang et al. 2010).

Thymus: The attention on a possible immune-regulatory function of Ephs and ephrins was hypothesized after the discovery of their expression in the thymus. (Gurniak et al. 1996). It relates to T-cell development and selection, thymus microenvironment and T-cell organization. For instance EphA4 knockout mice showed T cell maturation failures (decreased proportion of CD4+ CD8+ double positive cells), together with unstable thymic epithelium (Juan J. Muñoz et al. 2006). As regard Eph/ephrin-B system, it has been demonstrated a role for EphB2 in thymus-settling progenitors. Thymus does not possess self-renewing progenitors, but it receives lymphoid bone-marrow progenitors through the vasculature. EphB2 deficient bone-marrow progenitor cells displayed lower colonization properties than wild types (Juan Jose Muñoz et al. 2011).

Concerning thymocyte development, EphA *forward* signal modulates the selection of immature cells and TCR-mediated (T-Cell receptor) responses. In fact, ephrin-A1-Fc (EphA pathway stimulator) reduced IL-2 release by immature CD4+ CD8+ thymocyte; IL-2 is an autocrine factor of T-cell proliferation and differentiation; in parallel ephrin-A1-Fc blocked apoptotic TCR-based pathway. Normally this signal starts when thymocyte TCR binds self-antigens with high affinity aiming to eliminate self-responsive clones.

Taken together these findings demonstrated that EphA2 *forward* signal is involved in negative selection of self-responsive T-cells (Wu and Luo 2005).

Gastrointestinal tract: mostly all forms of Ephs and ephrins are expressed in human small intestine and colon (Hafner, Meyer, Langmann, et al. 2005). Here their engagement in cell organization has been identified in a study aimed at the patterning of the crypts. Crypts continuously face epithelial renewal of differentiated cells. EphB2 and EphB3 are highly expressed at the bottom of the crypts where proliferative cells inhabit. As cells differentiate and proliferate, they move up along crypts and villi. This movement is pushed by Wnt signalling which induces upregulation of EphB2/EphB3 in undifferentiated cells. In contrast differentiated cells downregulate EphB receptors and upregulate ephrin-B1 and ephrin-B2 (Figure 4). Differential expression along crypt-villus axis avoids intermingling of proliferative cells into the region of differentiated cells. In this way EphB/ephrin-B system keeps segregation of the dynamic intestinal cell populations. In fact intestinal cells presenting EphB activate metalloprotease ADAM10 to cell-cell contact region to cleave E-cadherins and break adherence junctions between stem cells and differentiated cells thus limiting their intermingling (Solanas et al. 2011). Typical behaviour is that one of Paneth cells that move towards the crypt at the base of the villus although they are differentiated. A concomitant gradient in EphB receptors expression appears; in fact, if EphB2 is highly expressed in stem cells, EphB3 is a specific B receptor of Paneth cells. EphB3 null mice presented Paneth cells in wrong sites and EphB2 null mice showed intermingling of epithelial intestinal cells (Batlle et al. 2002). Also EphA/ephrin-A system is differentially expressed along crypt-villus axis, that is EphA1 is present in crypts while EphA2 and ephrin-A1 at the top of villi (Kosinski et al. 2007). By the way EphA/ephrin-A involvement in the control of intestinal architecture still needs to be proven.

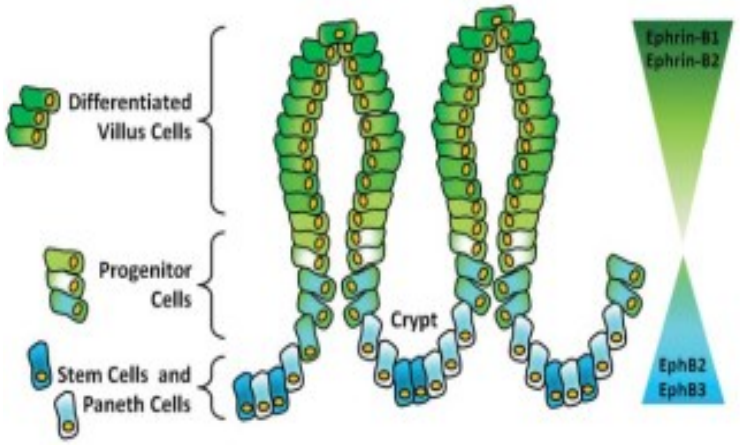


Figure 4: Differential expression of EphB2 EphB3 and ephrin-B1 ephrin-B2 along crypt-villus axis (Perez White et al. 2014)

However, studies on epithelial cell lines, including colon carcinoma cells demonstrated enhanced paracellular permeability after EphA2 stimulation with ephrin-A1-Fc. This event brings to the receptor phosphorylation and claudin-4 recruitment; claudin-4 cannot interact with zone-occludens-1 (ZO-1), essential cell-cell junction player, thus increasing cell-cell permeability (Tanaka et al. 2005).

2.2 Eph/ephrin system in inflammation

2.2.1 Inflammation and vascular modification

Inflammatory process is marked by tight interactions between endothelial cells and circulating immune cells as a necessary requirement for their local recruitment and activation. Pro-inflammatory cytokines released from engaged tissues rapidly provoke exhibition of adhesion proteins (I-CAM) on the luminal portion of endothelial cells for immune cells recruitment. Moreover cytokines (TNF-α, IL-1) weaken adherens and tight junctions between endothelial cells regulating vascular barrier.

Here, immune cells roll over initiated endothelial surface and get activated. In this way, chemotaxis starts through endothelial cells, taking advantage of inter-endothelial cell junctions impairment (Schnoor et al.2008).

Together with tight junctions (claudins, occludins and JAM-A), adherens junctions, whose principal component is VE-cadherin, are connected with actomyosin filaments influencing cell shape. All these proteins communicate with Rho family guanosine

triphosphatases (RhoA, Rac1,Cd42), known targets of Eph/ephrin signalling; if the two latter proteins stabilize cytoskeleton, RhoA disrupt actin filaments upon inflammatory stimuli like thrombin and VEGF (Coulthard et al. 2012) (Figure 5).

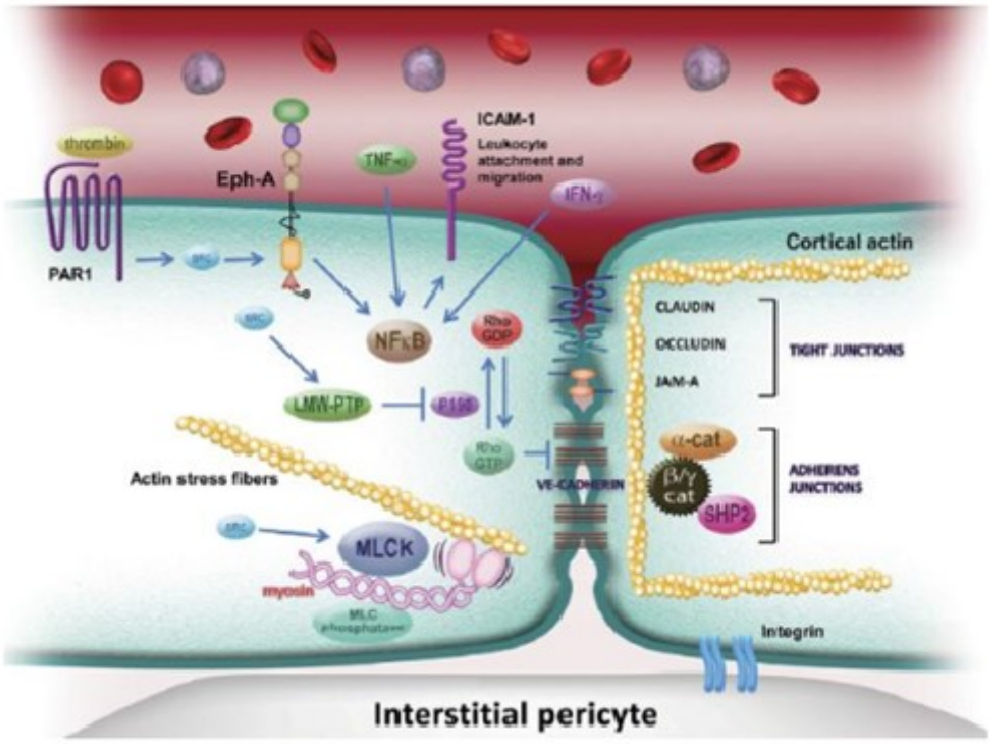


Figure 5: Systems of cell morphology modulation (Coulthard et al. 2012)

On this background there are increasing studies showing Eph/ephrin system ability to control different steps during inflammation. The starting evidence which identified a possible role of Ephs end ephrins in inflammation came from a vascular biology study showing the presence of a TNF-α responsive *ephrin-A1* gene in endothelial cells (Dixit et al. 1990).

Later, Larson and colleagues demonstrated that EphA2 signalling, stimulated by ephrin-A1-Fc exposure, increased pulmonary vascular permeability both in vivo and in vitro. This ligand-receptor couple expression raises in injured lungs, and EphA2 activation provoked tight and adherens junction disruption on human lung microvascular endothelial cells. In vivo, intravenous injection of ephrin-A1 increased the leakage of labelled albumin as a result of enhanced permeability (Larson et al. 2008).

Consistent with the demonstration that EphA2 and ephrin-A1 are associated with endothelial cell activation, ephrin-A1 mRNA expression has been found increased in LPS processing organs (lung, liver and brain) in rat models of inflammation (Larson et al. 2008).

Moreover, EphA2 *forward* signalling enhances endothelial permeability because of mislocation of the cell-cell junction proteins claudins-4, claudins-5, zonula occludens-1 and VE-cadherin as demonstrated in cell cultures after ephrin-A1-Fc exposure (Tanaka et al.2005).

As regard immune cells rolling and transmigration, EphA2 activation enhances the exposition of ICAM-1 on endothelial cell line (Chan e Sukhatme 2009), VCAMS and E-selectins on atherosclerosis-prone macrovascular endothelium (Funk et al. 2012).

In contrast with the findings observed for A system, there are no evidence about the correlation between inflammatory mediators and EphB/ephrin-B in endothelial homeostasis, although endothelium expresses EphB1, EphB2, EphB4 as receptors, and ephrin-B1 and ephrin-B2 as ligands. By the way, promoter gene of ephrin-B2 is responsive to the proinflammatory transcription factor NF-κB (Funk et al. 2013).

Consistent with potential role of ephrin-B2 in endothelial cell activation, a study carried out on HUVEC cells (Human umbilical vein endothelial cell) demonstrated that ephrin-B2 *reverse* signalling promoted cell pro-inflammatory differentiation and increased permeability in addition to E-selectin and VCAM-1 exposure (Liu et al. 2014).

Taken together these results particularly show that EphA2, ephrin-A1 and ephrin-B2 are involved in endothelial cell activation during vascular changes typical of inflammatory processes.

2.2.2 Inflammation and immune cell response

The activated endothelium modulates trafficking of leukocytes thanks to a coordinated action of adhesive molecules and chemoattractant agents. Tethering, rolling and firm adhesion of immune cells are progressive steps under the control of selectins, integrins and IgG-superfamily molecules in parallel with chemical gradients of chemokines, events that precede transmigration through the endothelium (Vestweber 2007).

Although the most important proteins involved in trafficking have been well defined, since their discovery, Ephs and ephrins have been proposed as cell surface molecules able to modulate immune cell trafficking.

In fact, the proof that ephrin-B2 expression changes on endothelial cells under shear stress (Goettsch et al. 2004) pushed towards the hypothesis that endothelial cells presenting ephrin-B2 interact with circulating cells expressing EphB receptors to promote trafficking.

More in detail, EphB receptors (especially EphB2 and EphB4) are expressed over hematopoietic cells derived from mouse bone marrow, mouse peripheral blood leukocytes, murine splenocytes and macrophages (F4/80+), as well in human CD14+ cells, human leukemic monocytes and macrophage cell lines . Pfaff and colleagues found pro-adhesive behaviour depending on direct monocyte-endothelium interaction respectively mediated by EphB/ ephrin-B2 link (Pfaff et al. 2008).

In the same direction goes a study performed on co-cultured monocytic THP-1 and an endothelial cell line. RNA silencing of ephrin-B2 on endothelial cells strongly inhibited diapedesis of THP-1 cells, abundantly expressing EphB2 (Liu et al. 2014). In turn, EphB *forward* signalling in monocytes seems to induce expression of proinflammatory chemokines. In particular ephrin-B2 expressed on endothelial cell interact with EphB2 and selectins of monocytes. The Eph/ephrin complex translocates towards cell-cell junctions thus leading diapedesis of monocytes (Braun et al. 2011) (Figure 6).

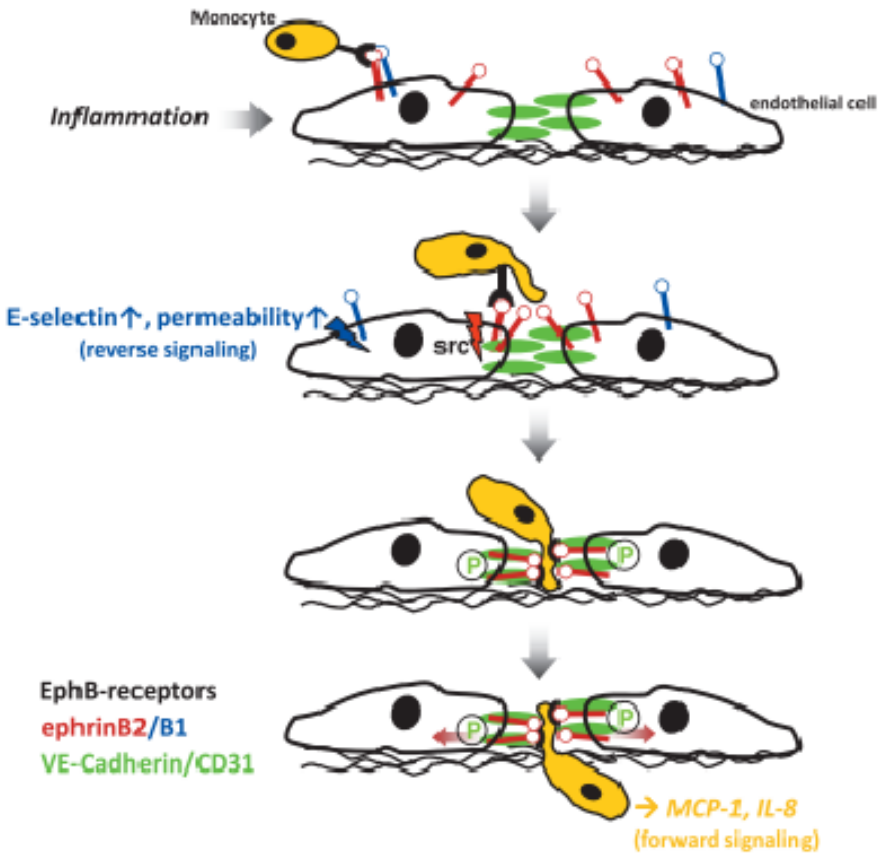


Figure 6: EphB/ephrin-B-mediated effects during inflammation (Liu et al. 2014)

Considering leukocyte biology involving EphA system, EphA2 expression is typical of dendritic cells and monocytes, EphA3 seems to be more present on lymphocytes while EphA4 and ephrin-A4, are present in different cells including T cells, B cells and monocytes (A. Sakamoto et al. 2011).

Beyond cell-specificity, Ephs and ephrins expression is not static, but rather changes following environmental conditions. For example culturing T cells for five days induces ephrin-A ligand expression (Funk et al. 2012).

EphA/ephrin-A interactions among T-cells and endothelium involve integrin mediated adhesion mechanisms. Activation of *reverse* signals of T cells with EphA2-Fc enhanced tethering to $\alpha 4\beta 1$ integrins and transmigration through endothelium (Trinidad et al. 2009). In contrast T cells treated with ephrin-A-Fc show reduced adhesion, demonstrating that *reverse* A-type signal spreading on T cells decreases interactions

with the endothelium (Sharfe et al. 2008). In parallel ephrin-A4-Fc stimulus enhanced EphA2 colocalization with ICAM-1 and VCAM-1 on endothelial cells (Trinidad et al. 2009). Taken together these studies provide evidence that EphA/ephrin-A system modulates lymphocyte adhesion.

2.3 Eph/ephrin involvement in acute and chronic diseases

2.3.1 Acute inflammatory diseases

One of the most commonly used models to study the acute inflammation is the ischemia/reperfusion technique. The ischemic event followed by blood reperfusion pushes to inflammatory reactions which are mediated by TNF-α, IL-1 and IL-6, overexpression of adhesion molecules and immune cells recruitment (Cannon et al. 2005).

In particular, renal EphA2 expression raised in murine models of ischemia-reperfusion injury (IRI) not only in vivo but also in vitro. In fact cultured renal tubular cells exposed to IRI presented the same EphA2 over expression (Baldwin et al. 2006).

Even cutaneous hypoxic models helped to understand Eph/ephrin engagement during hypoxia. In fact, in the mouse skin flap model of hypoxia, up-regulation of EphB4, ephrin-B2, EphA2, and ephrin-A1 was detected together with increased hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF). These data demonstrated that hypoxia is an important regulator of Ephs/ephrins expression (Vihanto et al. 2005).

Finally, effects dependent on EphA *forward* and *reverse* signal or on blocking both of them were explored in murine mesenteric ischemia-reperfusion (I/R) studies administering ephrin-A1-Fc, EphA2-Fc or monomeric EphA2. Stimulation of *forward* signal by ephrin-A1-Fc reverted intestinal and pulmonary injury caused by I/R, reducing leukocytes recruitment and IL-1β and TNF-α levels in the pulmonary district. The blockade of Eph/ephrin A system counteracted intestinal oedema and lung proinflammatory cytokines release (Vivo et al. 2017).

2.3.2 Chronic inflammatory pathologies

First evidence of Eph/ephrin potential involvement in chronic inflammatory diseases came from studies on cultured rat cardiac myocytes. These myocytes exposed to pro-inflammatory cytokines such as TNF-α and IL-1β showed altered expression of EphA3 (Li Y.Y. et al. 1998). Accordingly, also chronic heart failure is marked by high levels of circulating TNF-α and IL-1β which can influence EphA myocardial expression. This observation paved the way towards the study of Eph/ephrin system on multiple chronic inflammatory diseases as in rheumatoid arthritis (RA), osteoarthritis (OA), multiple sclerosis (MS), atherosclerosis (AS), and inflammatory bowel disease (IBD).

Rheumatoid arthritis is a chronic inflammatory disease which brings to tendons, cartilage and bone destruction due to abnormal immune responses and synovial proliferation (Gay et al.1993). Proliferation and activation of synovial cells, abnormal T cell homeostasis and dysregulation in peripheral tolerance seem to be the foundation of this pathology (Fox 1997). Specifically, synovial fibroblasts, peripheral blood lymphocytes and invading CD3-positive lymphocytes coming from RA patients express higher levels of ephrin-B1 compared with samples from healthy individuals. Moreover murine synovial cells released TNF-α and IL-6 in response to ephrin-B1-Fc treatment showing a direct role of EphB1 receptor in proinflammatory cytokines production (Kitamura et al. 2008). More recently the analysis of clinical parameters showed that ephrin-B1 upregulation on T-cells of RA patients correlates with the severity of the pathology (Hu et al. 2015).

Osteoarthritis is a pathology in which abnormal subchondral bone metabolism is crucial and, as mentioned, Ephs and ephrins are involved. In particular, in OA patients a subpopulation named “L-OA osteoblast”, with proresorption properties is particularly abundant and overexpresses EphB4. It has been proven that the activation of *forward* signalling on these osteoblasts down-regulated various bone remodelling factors such as PGE-2 and IL-17 reducing bone resorption. Moreover, also the production of proinflammatory IL-1 and IL-6 is favoured thus diminishing osteoclast survival and activity. In this sense EphB4 signalling stimulation could be identified as a therapeutic approach in OA (Kwan Tat et al. 2008).

Multiple sclerosis is a chronic and demyelinating disease characterised by nerve pain and paralysis; its etiology is still unclear: even though genetic and environmental factors are involved, a specific disease biomarker is not available to date. Given the importance of Eph/ephrin system in neurones development and maintenance, the expression of these receptors and ligands has been studied in active MS lesions as well as in normal-appearing white matter (NAWM) of Human CNS tissue samples. Reactive astrocytes and macrophages display a wide panel of A-type Eph and ephrins. Axonal EphA3, A4, A7 and ephrin-A1 were found overexpressed both in active lesions and in NAWM close to active lesions compared to control samples (Sobel 2005).

First evidence of Ephs and ephrins involvement in **Atherosclerosis** raised from genetic expression analysis in the atherosclerotic plaque. Human carotids of atherosclerotic patients showed altered expression of ephrin-B1 and EphB2 and the presence of macrophages presenting A and B Eph/ephrin (Funk et al. 2012; Aiji Sakamoto et al. 2008).

Moreover, there was an overexpression of ephrin-B2 in pro-atherosclerotic endothelium of murine aorta compared to healthy regions. Eph/ephrin-A system has been associated with plaque progression as well, as observed in ApoE or LDL receptor knockout murine models. In these cases, in the plaque, mRNA encoding EphA2 showed higher levels than in healthy mice (Yang et al. 2010). All these studies suggest that the Eph/ephrin system is crucial in plaque remodelling, development and macrophage recruitment.

Finally, the importance of Eph/ephrin system in intestinal epithelial cell differentiation within the crypts and its role in preventing local intermingling, pushed to investigate Ephs and ephrins expression in **Inflammatory bowel diseases**. Specifically, EphB2, and ephrin-B1/B2 were up-regulated in intestinal epithelial cells of mucosal lesions in patients with IBD compared to healthy donors. Moreover ephrin-B stimulation on IEC-6 cells (intestinal epithelial-6 cells) promoted higher wound closure capacity (Hafner et al. 2005). In fact it was demonstrated that Ephrin-B *reverse* signaling induces expression of wound healing associated genes such as *Cox-2*, *c-Fos*, *Egr-1*, *Egr-2*, and *MCP-1* involved in cell migration, antiapoptotic, host defence pathways (Hafner et al. 2005b).

TYPE	PATHOLOGY	MODEL	EFFECT	Reference
ACUTE	**Ischemia-reperfusion I/R**	Cultured renal tubular cells	EphA2 over expression	Baldwin et al. 2006
		Mouse skin flap model of hypoxia	EphB4, ephrin-B2, EphA2, and ephrin-A1 over expression	Vihanto et al. 2005
		Murine mesenteric I/R	*A-Forward* signal reverted intestinal and pulmonary injury	Vivo et al. 2017
CHRONIC	**Rheumatoid arthritis**	RA patients	-Invading lymphocytes expressing ephrin-B1 -EphB1 FW signal in synovial cells: cytokines release	Kitamura et al. 2008
	Osteoarthritis	OA patients	EphB4 FW signal on L-OA osteoblast: reduced bone resorption	Kwan Tat et al. 2008
	Multiple Sclerosis	MS patients	Reactive astrocytes and macrophages display a wide panel of A-type Eph and ephrins	Sobel 2005
	Atherosclerosis	AS patients	Macrophages presenting A and B Eph/ephrin in the atherosclerotic plaque	Funk et al. 2012 Aiji Sakamoto et al. 2008
		Murine AS model	Eph/ephrin-A system has been associated with plaque progression	Yang et al. 2010
	Inflammatory bowel diseases	IBD patients	EphB2, and ephrin-B1/B2 were up-regulated in mucosal lesions	Hafner et al. 2005
		IEC-6 cells	ephrin-B stimulation promoted higher wound closure capacity	Hafner et al. 2005b

Table: schematic table summarizing the role of the Eph/ephrin system in acute and chronic diseases

3 Human IBD and in vivo/in vitro models

3.1 Inflammatory bowel disease

Inflammatory bowel Disease (IBD) is a chronic inflammatory status comprising both Crohn's disease (CD) and ulcerative colitis (UC), pathologies characterized by alternating phases of clinical relapse and remission. Crohn's Disease can involve any intestinal portion, often in a discontinuous manner. Inflammation is mostly transmural and correlates with intestinal granulomas, strictures, and fistulas. On the other hand, ulcerative colitis might affect the colon, in part or entirely (pancolitis), with an uninterrupted pattern. In this case inflammation is local and confined within the mucosa (Abraham et al. 2009).

Microbiota, innate barriers of protection and innate and adaptive immunity balance are crucial in gut homeostasis. In fact the pathogenesis of IBD is the result of a multitude of altered relationships among susceptibility genes, host microbiome interactions, intestinal epithelial cells functions and immunity (K. L. Wallace 2014) (Figure 8).

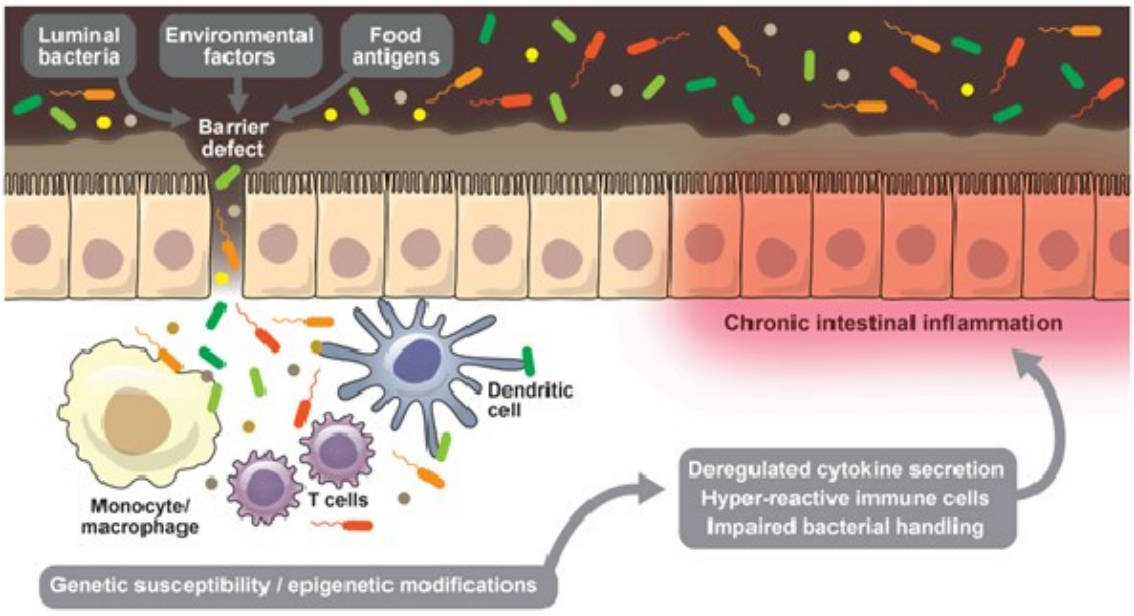


Figure 8: Current hypothesis of IBD pathogenesis (Swiss Med Wkly. 2018)

3.1.1 Pathogenesis and microbiota

The gut microbiota is not only composed of Bacteria, which represent the best characterized microbial population, but also of Virus, Archaea, Fungi and Protozoa. Indeed, the majority of data on IBD-associated dysbiosis are relevant to changes in Bacteria composition and load, however emerging studies are pointing to a role of Virus, Phage and Fungi in IBD pathogenesis (Matijasic et al. 2020). Intraluminal bacteria are in communication with intestinal epithelial cells and immune system providing key nutrients and host defence. The microbiota takes life at birth and develops quickly in few years. During adult life it is quite stable and defines a fingerprint of each individual but changes in quality and quantity could occur following environmental factors or in diseases (Eckburg et al. 2007). Simple evidence that correlates microbiota to IBD are the effectiveness of antibiotics in patients with IBD, or the bacterial dependent intestinal inflammation obtained in murine models of IBD (Elson et al. 2005a).

The importance of microbiota in IBD was strengthened since the discovery that IBD patients showed a different bacterial profile compared to healthy subjects, with a huge decrease in *Firmicutes* and *Bacteroidetes* (Frank et al. 2007). Concurrently in UC patients an increase in *Actinobacteria* and *Proteobacteria* was registered (Lepage et al. 2011). This profile is similar for CD, but in addition a new pathogen with high invasive behaviour belonging to E. coli class has been discovered. *Adherent-invasive E. coli* (AIEC) invades epithelium and survives and grows in macrophages participating to mucosal inflammation in CD (Martinez-Medina et al. 2009). Within this scenario it has been concurrently detected an increased production of serine protease activity in tissue biopsies of CD and UC patients (Denadai-Souza et al. 2018). In particular intestinal epithelial thrombin (F2), one of the serine proteases released in the gut, is thought to play a pivotal role in the physiological control of mucosal bacterial biofilm. In fact F2 is able to cleave microbiota biofilm-derived proteins, avoiding bacterial contact with epithelium, and limiting bacterial invasion (Motta et al. 2019).

3.1.2 Pathogenesis, mucus and epithelium

Intestinal epithelium is covered by a layer of mucus produced by Goblet cells along the mucosa. Mucus is a glycoprotein-based matrix which ensures spatial confinement

between epithelial cells and microbiota harbouring the gut. In fact, the inner layer of mucus is quite sterile and adherent, rich in antimicrobial peptides. On the other hand, the outer layer allows bacterial adhesion and interactions with the host. Biofilm encroachment over mucosal surfaces is one of the most important causes of long-lasting bacterial infections and defects in mucus stability which are associated with IBD (K. L. Wallace 2014). Indeed IBD patients can express a variant of *Muc2*, a gene encoding for a protein necessary for the correct mucus composition and again *Muc2* deficient mice develop spontaneous colitis (Elson et al. 2005b).

Moreover, CD is marked by Goblet cells depletion and by mucus layer impairments which permit a direct bacterial action on epithelial cells contributing to trigger an innate and adaptive immunity (Larsson et al. 2011).

Epithelium is the other player in gut homeostasis, considering that it is constituted by absorptive enterocytes, Goblet cells producing mucus, hormone producing enteroendocrine cells and Paneth cells producing antimicrobial and growth factors. Intestinal epithelium interplays between immune cells, harbouring the lamina propria, and microbiota (K. L. Wallace 2014). For instance, interactions between bacteria and enterocytes as well as immune cells happen through Pattern Recognition Molecules Signal receptors, such as NOD-like receptors (NLRs). Gene mutation of these receptors correlates with susceptibility to IBD. In addition NOD1 and NOD2 knockout mice displayed a reduced expression of E-Cadherin with increased epithelial permeability and reduced antimicrobial peptides release (Natividad et al. 2012). Hence epithelial paracellular space, controlled by tight junctions, regulates the activation of entire immune machinery.

3.1.3 Pathogenesis, innate and adaptive immunity

Although it is well known that adaptive immune system is the principal actor of IBD pathogenesis, also innate immune cells participate in the intestinal inflammation through macrophage, monocyte, neutrophil, DC, and natural killer cell (NK cells), eosinophil, and basophil reactivity. The latter is a non-specific immunity and does not have links with immune memory processes. On the contrary, the former is specific, produces long lasting immunity through the over activation of T-helper (Th) cells or the

reduced activity of anti-inflammatory T-regulatory cells (T-regs). Immune system activation brings to register in the intestinal mucosa high levels of TNF-α, Interleukin-1β, interferon-ɣ and cytokines of IL-23—Th17 pathway (Abraham et al. 2009).

More in detail, first immune barrier is the innate one, thanks to the capacity to display receptors in order to identify general microbial patterns. In fact, a constant sampling of intestinal bacterial antigens is provided not only by the epithelium but mostly by dendritic cells. These are antigen presenting cells that, following the antigen recognition, migrate in the secondary lymphoid organs e.g. Peyer's patches and mesenteric lymph nodes. Here the communication with CD4+ Naïve T cells triggers the adaptive immune response. Moreover, NOD2 is expressed by Paneth cells, macrophages and again dendritic cells. Its activation by microbial peptidoglycan provokes TNF-α, interleukin-1β release via factor NF-κB and mitogen-activated protein (MAP) kinase signalling (Abrahamet al.2006).

After T cell activation in secondary lymphoid tissues, pro inflammatory cell subtypes such as Th1, Th2, and Th17 are involved. In normal conditions their activity still exists and continually defends the epithelium from pathogens, but their expansion and over activation correlates with inflammation in IBD. Indeed, impairments of subgroups of CD4+ T cells were registered in the lamina propria of IBD patients together with increased Th17-derived interleukin-17 and the Th1-derived INF-ɣ and TNF-α. In contrast, ulcerative colitis is marked by intestinal increase of Th2 cytokines along with interleukin-17 (Fujino et al. 2003).

3.2 In vivo/in vitro models for IBD studies

A disease model should pursue three main properties: face validity, construct validity, and predictive validity. The first one means that an animal model should reproduce clinical conditions of a pathology as much as possible; the second one points at investigating a mechanistic relationship with the pathology by modulating several involved pathways; the latter would pursue reliability, aiming at the discovery of new therapeutic approaches (Mayer e Collins 2002). So far, animal models act as leading source of knowledge about biochemical systems which cannot be entirely mimicked in

vitro. Following construct validity and predictivity principles, animal models should widely investigate the efficacy of a test compound through the assessment of different endpoints. In IBD studies there are common endpoints assessing general health conditions such as animal weight, disease activity index or macroscopic score. Conversely, specific cytokines and immune cells could be followed as specific pathological endpoint.

In the last decades more than fifty animal models of IBD were proposed and categorised in several classes: chemically-induced, genetically-engineered, spontaneous and immune-mediated models (Mizoguchi et al. 2010).

Ethical issues surrounding the use of animals to interpolate human pathologies recently pushed to opt for in vitro/ex vivo models which allow to respect 3R principles, Replacement, Reduction, Refinement.

3.2.1 TNBS-induced colitis

2,4,6-trinitrobenzene sulfonic acid is a haptenating agent intrarectally administered in rodents to make epithelial proteins immunogenic to the immune system thus initiating mucosal inflammation in murine strains. The evoked immune response provokes CD-like severe ulcerations of the colon mucosa as well as induction of severe diarrhea, weight loss, and rectal prolapse. This condition is mostly due to a Th1-dependent response and is marked by infiltration of the lamina propria by CD4+ T cells, neutrophils, and macrophages with high local levels of TNFα, IL-1β, IL-12, IL-17, IL-18 and IL-6. Reproducibility and cost effectiveness are the advantages that characterise this mouse IBD model, widely used in basic and preclinical research. Even chronicity is allowed through multiple enemas and in chronic inflammation also Th17 counterpart is stimulated as well as the increased production of IL-17, IL-12, IL-10 and macrophage inflammatory protein-2 (Dothel et al. 2013). This model is widely used in immunological studies to clarify oral tolerance, intestinal fibrosis and spontaneous resolution mechanisms (Kiesler, Fuss, e Strober 2015).

3.2.2 DSS-induced colitis

Dextran sulfate sodium, DSS, is a polysaccharide able to induce loss of barrier integrity and for this reason it is widely used to study mechanisms of compounds that lead to

mucosal repair (Kiesler et al. 2015). The exact modality by which DSS produces intestinal inflammation is still unclear but there are several hypothesis; DSS could have a direct cytotoxicity on intestinal epithelium or it could produce a degradation of zonula occludens-1, one of the major components of tight junctions (Poritz et al. 2007).

DSS is added to the drinking water and different concentrations are generally used (2-5% w/V). Rodents are exposed to DSS for short periods (5-7 days), thus inducing an acute colitis marked by bloody diarrhoea, weight loss and granulocytes infiltration in the intestinal mucosa. These events are associated with a Th-1 mediated response with high release of INF-ɣ in the colon (Kim et al. 2011).

In contrast, repeated DSS cycles, followed by washout periods, switch inflammation into a Th2-mediated one, resembling features of human UC such as high levels of IL-4, IL-5 and IL-6 (Perše e Cerar 2012).

3.2.3 T-cell transfer model

Since no single animal model can entirely reproduce UC or CD pathologies and in order to have more translational power, immune-mediated models have been introduced. This class of models can employ immunological modulation to induce colitis in rodents. Hence, among immune-mediated systems to produce IBD, it has been recently developed T-cell transfer model in mice. A subpopulation of CD4+ cells with a CD45^{RB}high profile from wild type donors is intravenously injected to T and B cells immunocompromised mice. This manipulation brings to a crushing disease and colitis after 3-5 weeks following the transfer (Lindebo Holm et al. 2012). Cell subpopulation sorting seems to be necessary because there are proofs that the counterpart CD45^{RB}low cells inhibits the pro-inflammatory action of CD45^{RB}high cells (Powrie et al. 1995). The induced immune response is mainly Th1 mediated since the resolution could be obtained using treatments with anti-TNFα MAbs, or anti-IFNγ MAbs (Powrie et al. 1994). Histopathological analysis revealed wide intestinal inflammation in recipient mice with involvement from the cecum to the rectum. It could be transmural or restricted to the mucosa depending on mice strain susceptibility. Typical clinical signs are mucin depletion, epithelial hyperplasia, and increase in intraepithelial lymphocyte number (De Winter et al. 1999). In common with other models, inflammation is driven by bacteria,

indeed it does not occur in germ-free mice. So far it seems that T-cell transfer model finds a tight correlation with the presence of common commensal bacteria in the stimulation of the Th1 response (Powrie et al. 1995). Beyond the well-known immunological pathways involved, other main advantages are reproducibility and a predictable time course which makes this model very attractive for the study of T cell role in intestinal inflammation (De Winter et al. 1999).

3.2.4 Organoids

Intestinal epithelium plays different roles in gut homeostasis such as barrier function, digestion and absorption of substrates, microbiota modulation and contribution in host immune responses.

In order to investigate intestinal epithelial functions, cell culture methods have been introduced. Although immortalized cell cultures are employed in permeability and transport studies, there is evidence of protein mislocation and difference in protein expression compared to the native of the intestinal epithelium; moreover immortalized cells lack intestinal specificity being a single cell line culture, far away from the complexity of the multicellular intestinal identity (In et al. 2016). Because of these limitations organoids have been recently proposed as a better system to model the human epithelium. Enteroids and colonoids are ex vivo primary cultures obtained from small or large intestine respectively. Biological samples can be human or murine and it is possible to extract the crypts, the functional intestinal units, from healthy or diseased biopsies (M. Li et al. 2019).

Initially developing colonoids are spheroids, they have typical polarization with their apical side facing in, and cadherin-1, β-1 catenin expression in the basolateral side. In this state they are considered not-differentiated, keeping a high proliferation profile. Progressive organoid treatment with growth factors such as epidermal growth factor (EGF), Noggin, and R-spondin-1 promotes spheroid progression and differentiation in 3D intestinal organoids marked by neo crypts formation. Intestinal stem cell niches are physiologically supported by WNT3A produced by Paneth cells and their low abundance in organoid development needs to be compensated by exogenous WNT3A in the medium. Final organoids are constituted by different cell types like stem cells, Paneth

cells, enterocytes, Goblet cells and enteroendocrine cells. Moreover myofibroblasts and smooth muscle cells are present (Watson et al. 2014).

To summarize there are several advantages in the use of organoid model such as the possibility to have a diverse cell environment in order to model either proximal or distal gut portions. Although it cannot represent alone the intestinal complexity due to the lack of enteric plexuses, peristalsis, blood flow or microbiota (In et al. 2016), organoids are a powerful tool for IBD research and for the personalized medicine. In fact it could be possible to alter the phenotypic appearance of growing organoids through incubation with TNF-α (Grabinger et al. 2014), one of the main pro-inflammatory cytokines registered in the intestinal mucosa of CD patients.

Moreover, a recently developed model based on organoid culture is the production of 2D monolayers from 3D organoid systems. This improvement increases the possibility to further manipulations, sacrificing crypt-histology. The advantage consists in the capacity to polarize gut cell lining on transwell plates, providing a lumen (apical compartment), and a basolateral region (basal compartment). This organoid application could allow to co-culture organoids with patient-derived immune cells, or microbiota (Angus et al. 2020).

AIM

Eph receptor tyrosine kinases and their cell-bound ephrin ligands participate to many physiological processes in developmental and adult organs. Interestingly, the discovery that *ephrin-A1* is a TNF-α responsive gene in endothelial cells created a link between this system and the pathophysiological events occurring during inflammation (Dixit et al. 1990). Indeed several studies demonstrated molecular mechanisms of Eph/ephrin involvement in endothelial activation as well as in the recruitment of immune cells (Larson et al. 2008; Liu et al. 2014). This is the case of monocyte adhesion over the endothelium, which is mediated by EphB4/ephrin-B2 interactions respectively (Pfaff et al. 2008). Therefore, Eph/ephrin system has become an intriguing point of study in acute and chronic pathologies in order to identify potential pharmacological targets. Although there is increasing evidence that Eph/ephrin is involved in chronic inflammatory pathologies such as rheumatoid arthritis (Kitamura et al. 2008), atherosclerosis (Aiji Sakamoto et al. 2008), multiple sclerosis (Sobel 2005), little research has been performed in the intestinal field. What is known is that EphB2/B3 are highly expressed on stem cells harbouring the crypts, while ephrin-B1/B2 expression is typical of differentiated cells. This differential expression along the crypt-villus axis avoids intermingling of proliferative cells into the region of differentiated cells (Solanas et al. 2011). Hafner and colleagues demonstrated that some members of B family are increased in mucosal lesions of IBD patients, probably due to a potential role in wound healing capacity (Hafner et al. 2005). A similar differential expression has been documented also for the A-system proteins (Kosinski et al. 2007), but on the contrary, the evidence that type-A signalling pathway is affected by inflammatory bowel disease is lacking.

Starting from these premises, the aim of the present project was **firstly** to investigate the effects of the blockade of B-type Eph/ephrin signalling on intestinal inflammation with a particular focus on the responses of the immune system. A **second** purpose was to get a deeper insight into the responses evoked by the blockade or by the unidirectional activation of EphA/ephrin-A *forward* and *reverse* signalling in the same experimental conditions.

Hence, a murine model of CD, TNBS-induced colitis, has been used in order to evaluate the effects on the local and systemic responses produced by B-type Eph/ephrin blockade through the administration of monomeric EphB4.

Moreover, the interest toward the Eph/ephrin system has been further boosted by UniPR1331, a small molecule acting as a potent Eph-ephrin antagonist endowed with good oral bioavailability (Castelli et al. 2015). This compound was tested in the same experimental model and compared with Sulfasalazine used as positive control.

In order to explore the effects of A-type Eph/ephrin modulation, the soluble extracellular EphA2 domain was administered to selectively block type-A signalling. Finally, ephrin-A1-Fc, stimulating EphA *forward* signalling and inhibiting ephrin-A *reverse* one, and EphA2-Fc, inhibiting EphA *forward* signalling and stimulating ephrin-A *reverse* one, were tested in the TNBS-induced colitis. As indicators of colitis severity, both clinical and inflammatory markers were evaluated in mice exposed to TNBS. Furthermore, possible changes in the expression levels of A and B members mRNA and the corresponding proteins in the colon have been explored. In order to investigate whether pharmacological modulation of Eph/ephrin system could affect TNF-α release, murine splenic mononuclear cells were stimulated with phorbol myristate acetate (PMA) and ionomycin (I) and treated with Eph/ephrin modulators. In this case cyclosporine A was used as positive control.

Then, in order to plan future studies focused on the Eph/ephrin involvement in epithelial dysfunctions, a murine colonoid culture was developed.

After setting up a colonoid culture, EphB2 receptor was identified in growing organoids using immunocytofluorescence techniques.

Considering that IBD is the result of a multitude of factors, such as microbiota impairments, preliminary additional investigations were devoted to unravel the potential link between gut bacterial homeostasis and intestinal epithelial thrombin.

In fact, an increased level of active thrombin has been found in the intestinal mucosa of IBD patients (Motta et al. 2020).

Hence, intestinal bacteria coming from healthy human colon biopsies were exposed to increasing concentration of thrombin. Unexposed and thrombin-exposed microbes

were co-incubated with Caco-2 cells to study the effect of high levels of thrombin on bacterial adhesion. These last and preliminary experiments were carried out under the supervision of Dr. Nathalie Vergnolle, Director at Institut de Recherche en Santé Digestive, INSERM, Toulouse (France).

METHODS

1 In vivo experimental model

1.1 Animals

Female C57BL/6J mice (8-12 weeks old) (Charles River Laboratories, Calco, Italy), weighing 18-22 g, were housed and kept 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 performed between 9:00 a.m. and 12:00 a.m. and all efforts were made to minimize animals' suffering. Animal experiments were conducted 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).

1.2 Induction and assessment of colitis

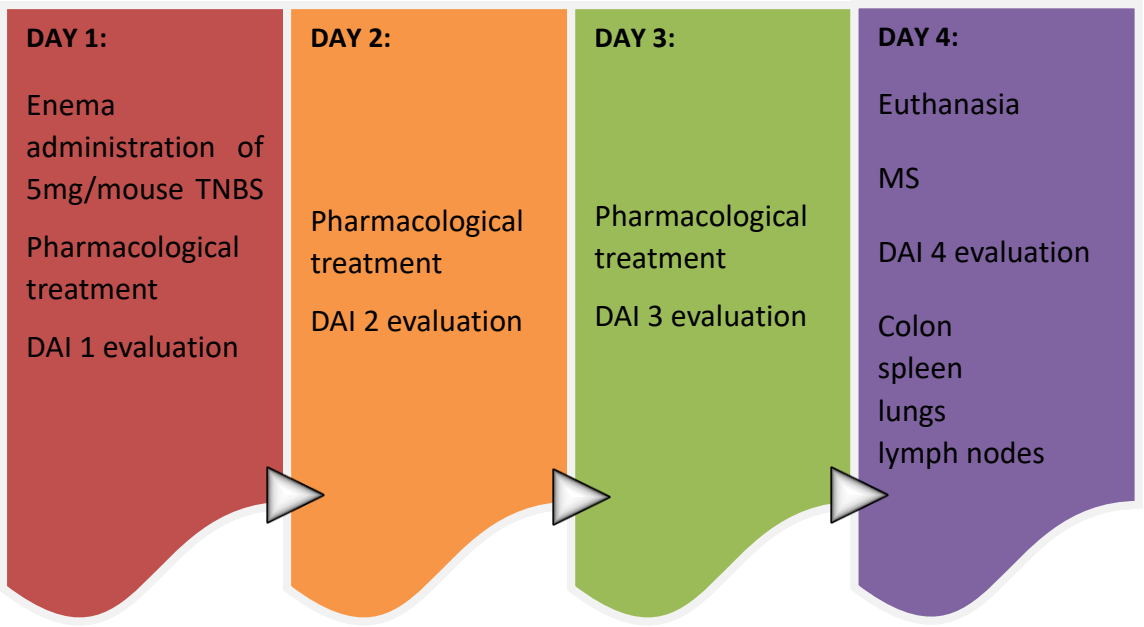
1.2.1 TNBS colitis induction

Mice were fasted for 4 hours with free access to water and a 3 cm long PE-50 tubing attached to a syringe was inserted into the anus. Colitis has been induced following the protocol already optimized on female mice in our laboratories. Briefly, 50 µL of a 10% (w/V) 2,4,6-TrinitroBenzene Sulfonic acid (TNBS) solution (5mg/mouse) in 50% ethanol was instilled by enema administration and mice were kept in a head-down position for 3 minutes to avoid leakage of TNBS. Acting as haptenating agent, TNBS is able to reproduce an acute form of colitis. Animals were suppressed by CO_2 inhalation three days after TNBS administration. Subcutaneous (s.c.) or oral (p.o.) pharmacological treatments started 8 hours after the induction of colitis and were applied daily or twice daily until euthanasia.

1.2.2 Colitis Assessment

Disease Activity Index (DAI) was determined daily by unaware investigators considering body weight, stools consistency and rectal bleeding. Following euthanasia, the macroscopic damage (MS) was determined by inspecting colonic mucosa. Colon weight and length were measured in order to determine the intestinal wall thickening, expressed as weight/length ratio (Bischoff et al. 2009). Colon, lungs, spleen and

mesenteric lymph nodes were collected for subsequent biochemical or flow cytometry analyses.



Schematic representation of TNBS experimental protocol

1.2.3 Pharmacological treatments

Pharmacological treatments started 8 hours after TNBS colitis induction and were applied once or twice daily by subcutaneous (s.c.), or oral administration (p.o.). Animals were randomly divided in the following different experimental groups (n=6-10):

- Normal [N]: 0.9% NaCl (saline) i.r. + saline 10 ml/kg/die s.c.;
- Colitis [C]: TNBS i.r + saline s.c.;
- EphB4-5: TNBS i.r + EphB4 5 µg/kg/die s.c.;
- EphB4-10: TNBS i.r + EphB4 10 µg/kg/die s.c.;
- EphB4-20: TNBS i.r + EphB4 20 µg/kg/die s.c.;
- Uni10: TNBS i.r + UniPR1331 10 mg/kg/bid p.o.;
- Uni25: TNBS i.r + UniPR1331 25 mg/kg/bid p.o.;
- EphA2: TNBS i.r + EphA2 20 µg/kg/die s.c.;
- ephrin-Fc 16: TNBS i.r + ephrin-A1-Fc 16 µg/kg/die s.c.;
- ephrin-Fc 50: TNBS i.r + ephrin-A1-Fc 50 µg/kg/die s.c.;
- EphA2-Fc: TNBS i.r + EphA2-Fc 30 µg/kg/die s.c.;
- Sulfa: TNBS i.r + sulfasalazine 50 mg/kg/die p.o.;

The dosage of EphB4 was chosen on the basis of the effects obtained in dose-response preliminary experiments. Dosages of ephrin-A1-Fc, EphA2 and EphA2-Fc applied were equimolar to the effective dose of EphB4. Later, ephrin-A1-Fc was increased of three times, up to 50 μg/kg, to find possible dose dependent effects. The dosage of UniPR1331 was chosen on the basis of Festuccia et al., 2018, and the dose of sulfasalazine was chosen according to Grandi et al., 2017.

1.2.4 Evaluation of Inflammatory Parameters

Disease Activity Index (DAI)

DAI is a parameter which correlates with the severity of the disease; it is calculated on the total score resulting from body weight loss, rectal bleeding and stool consistency according to Cooper's modified method (Cooper et al. 1993).

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%).

Macroscopic Score (MS)

Following euthanasia, the colon was extracted, opened longitudinally, washed and the mucosa was inspected in order to assign a macroscopic score. MS was determined as a sum of scores attributed as follows (J. L. Wallace et al. 1989; Khan et al. 2002):

- Presence of strictures and hypertrophic zones (0, absent; 1, 1 stricture; 2, 2 strictures; 3, more than 2 strictures);
- Mucus (0, absent; 1, present);
- Intraluminal hemorrhage (0, absent; 1, present);
- Erythema (0, absent; 1, presence of a crimsoned area < 1 cm^2; 2, presence of a crimsoned area > 1 cm^2);
- Ulcerations and necrotic areas (0, absent; 1, presence of a necrotic area < 0,5 cm^2; 2, presence of a necrotic area < 1 cm^2; 3, presence of a necrotic area < 1,5 cm^2; 4, presence of a necrotic > 1,5 cm;

- 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);

The maximum reachable score was 14.

Colonic length and thickness

To evaluate the fibrotic material deposition induced by TNBS-induced inflammation, colon length and thickness were measured. The latter was estimated calculating the ratio weight (mg)/length (cm) (Bischoff et al. 2009).

Colonic and Pulmonary Myeloperoxidase Activity (MPO)

MPO activity, a marker of tissue granulocytes accumulation, was determined in the colon and lungs as described by Ivey et al. 1995). Briefly, frozen tissues were homogenized in 9 volumes of *A solution* containing 1µg/ml aprotinin and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was discarded and pellets were homogenized again in 4 volumes of *B solution* containing 1µg/ml aprotinin with 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed in liquid nitrogen for 15 minutes and in water bath 37°C for 10 minutes, three times and centrifuged at 12000 rpm for 30 min at 4°C. MPO-rich supernatant was saved for assay. The assay was carried out by measuring the change in absorbance at 690 nm using *C solution*. 15 µL of the supernatant were made to react with 285 µL of reactive C solution.

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.

A Solution: 0.02 M $NaPO_4$ buffer (pH 4.7), 0.1 M NaCl and 0.015 M Na_2EDTA,
B Solution: 0.05 M $NaPO_4$ (pH 5.4) with 0.5% hexadecyltrimethylammonium bromide
C Solution: potassium phosphate 0.08 M (pH 5.4), TMB 1.6 M, H_2O2_2 0.3 mM, N,N-dimethylformamide, 40% Dulbecco's PBS

Colonic Interleukin-1β Levels

After euthanasia colons were excised to measure interleukin-1β levels determined using a commercially available Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (IL-1β Mouse SimpleStep ELISA™ kit; Abcam Biochemicals™, Cambridge, UK). Samples were homogenised for 1 minute in 700 µL of extraction buffer in accordance to the manufacturer's protocols. Samples were then centrifuged for 30 minutes at 12000 rpm. Supernatant were used to determine total protein content using Pierce bicinchoninic acid (BCA), protein assay kit (TermoFisher Scientific Inc., Waltham, MA, USA). Colonic concentrations of IL-1β were assessed in duplicate in 100 µl of sample: absorbance was spectrophotometrically measured at 450 nm. The assays sensitivity for IL-1β was 5 pg/mL. Results were expressed as pg/mg protein.

1.3 Isolation of splenocytes and mesenteric lymph nodes

Spleen and mesenteric lymph nodes (MLN) were excised after euthanasia; adipose tissue was removed and lymphoid tissues were mechanically broken up through a 70 µm cell strainer. Splenic samples were washed with PBS containing 0.6 mM EDTA (PBS-EDTA), while lymph nodes with Hank's Balanced Salt Solution (HBSS). Splenocytes were incubated with 1 mL of NH_4Cl lysis buffer (0.15 M NH_4Cl, 1mM $KHCO_3$, 0.1 mM EDTA in distilled water) for 30-60 seconds, in the dark to lyse erythrocytes and then diluted up to 6 ml with PBS-EDTA. After centrifugation at 1000 rpm for 10 minutes at 4°C, pellets from the spleen were resuspended in 5ml of cell staining buffer (PBS containing 0.5% fetal bovine serum and 0.1% sodium azide) and MLN pellets in 1 ml of cell staining buffer. Cell suspensions were stained with fluorescent antibodies.

1.4 Immunofluorescence and flow cytometry analysis

Non-specific sites were saturated with anti-mouse CD16/32 0,5 mg/ml for 10 minutes at 4°C in the dark. The following fluorescent antibodies were used: Phycoerythrin-Cyanine 5 (PE-Cy5)-conjugated anti-mouse CD3ε (0.25 µg/10^6 cells) emitting red fluorescence, Fluorescein Isothiocyanate (FITC) anti-mouse CD4 (0.25 µg/10^6 cells)

emitting green fluorescence and PE anti-mouse CD8a (0.25 μg/10^6 cells) emitting yellow fluorescence.

Cells were incubated for 1 hour at 4°C in the dark, washed with PBS and resuspended in cell staining buffer for flow cytometry (FACS) analysis. Cells viability was measured through propidium iodide (PI) assay: PI is able to highlight dead or apoptotic cells through binding to nuclear DNA. Cells were incubated with 10 μg/mL PI for 1 minute in the dark, at room temperature and then analysed using Guava easyCyteTM and InCyteTM software. Forward Scatter (FSC) and Side Scatter (SSC) were used to define physical cell properties and FSC low/SSC low identified the lymphocyte region. T-cells subpopulations were determined by counting CD4+ and CD8+ cells within CD3+ lymphocytes.

Cells viability was determined by assessing PI- cells; all PI+ lymphocytes were excluded from the analysis.

1.5 Reverse Transcription Polymerase Chain Reaction and agarose gel

Total RNA was extracted from murine colons using Qiagen RNeasy Protect Mini Kit (Qiagen, Hilden, DE). RNA amount was measured by Nanodrop ND-1000 (Termo Fisher Scientifc Inc, Waltham, MA). 1 μg of RNA was reverse transcribed into complementary DNA (cDNA) and amplifed using OneStep RT-PCR kit (Qiagen, Hilden, DE), according to the manufacturer's protocol. The following primers were used:

- ephrin-B2, 5'-ACCCACAGATAGGAGACAAA-3' (forward),
- 5'-GGTTGATCCAGCAGAACTTG-3' (reverse);
- EphB4, 5'-AGCCCCAAATAGGAGACGAG-3' (forward),
- 5'-GGATAGCCCATGACAGGATC-3' (reverse);
- ephrin-A1, 5'-CATCATCTGCCCACATTACG-3' (forward),
- 5'-AGCAGTGGTAGGAGCAATAC-3' (reverse);
- EphA2, 5'-GAGTGTCCAGAGCATACCCT-3' (forward),
- 5'-GCGGTAGGTGACTTCGTACT-3' (reverse);
- GAPDH, 5'-GACTCCACTCACGGCAAATT-3' (forward),
- 5'-TCCTCAGTGTAGCCCAAGAT-3' (reverse);

All constructs were amplified according to already published works (Ogawa et al. 2006; Mukai et al. 2017) and the following conditions were applied:

GENE	Denaturation	Annealing	Extension	Cycles
Ephrin-B2	45 s at 94°C	45 s at 53°C	90 s at 72°C	36
EphB4	10 s at 94°C	45 s at 53°C	3 min at 68°C	36
Ephrin-A1	45 s at 94°C	45 s at 60°C	60 s at 72°C	36
EphA2	45 s at 94°C	45 s at 62.5°C	60 s at 72°C	36
GAPDH	45 s at 94°C	45 s at 53°C	90 s at 72°C	30

PCR products were separated on 1% agarose gels and acquired with ChemiDoc Imaging System following RedSafe staining and analysed by Image Lab software, version 5.0. mRNA expression was assessed from 4 to 6 samples after normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control.

1.6 Western Blotting

Colons of mice were lysed to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Semi-dry transfer was used to electro-blot gels onto a nitrocellulose membrane by a mixture of tris-glycine transfer buffer and 20% methanol. After blocking with bovine serum albumin 5% for 1 h at room temperature, membranes were incubated at 4°C with primary antibodies against EphB4, ephrin-B2 and β-actin. Following washing with tris-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized using a chemiluminescence detection kit (Termo Fisher Scientifc Inc). The intensity of the bands of Eph/ephrin proteins were normalized to the signal intensity of β-actin obtained in the same lane.

2 In vitro experimental models

2.1 Mononuclear Cells Culture and TNF-α Levels determination

Splenocytes were obtained from healthy mice as previously described. Mononuclear cells were isolated through 40%/80% Percoll density gradient centrifugation. After centrifugation at 1000 RCF for 20 min at 20°C mononuclear cells were collected and washed in RPMI-1640. Cells were finally resuspended in medium containing heat-inactivated 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin up to 1*10^6 cell/ml.

200 µl containing 200 000 cells were plated on U-bottom 96-well plates and incubated for 24h at 37°C. In the last 4 hours cells were treated with Ionomycin (500 ng/mL) and PMA (50 ng/mL) or vehicle (Y. Li et al. 2015). Simultaneously Eph/ephrin modulators were added to the culture media:

- EphB4 0.01-0.1 µg/ml
- UniPR1331: 10-30 µM
- EphA2 0.25-1 µg/ml
- ephrin-A1-Fc 0.25-0.5-1 µg/ml
- EphA2-Fc 0.5-1-2 µg/ml
- IgG1-Fc 0.3-0.6 µg/ml
- Cyclosporine A (CysA) 1µg/ml as positive control

EphB4 concentrations were chosen basing on the binding affinity study performed by Grandi-Zini et al., 2018. Ephrin-A1-Fc concentrations were tested according to affinity data on murine lymphocytes published by Sharfe et al., 2008. EphA2 and EphA2-Fc concentrations were chosen to be equimolar to ephrin-A1-Fc".

TNFα levels were determined in 100 µl of culture media using commercially available ELISA kit (Mouse TNF-α ELISA Ready-SET-Go!, eBioscience™) The absorbance was measured spectrophotometrically at 450 nm.

Viability of mononuclear cells was determined after incubation with the treatments through flow cytometry by assessing the number of cells identified inside the

lymphocyte region (FSC low/SSC low). Moreover, in this region the percentage of pi-cells was calculated.

2.2 Organoid culture

Samples of colons were harvested from the 2/3 ends of descendant portions of male C57BL/6J mice (6-10 weeks old). The colons were opened longitudinally, washed in PBS, cut in small segments and incubated with 3 mM EDTA plus 3 mM dithiothreitol (DTT) and 10 µM Y-27632 at 4 °C for 10 min, under orbital shaking. After transfer in cold PBS, segments of colons were shaken vigorously for 2 min to isolate crypts in the supernatants which were centrifuged at 45g for 5 min at 4°C. After two washes with cold PBS, 200 or 50 crypts were embedded in 25 µl Matrigel and seeded in 48-well plates or 8-well Lab-Tek. 10 minutes after Matrigel polymerization at 37 °C, 250 µl DMEM F12 supplemented with 100 U/ml penicillin/streptomycin, 2mM Glutamax, 10 mM Hepes, N2 (1/100), B27 (1/50) (from Thermo Fisher Scientific), 100 ng/ml Wnt3a (RD Systems), 50 ng/ml EGF (Gibco, Thermo Fisher Scientific), 100 ng/ml noggin (Peprotech, Neuilly sur Seine, France), and 1 µg/ml R-spondin-1 (RD Systems). Obtained colonoids were observed daily using an Apotome microscope (Zeiss Axio-observer, HXP120) to follow their growth and viability. Blurry and dispersed structure were considered as dead organoids. For immunostaining, colonoids were seeded in eight-well Lab-Tek and fixed in 2% paraformaldehyde (20 min at 37°C), washed three times in PBS (15 min), and permeabilized and blocked with 0.5% Triton X-100 plus 1% BSA (40 min at RT). Primary goat-EphB2 antibody (R&D System AF467) or its goat-isotype were incubated overnight at 4 °C. Then, after three washes with PBS, actin filaments and nuclei were stained with 1:500 and 1:1000 of phalloidin and DAPI respectively diluted in PBS. Lab-tek was washed again three times with PBS and sealed using droplets of Vectashield mounting medium and observed by confocal laser scanning (Zeiss LSM710).

3 Study on human microbiota

3.1 Adhesion assay

2*10^5 Caco-2 cells were plated into 12-well plates or transwell plates in DMEM + 10 % FCS + 1% pen/strep. When a full layer was reached, cells were exposed to microbiota coming from healthy human colon biopsies. The intestinal biofilm was previously seeded into the Calgary Biofilm Device and exposed to various concentrations of thrombin (10 to 1000 mUnit/ml) or its vehicle. Bacterial adhesion was measured after 90 minutes of incubation with Caco-2 cells and quantified by plating on agar. Briefly, supernatants were discarded and cells were washed with PBS + 0,1% TritonX100. The resulting suspension was plated on agar plates in order to count each colony forming unit after 1 day of bacterial growth.

3.2 FISH staining

The membranes were cut from trans-well plates and exposed to Fish (Fluorescent In-Situ Hybridization) analysis. Membranes were fixed in 4% paraformaldehyde for 10 minutes at rt and incubated with 1 ml of 10 mg/ml Lysozyme buffer for 10 minutes at 37°C. After washing with distilled water cells were exposed to the hybridization buffer to stain eubacteria (900 mM NaCl, 20 mM TrisHCl, 0.01 % SDS and 5 ng/ml of fluorescent EUB338-Cy5) for 3 hours, in the dark at 46°C. Membranes were washed two times in Saline Sodium Citrate buffer and incubated with 1:1000 dilution of Alexa Fluor 488 WGA, to stain sugar-rich structure, for 15 min at RT. After washing with distilled water, membranes were put on glasses and sealed using cover slips through mounting medium containing DAPI staining and observed by confocal laser scanning (Zeiss LSM710).

Statistics

All data are presented as means ± SEM. Experimental groups were compared using the analysis of variance (one-way or two-way ANOVA) followed by Bonferroni's post-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 5 software (GraphPad Software Inc. San Diego, CA, USA).

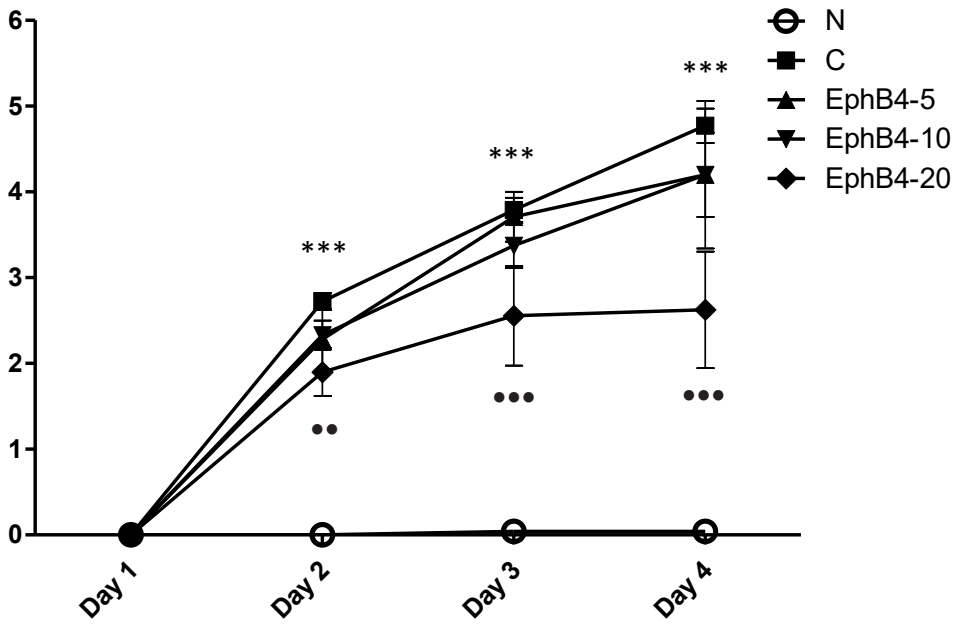
RESULTS

IN VIVO and EX VIVO

EphB/ephrin-B blockade in TNBS colitis:

In the first part of the project, the TNBS model of colitis was used to assess whether the pharmacological blockade of EphB/ephrin-B signalling could influence local and systemic inflammatory parameters. Female C57BL/6 mice were challenged with TNBS administration and successively treated for three days with saline or with increasing dosages of the monomeric protein EphB4: EphB4 5µg/kg, EphB4 10µg/kg, EphB4 20µg/kg s.c. Markers of local and systemic inflammation were detected at the end of the experimental period. In addition, flow cytometric analysis of splenic and mesenteric lymph nodes cells was used to define the percentages of CD4+ and CD8+ populations.

Disease activity index



Graph 1: Disease activity index-DAI

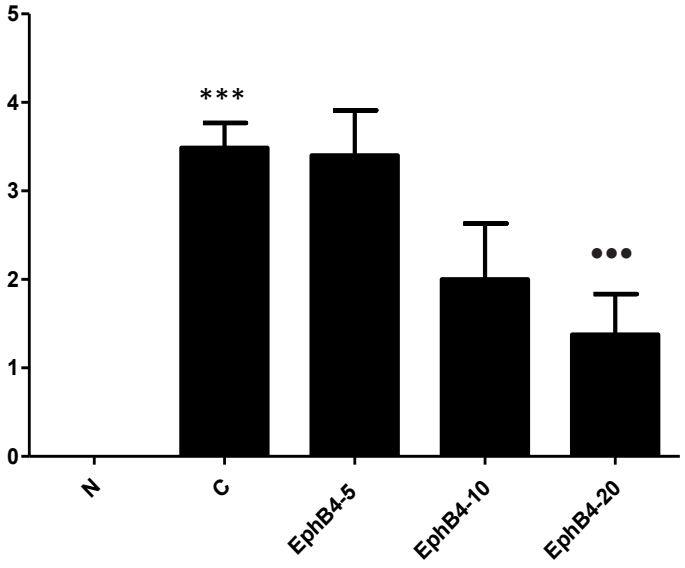
Disease activity index assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 values per group)

*** P<0.001 vs N; ●● P<0.01 vs C; ●●● P <0.001 vs C

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 induced colitis. For each animal, the weight loss and the presence of soft stools or diarrhoea were 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 N animals DAI remained 0 throughout the whole period, whilst TNBS group registered higher scores on days 2-3-4 compared to N group. This was the sign of the extremely significant worsening of animals' condition with respect to N mice (P<0.001 vs N). Only EphB4 20 µg/kg was able to reduce DAI score compared to C, starting already from day 2 (P<0.01 vs C).

Macroscopic score



Graph 2: Macroscopic score-MS

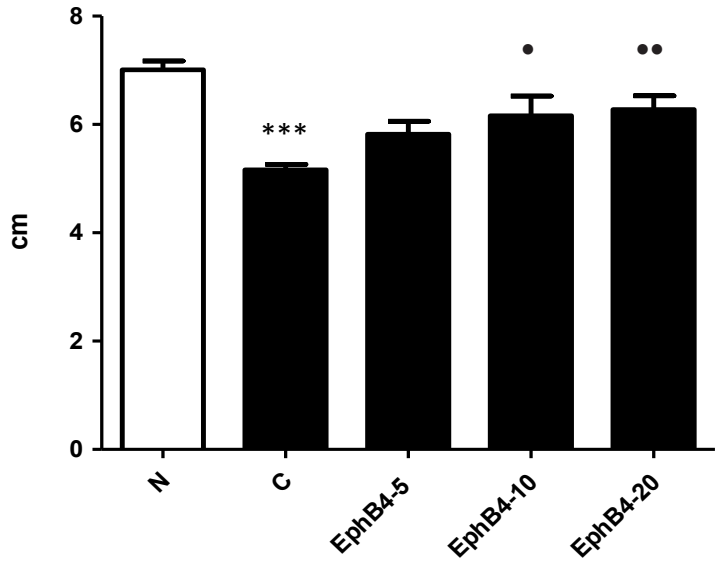
Macroscopic score assessed in vehicle-treated normal mice (N) and in colitic mice treated subcutaneously with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 values per group)

*** P<0.001 vs N; ●●●P<0.001 vs C

One-way ANOVA + Bonferroni's post test

Macroscopic score was determined right after colon removal to determine the local damage induced by TNBS administration. The presence of ulcers, erythema and colon strictures contributed to define the score. N animals scored 0, while C group score was significantly increased (P<0.001 vs N) (Graph 2). EphB4 exerted a dose-dependent protective effect reaching an extremely effective protection of colon mucosa at the highest dose of 20 µg/kg (P<0.001 vs C).

COLONIC LENGTH



Graph 3: Colonic length

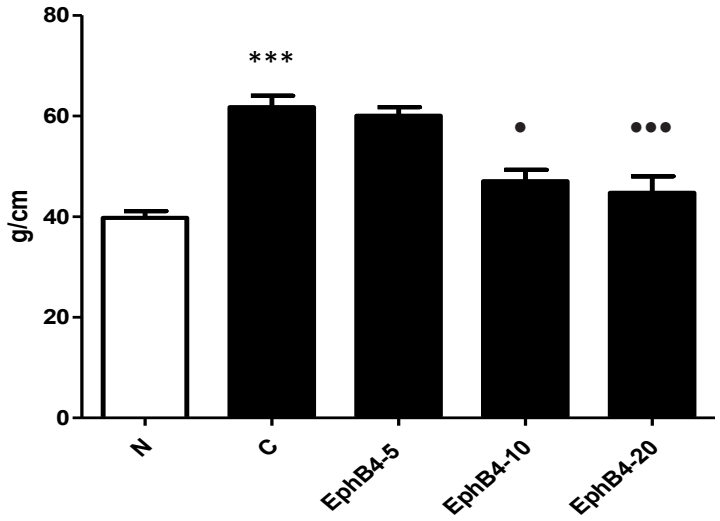
Colonic length assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 values per group)

***P<0.001 vs N; ●● P<0.01 vs C; ● P <0.05 vs C

One-way ANOVA + Bonferroni's post test

Colitis induced an important shortening of colons in C mice compared to N animals (P<0.001 vs N) (Graph 3). Colonic shortening was prevented by EphB4 both at 20 µg/kg and 10 µg/kg in a dose-dependent manner (P<0.01, P<0.05 vs C respectively).

COLONIC THICKNESS



Graph 4: Colonic thickness

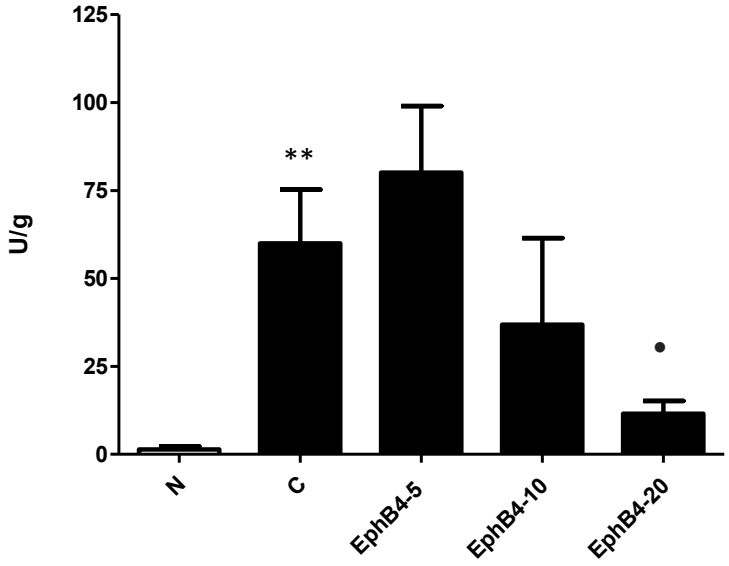
Colonic thickness assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 per group)

*** P<0.001 vs N; ** P<0.01 vs N; ●●● P<0.01vs C; ● P <0.05 vs C

One-way ANOVA + Bonferroni's post test

TNBS provoked a huge thickening of colonic wall compared to N mice (P<0.001 vs N) (Graph 4). Colonic thickening was prevented by both EphB4 20 µg/kg and EphB4 10 µg/kg in a dose-dependent manner (P<0.001, P<0.05 vs C respectively). EphB4 5 µg/kg was completely ineffective and reached thickening values similar to those of C group.

COLONIC MYELOPEROXIDASE ACTIVITY



Graph 5: Colonic MPO

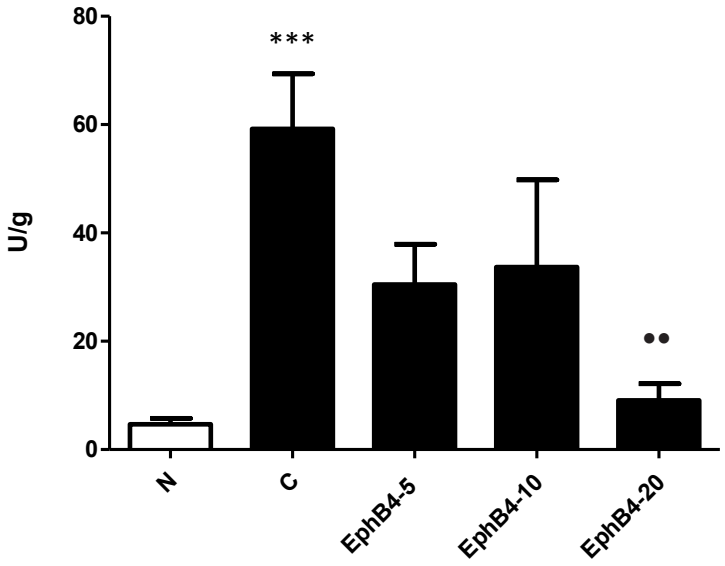
Colonic MPO assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 per group)

**P<0.01 vs N; ●P <0.05 vs C

One-way ANOVA + Bonferroni's post test

Myeloperoxidase (MPO) is a parameter which represents the amount of infiltrated neutrophils within the inflamed tissue (Graph 5). Colitis induced a remarkable recruitment of neutrophils within colonic mucosa of C mice compared to N group (P<0.001 vs N). Neutrophils infiltration was dampened only by EphB4 20 µg/kg (P<0.05 vs C).

PULMONARY MYELOPEROXIDASE ACTIVITY



Graph 6: Pulmonary MPO

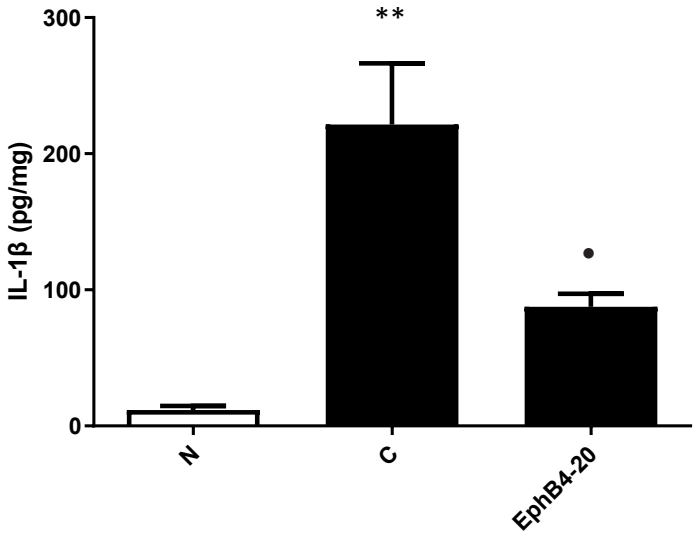
Pulmonary MPO assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 5, 10 or 20 µg/kg (EphB4-5, EphB4-10, EphB4-20) (n=6-10 per group)

*** P<0.001 vs N; ●● P <0.01 vs C

One-way ANOVA + Bonferroni's post test

The assessment of MPO levels within the lungs gives information about the systemic inflammation following colitis induction. As shown by Graph 6, it is clear that inflammation-induced recruitment of neutrophils was increased in C group (P<0.001 vs N) and effectively limited by the highest dose of EphB4 treatment (P<0.01 vs C)

COLONIC IL-1β LEVELS



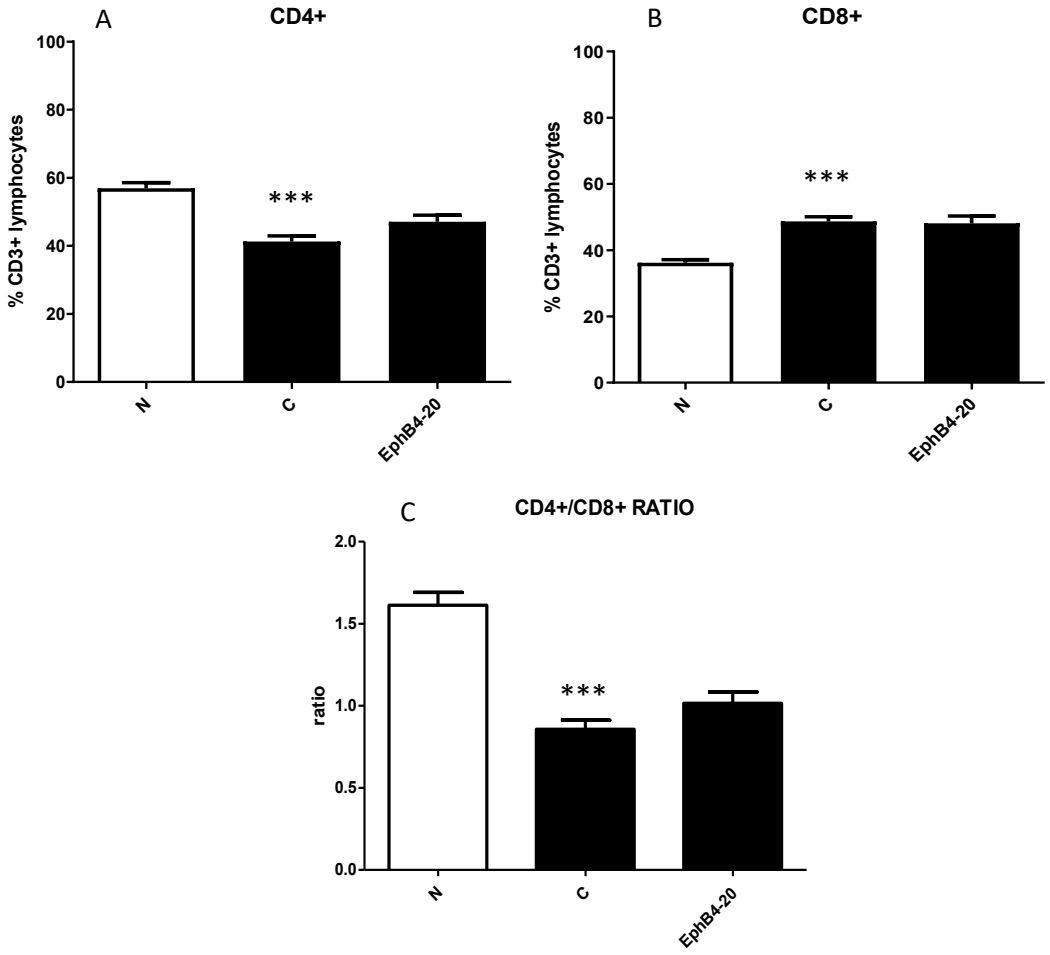
Graph 7: Colonic IL-1β level determination

Colonic IL-1β assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 20 µg/kg (EphB4-20) (n=6-10 per group) ** P<0.01 vs N; ●P <0.05 vs C

One-way ANOVA + Bonferroni's post test

As a result of the induced colitis, colonic IL-1β levels were increased in C mice compared with the N group (P<0.01 vs N). Colonic IL-1β was significantly attenuated by 20 µg/kg EphB4 as shown in graph 7 (P <0.05 vs C) (Graph7).

SPLENIC T LYMPHOCYTES



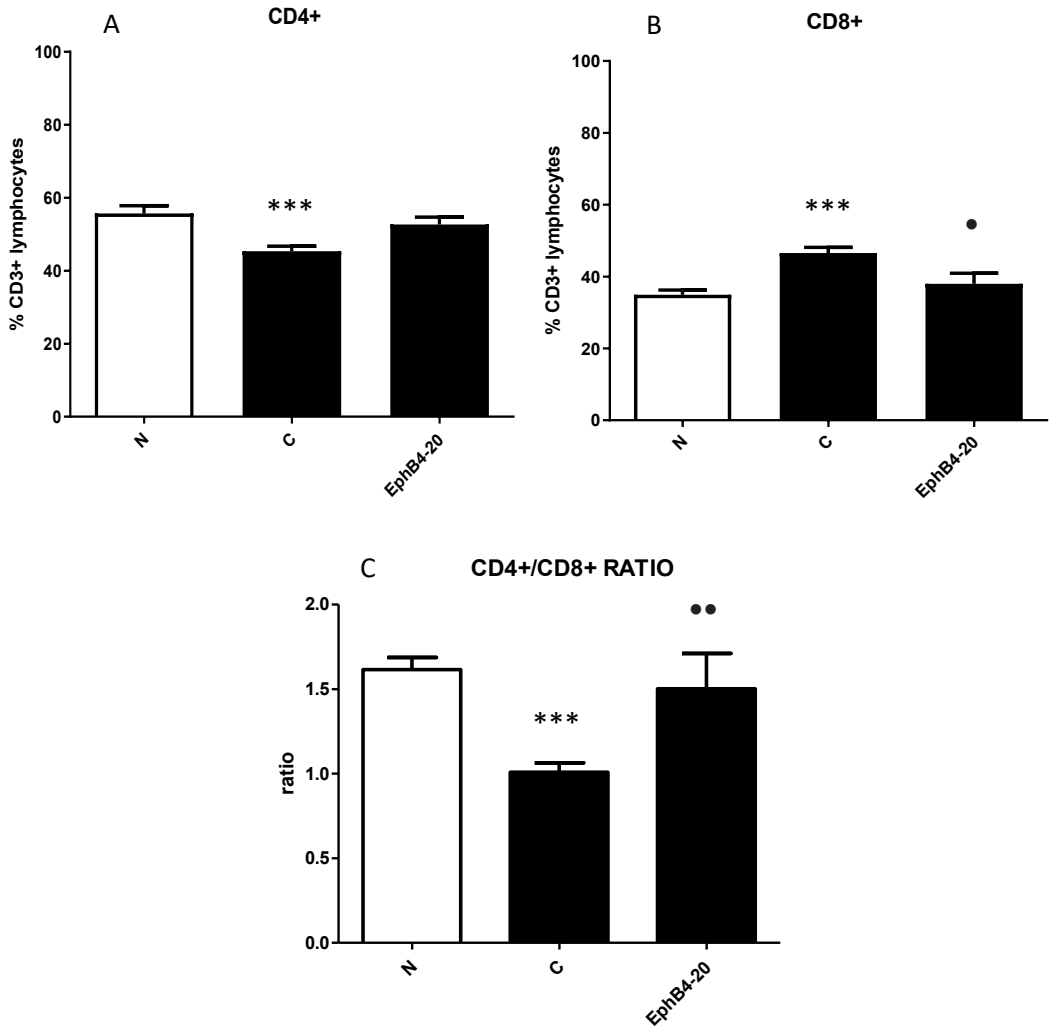
Graph 8: Flow cytometric analysis of splenic T cells

Percentage of splenic CD4+(A), CD8+(B) cells and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 20 µg/kg (EphB4-20) (n=6-10 per group) *** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

Flow cytometry is a technique that allows the identification of different cell subtypes in a complex population. Percentages of CD3+CD4+ (T helper cells) (A), CD3+CD8+ (T cytotoxic cells) (B) subpopulations and their ratio (C), obtained from spleens are presented. Colitis produced a reduction of the percentage of T CD4+ cells in favour of an increase in the percentage of CD8+ subpopulation in the spleen compared to N mice (P<0.001 vs N) (Graph 8A and B). Colitis reduced the CD4+/CD8+ ratio (Graph 8C). EphB4 20 µg/kg was not able to counteract colitis induced changes in splenic T cell profile.

MESENTERIC LYMPH NODES T CELLS



Graph 9: Flow cytometric analysis of mLN T cells

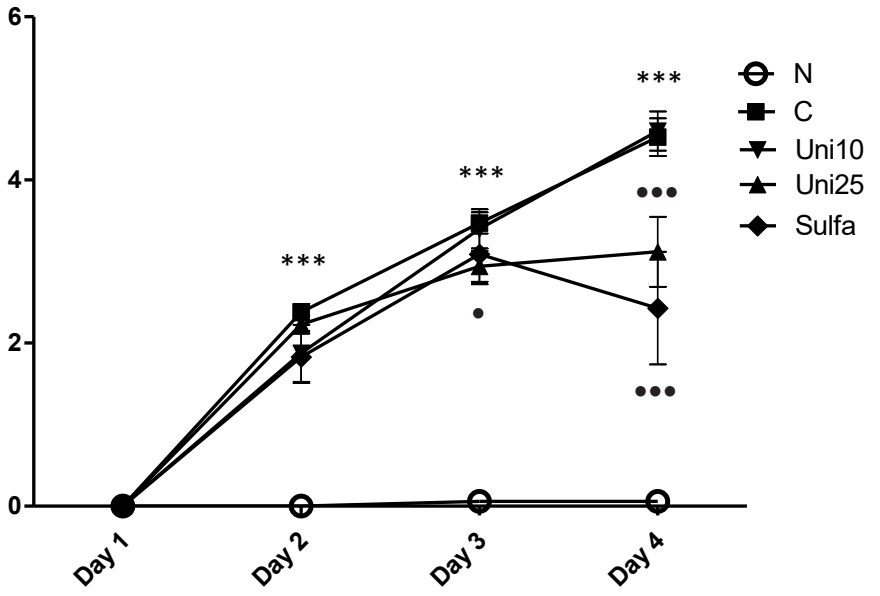
Percentage of mLN CD4+(A), CD8+(B) and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously treated with vehicle (C) or EphB4 at 20 µg/kg (EphB4-20) (n=6-10 per group) ***P<0.001 vs N; ●●P<0.01 vs C; ●P<0.05 vs C One-way ANOVA + Bonferroni's post test

Similarly to the spleen results, in C group there was a reduction of the percentage of T CD4+ cells in favour of an increase in the percentage of CD8+ subpopulation in the mLN compared to N mice (P<0.001 vs N) (graph 9A and B). As a result, colitis reduced the CD4+/CD8+ ratio (graph 9C). EphB4 was able to restore the percentage of CD8+ and the ratio CD4+/CD8+ in mLNs (P<0.05 vs C; P<0.01vs C respectively) (Graph 9B and C).

Eph/ephrin blockade in TNBS colitis: the small molecule UniPR1331

Eph/ephrin blockade has been obtained also by administering UniPR1331, a small molecule acting as a potent Eph-ephrin antagonist endowed with good oral bioavailability. This compound was tested in TNBS-induced inflammation, and local and systemic inflammatory parameters were compared with Sulfasalazine used as positive control. The dosages of UniPR1331 10mg/kg and 25mg/kg p.o. (Uni10, Uni25) were chosen on the basis of safety and efficacy findings reported by Festuccia et al., 2018, and the dose of sulfasalazine 50 mg/kg (Sulfa) p.o. was selected according to previous experiments (Grandi et al. 2017).

Disease activity index



Graph 10: Disease activity index-DAI

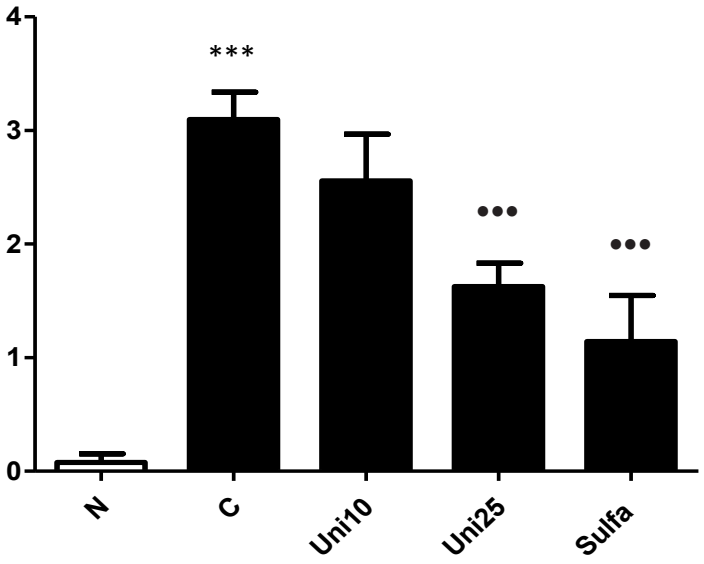
Disease activity index assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine (Sulfa) (n=6-12 values per group)

***P<0.001 vs N; ●●●P<0.001 vs C; ●P<0.05 vs C

Two-way ANOVA + Bonferroni's post test

Sulfasalazine at 50 mg/kg was able to highly dampen the disease activity index at day 4 compared to C group (P<0.001 vs C). The same result was shown by 25 mg/kg UniPR1331 not only at day 4 (P<0.001 vs C), but also at day 3 (P<0.05 vs C) (Graph 10).

Macroscopic score



Graph 11: Macroscopic score-MS

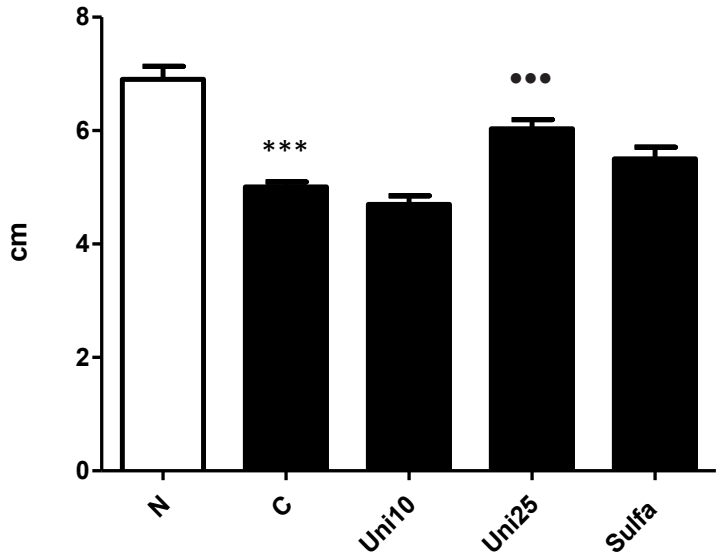
Macroscopic score assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa) (n=6-12 values per group)

*** P<0.001 vs N; ●●●P<0.001 vs C

One-way ANOVA + Bonferroni's post test

The increased macroscopic score induced by TNBS (P<0.001 vs N) was reduced by both UniPR1331 25 mg/kg and sulfasalazine 50 mg/kg (P<0.001 vs C). UniPR1331 at lower dose did not prevent mucosal damage associated with the colitis (Graph 11).

COLONIC LENGTH



Graph 12: Colonic length

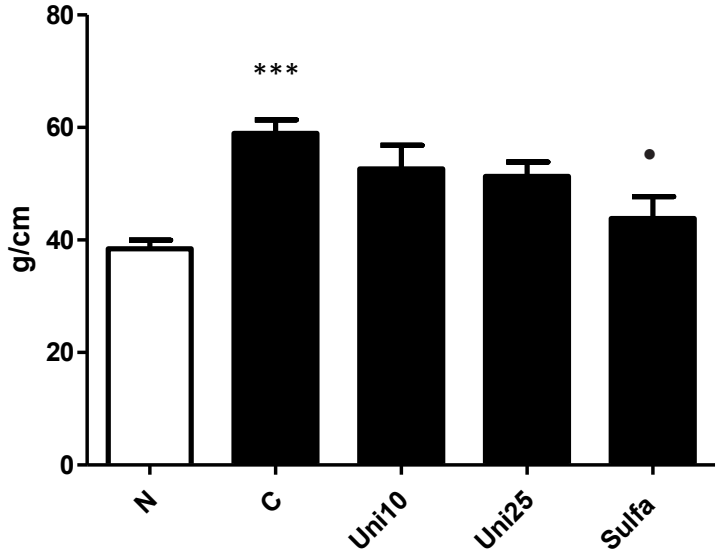
Colonic length assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa) (n=6-12 values per group)

*** P<0.001 vs N; ●●●P<0.001 vs C

One-way ANOVA + Bonferroni's post test

Colon shortening produced by TNBS (P<0.001 vs N) was weakly antagonized by sulfasalazine. By contrast, UniPR1331 25 mg/kg strongly counteracted the shortening (P<0.001 vs C), while being inactive at the lower dose (Graph 12).

COLONIC THICKNESS



Graph 13: Colonic thickness

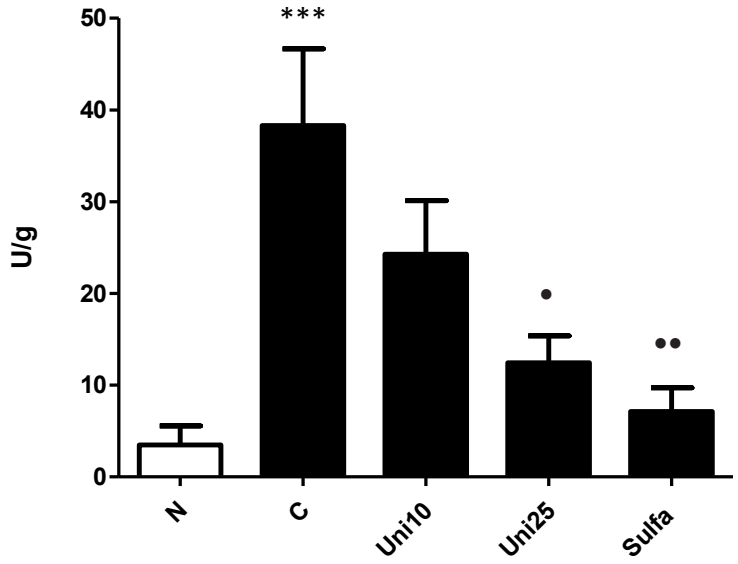
Colonic thickness assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa) (n=6-12 values per group)

*** P<0.001 vs N; ●P<0.05 vs C

One-way ANOVA + Bonferroni's post test

Sulfasalazine was able to significantly reduce the colonic thickening induced by the intestinal inflammation (P<0.05 vs C). Both dosages of UniPR1331 were ineffective in counteracting mucosal thickening typical of TNBS-challenged mice (P<0.001 v N) (Graph 13).

COLONIC MYELOPEROXIDASE ACTIVITY



Graph 14: Colonic MPO

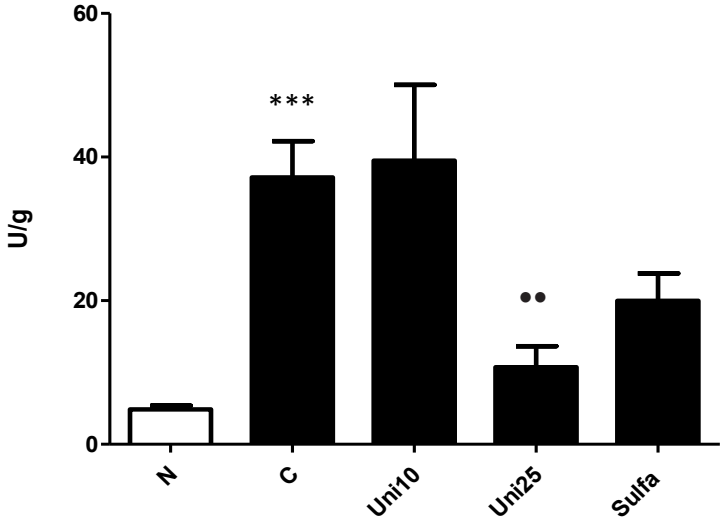
Colonic MPO assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa) (n=6-12 values per group)

*** P<0.001 vs N; ●P<0.05; ●●P<0.01vs C

One-way ANOVA + Bonferroni's post test

The huge neutrophil recruitment in the colon produced by TNBS (P<0.001 vs N) was completely counteracted by sulfasalazine (P<0.01 vs C). The same result was shown by UniPR1331 25 mg/kg (P<0.05 vs C); UniPR1331 was ineffective at 10 mg/kg (Graph 14).

PULMONARY MYELOPEROXIDASE ACTIVITY



Graph 15: Pulmonary MPO

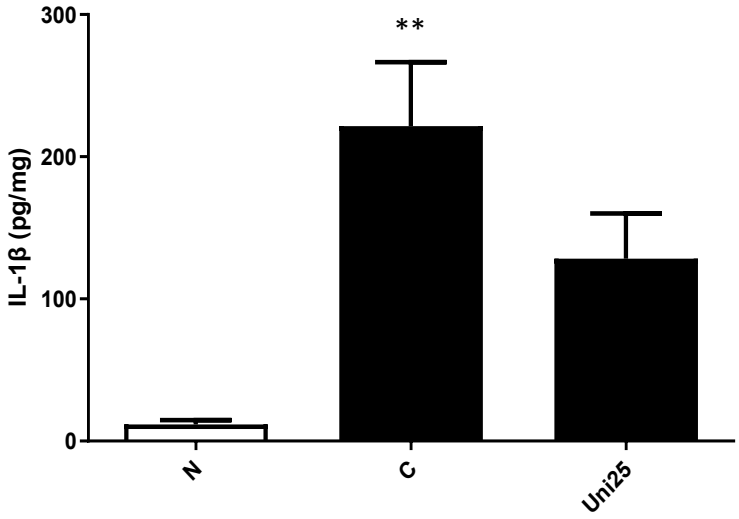
Pulmonary MPO assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa) (n=6-12 values per group)

*** P<0.001 vs N; ●●P<0.01 vs C

One-way ANOVA + Bonferroni's post test

TNBS produced a massive neutrophil recruitment in the lungs (P<0.001 vs N). It was strongly counteracted by UniPR1331 25 mg/kg (P<0.01 vs C), while UniPR1331 10 mg/kg was ineffective. Sulfasalazine weakly dampened MPO activity in the lungs (Graph 15).

COLONIC IL-1β LEVELS



Graph 16: Colonic IL-1β level determination

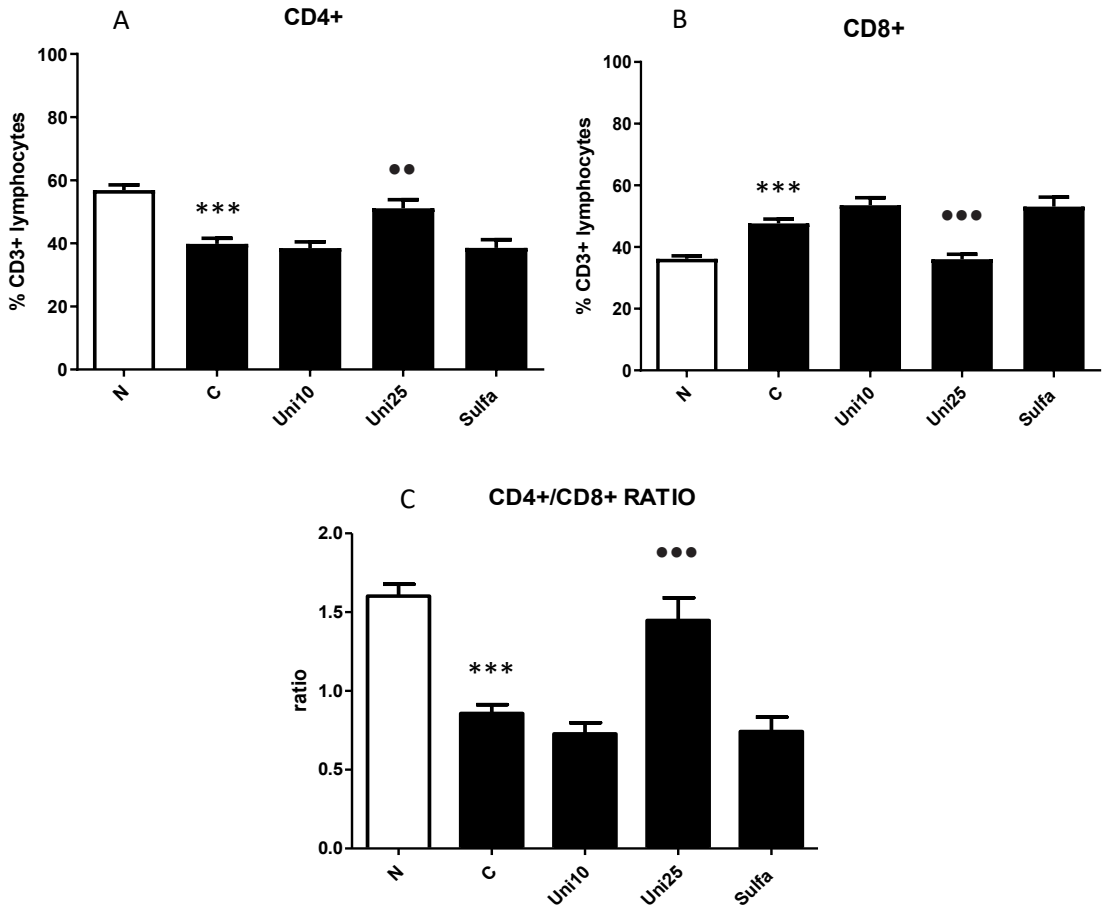
Colonic IL-1β assessed in vehicle-treated normal mice (N) and in colitic mice orally administered with vehicle (C), UniPR1331 at 25 mg/kg (Uni25)

(n=6-12 values per group) ** P<0.01 vs N

One-way ANOVA + Bonferroni's post test

IL-1β level was very high in the colon, confirming the inflammatory status of C mice (P<0.01 vs N). UniPR1331 attenuated colonic IL-1β concentrations, even though not producing a significant reduction (Graph 16).

SPLENIC T LYMPHOCYTES



Graph 17: Flow cytometric analysis of splenic T cells

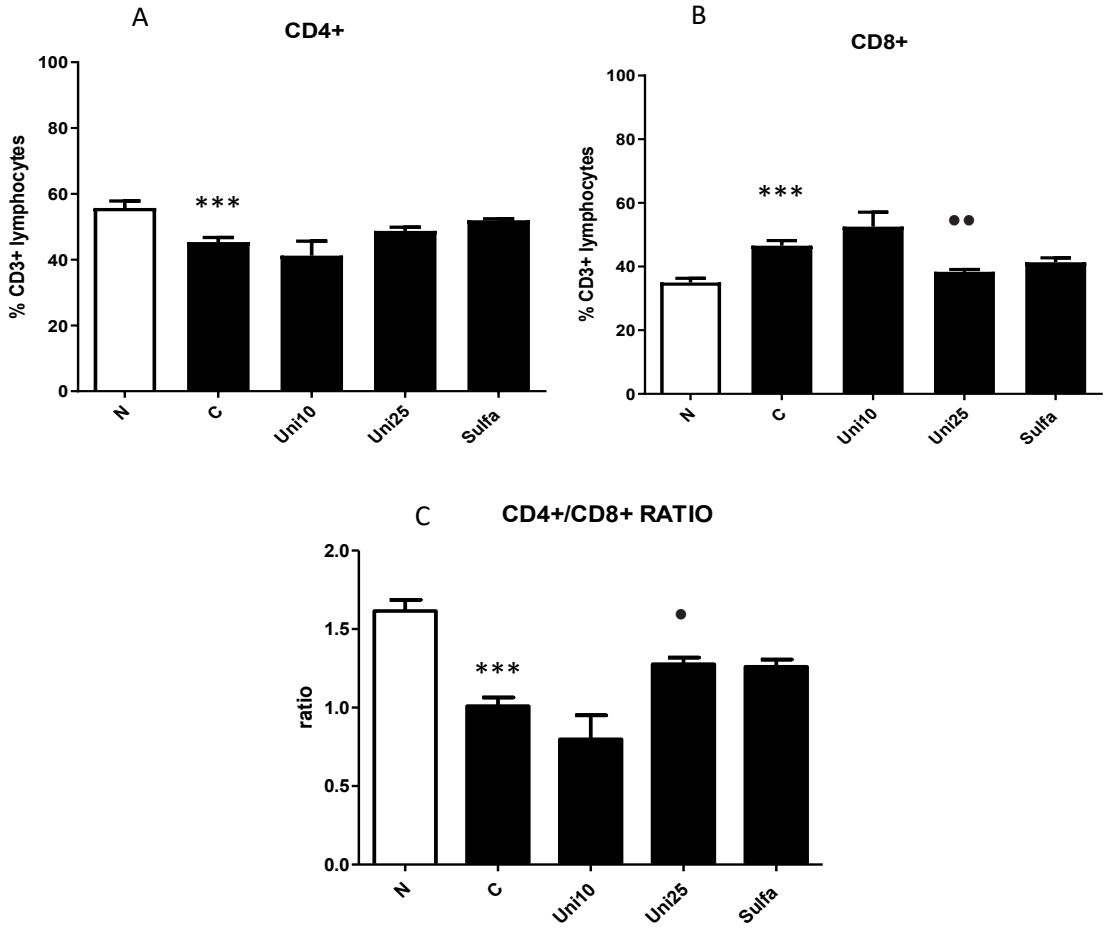
Percentage of splenic CD4+(A), CD8+(B) and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa)

(n=6-12 values per group) *** P<0.001 vs N; ●● P<0.01 vs C; ●●● P<0.001 vs C

One-way ANOVA + Bonferroni's post test

The reduction of the ratio CD4+/CD8+ in C mice (P<0.001 vs N), explained by the decresase of the percentage of CD4+ (P<0.001 vs N) in favour of an increase in CD8+ (P<0.001 vs N) in the spleen, is reversed by UniPR1331 at 25 mg/kg (P<0.001 vs C), while the lower dose was ineffective. Also sulfalsalazine was not able to counteract the reduction of CD4+/CD8+ ratio (Graph 17A, B, C).

MESENTERIC LYMPH NODES T CELLS



Graph 18: Flow cytometric analysis of mLN T cells

Percentage of mLN CD4+(A), CD8+(B) and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C), UniPR1331 at 10 or 25 mg/kg (Uni10, Uni25) or sulfasalazine 50 mg/kg (Sulfa)

(n=6-12 values per group) *** P<0.001 vs N; ● P<0.05 vs C; ●● P<0.01 vs C

One-way ANOVA + Bonferroni's post test

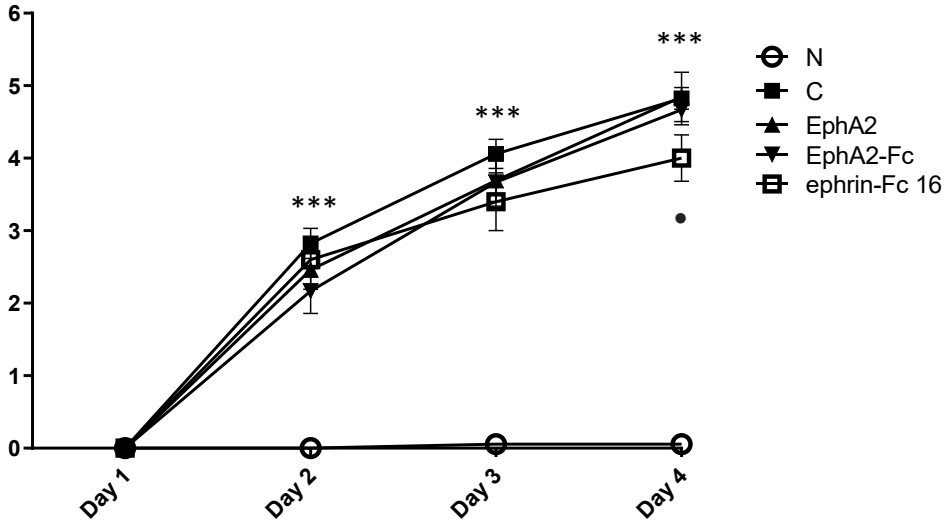
The same reduction of CD4+/CD8+ ratio, detected in the spleen, was observed also in mLN for C group compared to N mice (P<0.001 vs N). Only UniPR1331 counteracted this decrease (P<0.05 vs C), especially acting on CD8+ population (P<0.01 vs C). UniPR1331 at 10 mg/kg and sulfasalazine were ineffective (Graph 18).

EphA/ephrin-A modulation in TNBS colitis:

A second purpose of this project was to get a deeper insight into the responses evoked by the blockade or by the unidirectional activation of EphA/ephrin-A f*orward* and *reverse* signalling in TNBS-induced colitis. For this reason, EphA2 20 µg/kg s.c., EphA2-Fc 30 µg/kg s.c. and ephrin-A1-Fc 16 µg/kg s.c. (EphA2, EphA2-Fc, ephrin-Fc 16) were tested on TNBS-exposed mice.

The applied doses of EphA/ephrin-A recombinant proteins were equimolar to the previously tested EphB4 20 µg/kg.

Disease activity index



Graph 19: Disease activity index-DAI

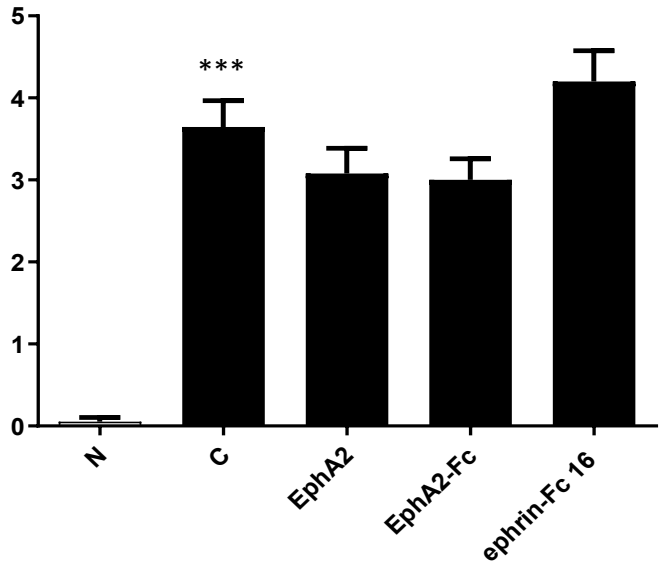
Disease activity index assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N; ● P<0.05 vs C

Two-way ANOVA + Bonferroni's post test

In N animals, DAI remained 0 throughout the whole period, whilst TNBS group marked the highest score on day 4, sign of the extremely significant worsening of animals' conditions with respect to N mice (P<0.001 vs N). Monomeric EphA2 and EphA2-Fc did not influence DAI score, but ephrin-A1-Fc 16µg/kg ameliorated animals' general conditions at day 4 (P<0.05 vs C) (Graph 19).

Macroscopic score



Graph 20: Macroscopic score-MS

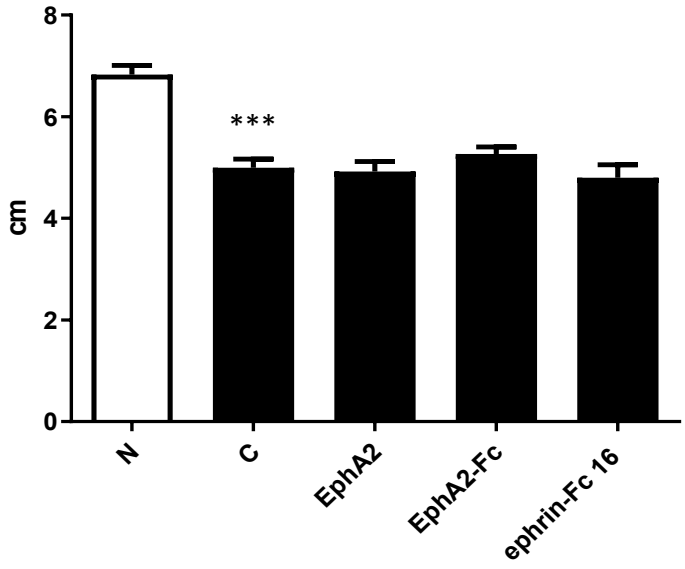
Macroscopic score assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N;

One-way ANOVA + Bonferroni's post test

None of the EphA/ephrin-A recombinant proteins changed mucosal damage associated with the colitis (Graph 20).

COLONIC LENGTH



Graph 21: Colonic length

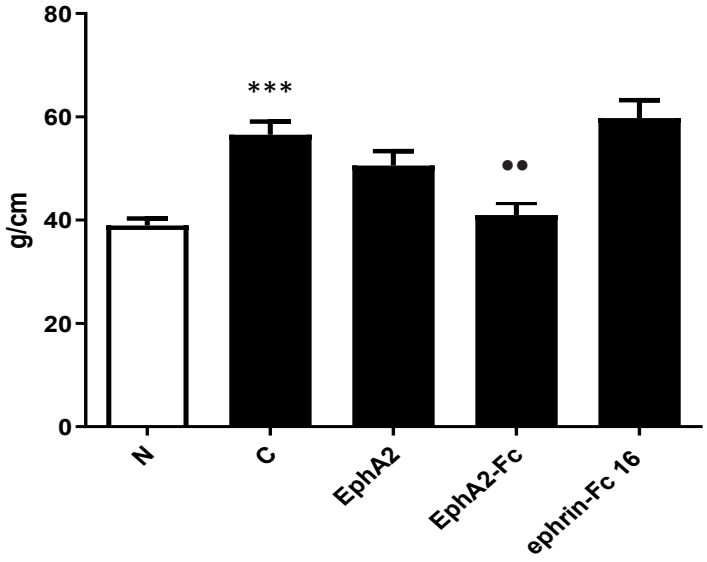
Colonic length assessed in vehicle-treated normal mice (N) and in colitis mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

None of the EphA/ephrin-A recombinant proteins counteracted the colonic shortening induced by TNBS administration (Graph 21).

COLONIC THICKNESS



Graph 22: Colonic thickness

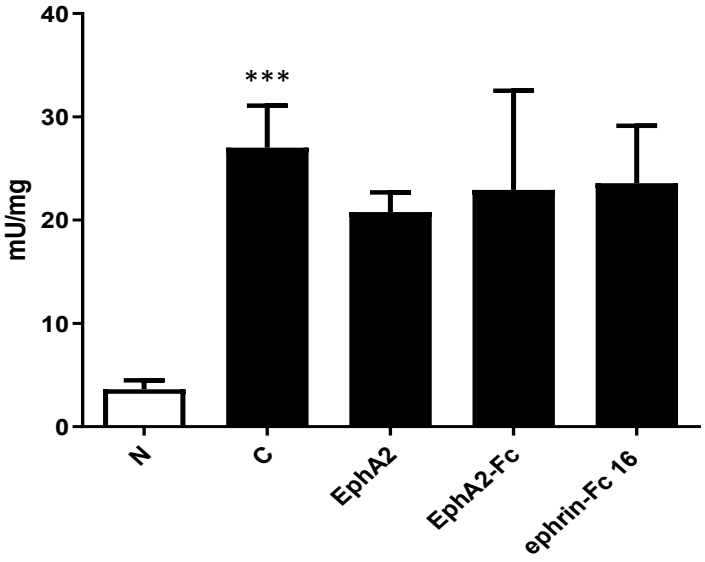
Colonic thickness assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N; ●● P<0.01 vs C

One-way ANOVA + Bonferroni's post test

Neither EphA2 nor ephrin-A1-Fc counteracted the colonic thickening induced by TNBS administration. Only EphA2-Fc reduced the colon thickness compared to C mice (P<0.01 vs C) (Graph 22).

COLONIC MYELOPEROXIDASE ACTIVITY



Graph 23: Colonic MPO

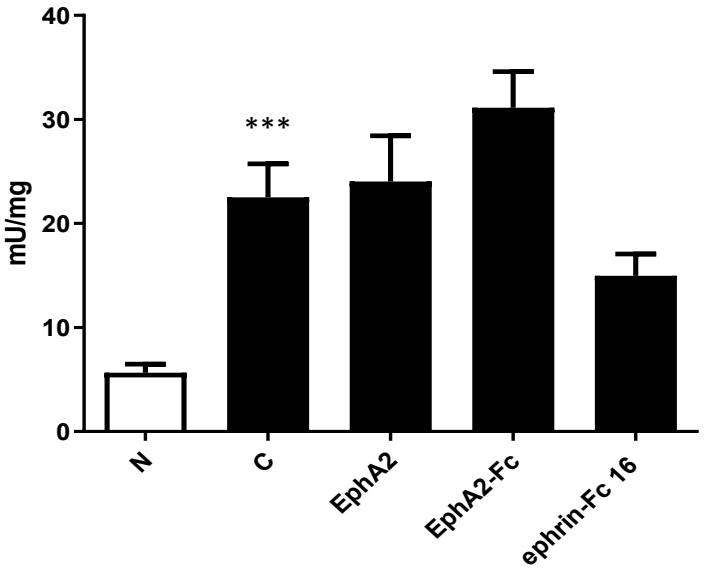
Colonic MPO assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

The neutrophil recruitment in the colon produced by TNBS (P<0.001 vs N) remained unchanged in EphA/ephrin-A recombinant proteins treated mice (Graph 23).

PULMONARY MYELOPEROXIDASE ACTIVITY



Graph 24: Pulmonary MPO

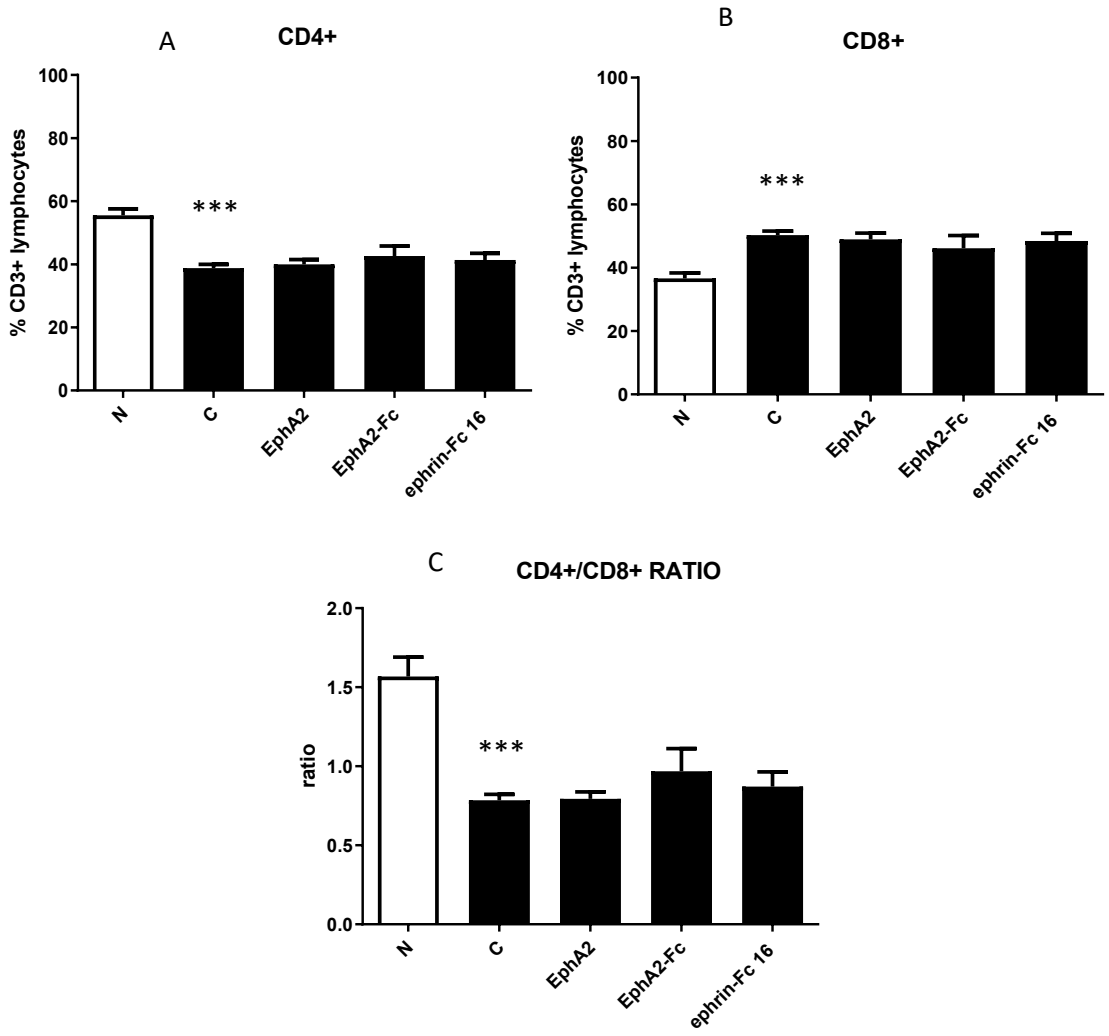
Pulmonary MPO assessed in vehicle-treated normal mice (N) and in colitis mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

TNBS produced a big neutrophil recruitment in the lungs compared to Normal mice (P<0.001 vs N). Although Fc-ephrinA1 seemed to weakly reduce MPO activity none of the EphA/ephrin-A recombinant proteins influenced this systemic inflammatory parameter (Graph 24).

SPLENIC T LYMPHOCYTES



Graph 25: Flow cytometric analysis of splenic T cells

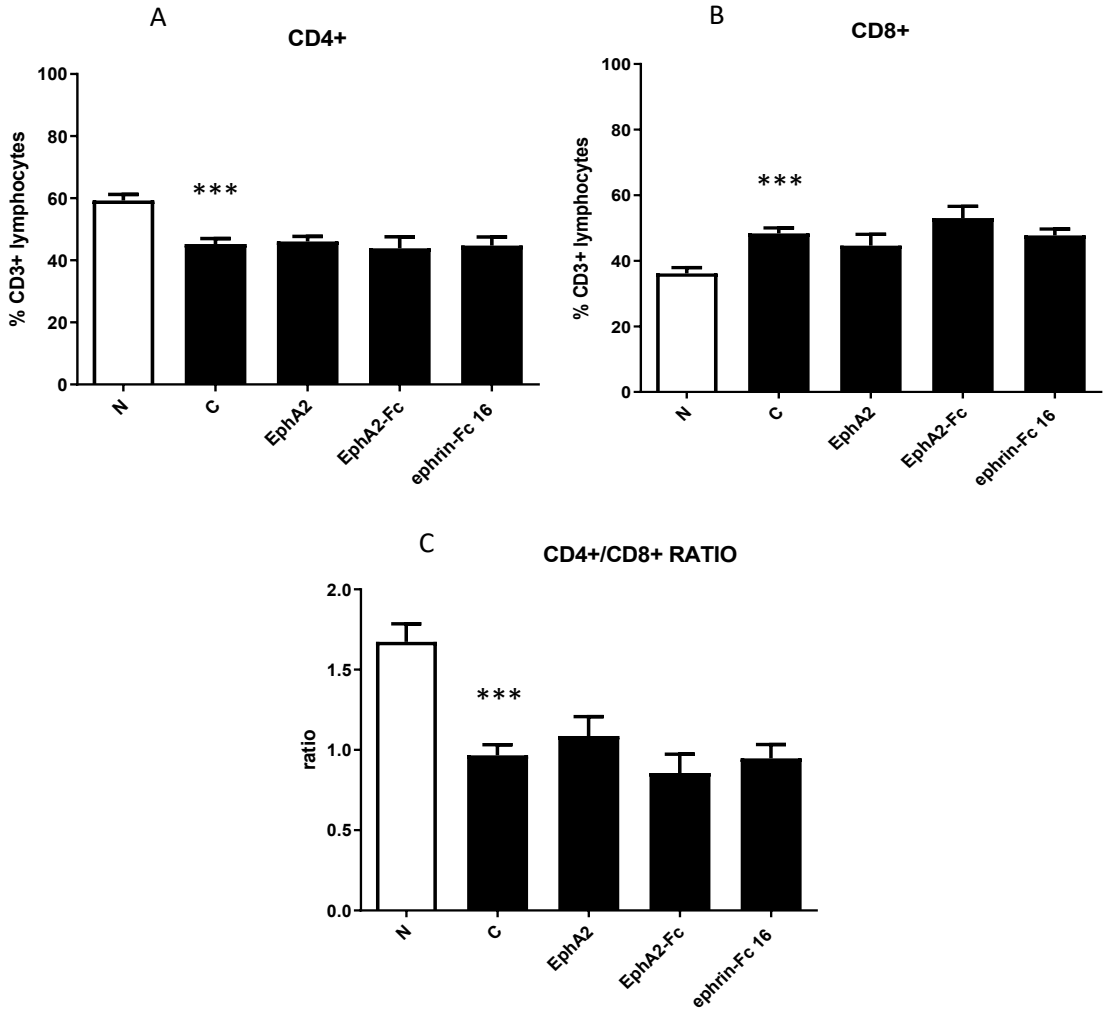
Percentage of splenic CD4+ (A), CD8+ (B) and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitis mice subcutaneously administered with vehicle (C) or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

None of the EphA/ephrin-A recombinant proteins tested were able to counteract the reduction of CD4+/CD8+ ratio in the spleen induced by TNBS (Graph 25 A, B, C).

MESENTERIC LYMPH NODES T CELLS



Graph 26: Flow cytometric analysis of mLN T cells

Percentage of mLN CD4+(A), CD8+(B) and ratio CD4+/CD8+(C) assessed in vehicle-treated normal mice (N) and in colitis mice subcutaneously administered with vehicle (C), or EphA2 20 µg/kg (EphA2), EphA2-Fc 30µg/kg (EphA2-Fc) and ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16)

(n=6-12 values per group) *** P<0.001 vs N

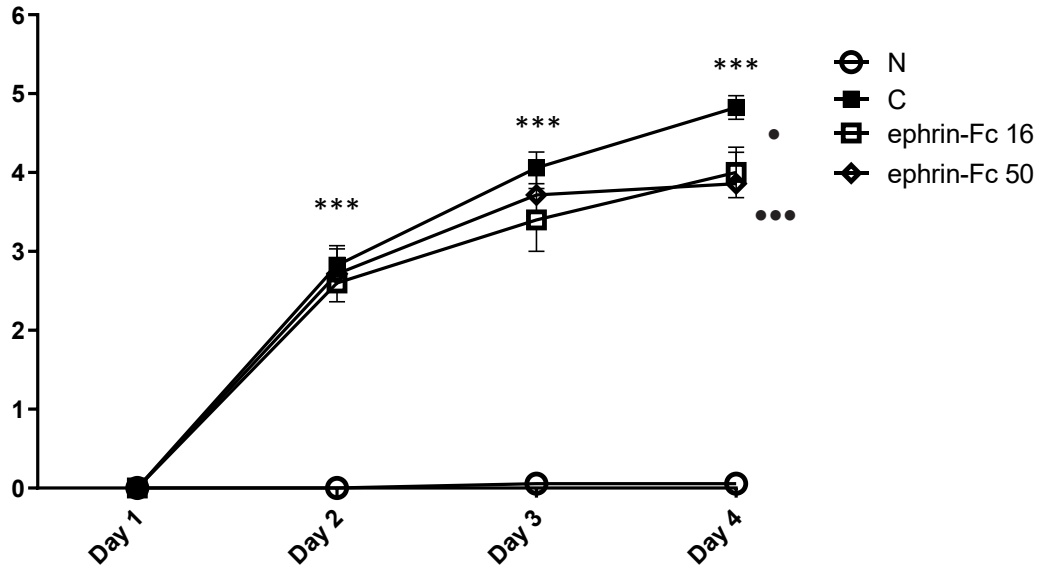
One-way ANOVA + Bonferroni's post test

None of the EphA/ephrin-A recombinant proteins tested were able to counteract the reduction of CD4+/CD8+ ratio in mesenteric lymph nodes induced by TNBS (Graph 26 A, B, C).

TNBS colitis: Ephrin-A1-Fc 16 µg/kg and 50 µg/kg

Ephrin-A1-Fc 16 µg/kg s.c. showed some promising results, being able to ameliorate the general status of mice considering DAI score at day 4 (Graph 19). Moreover, it weakly dampened pulmonary MPO activity (Graph 24). Therefore, the dose of ephrin-A1-Fc was increased at 50 µg/kg to investigate whether additional beneficial effects were possible.

Disease activity index



Graph 27: Disease activity index-DAI

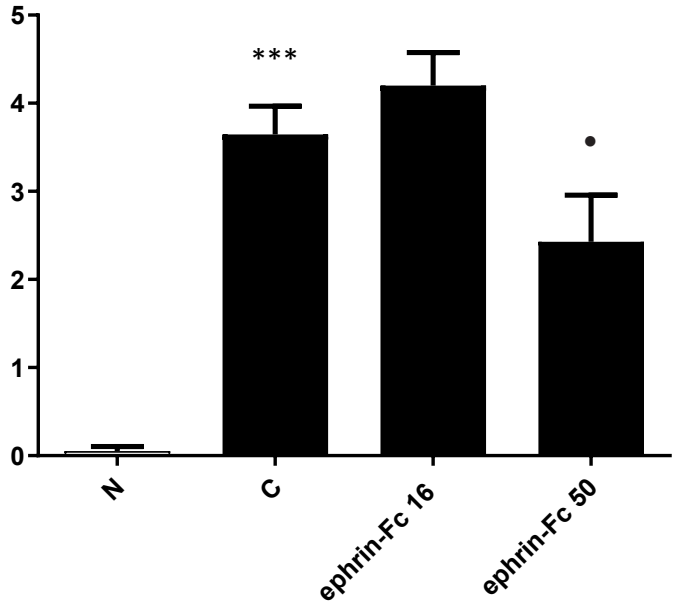
Disease activity index assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16) and ephrin-A1-Fc 50 µg/kg (ephrin-Fc 50) (n=6-12 values per group)

*** P<0.001 vs N; ● P<0.05 vs C; ●●● P<0.001 vs C

Two-way ANOVA + Bonferroni's post test

The amelioration of general mice health status at day 4, already verified for ephrin-A1-Fc at 16 µg/kg (P<0.05 vs C), remained evident after the administration of ephrin-A1-Fc at 50 µg/kg (P<0.001 vs C) compared to vehicle-treated inflamed mice (Graph 27).

Macroscopic score



Graph 28: Macroscopic score-MS

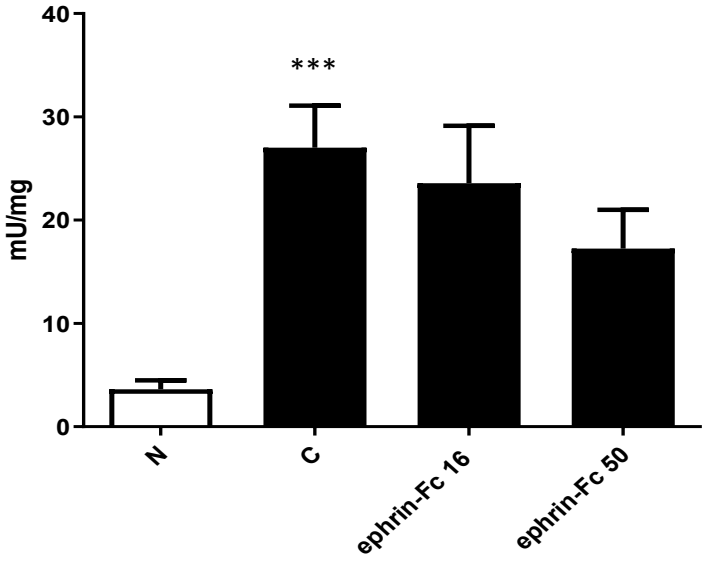
Macroscopic score assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) or ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16) and ephrin-A1-Fc 50 µg/kg (ephrin-Fc 50) (n=6-12 values per group)

*** P<0.001 vs N; ● P<0.05 vs C

One-way ANOVA + Bonferroni's post test

Differently from the lower dose of 16 µg/kg, ephrin-A1-Fc at 50 µg/kg significantly reduced macroscopic score compared to C group (P<0.05 vs C) (Graph 28).

COLONIC MYELOPEROXIDASE ACTIVITY



Graph 29: Colonic MPO

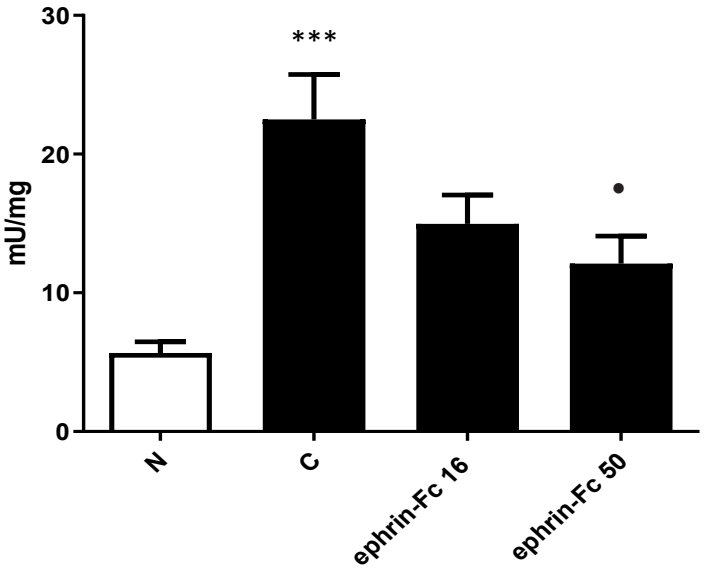
Colonic MPO assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16) and ephrin-A1-Fc 50 µg/kg (ephrin-Fc 50) (n=6-12 values per group)

*** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

As for macroscopic score, also in the case of colonic MPO, ephrin-A1-Fc was inactive at 16 µg/kg but it seemed to weakly dampen the colonic MPO activity at 50 µg/kg (Graph 29).

PULMONARY MYELOPEROXIDASE ACTIVITY



Graph 30: Pulmonary MPO

Pulmonary MPO assessed in vehicle-treated normal mice (N) and in colitic mice subcutaneously administered with vehicle (C) ephrin-A1-Fc 16 µg/kg (ephrin-Fc 16) and ephrin-A1-Fc 50 µg/kg (ephrin-Fc 50) (n=6-12 values per group)

*** P<0.001 vs N; ● P<0.05 vs C

One-way ANOVA + Bonferroni's post test

Differently from the dose of 16 µg/kg, ephrin-A1-Fc at 50 µg/kg significantly reduced the pulmonary MPO activity compared to C group (P<0.05 vs C) (Graph 30).

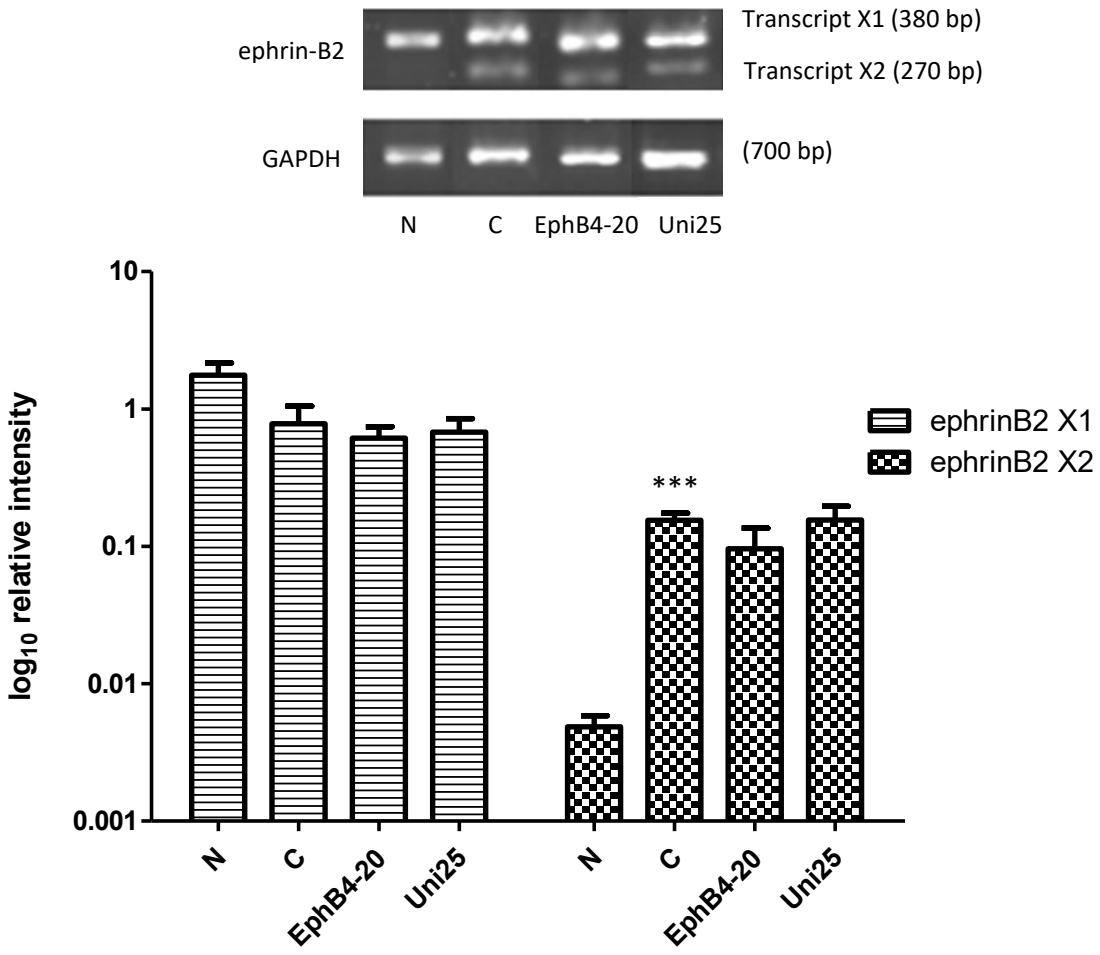
EphrinA1-Fc at 50 µg/kg did not affect any other inflammatory parameter considered (data not shown).

Eph/ephrin expression in TNBS colitis

In order to assess whether the beneficial action provided by EphB4 was associated with changes in the expression levels of ephrin-B2 and of its preferential ligand EphB4 in the colon, their mRNA and protein levels were determined in vehicle- and drug-treated colitic mice. Same genes and proteins were investigated in the colons of UniPR1331 treated mice.

Moreover, in order to investigate whether EphA/ephrin-A mRNA could change in inflamed colon, the expression EphA2 and ephrin-A1 was investigated. These genes are physiologically expressed in the colon (Kosinski et al. 2007), but there is still no evidence of their involvement in IBD.

Colonic Ephrin-B2 mRNA expression



Graph 31: ephrin-B2 mRNA expression

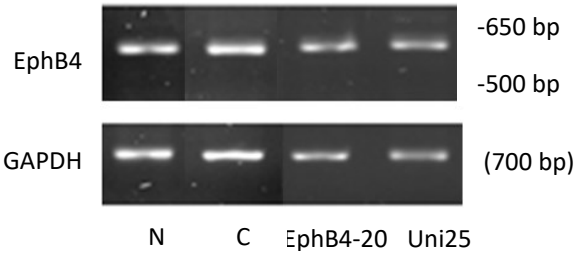
Ephrin-B2 mRNA expression assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C) or EphB4 20 µg/kg (EphB4-20) and UniPR1331 25 mg/kg (Uni25) (n=5-7 values per group)

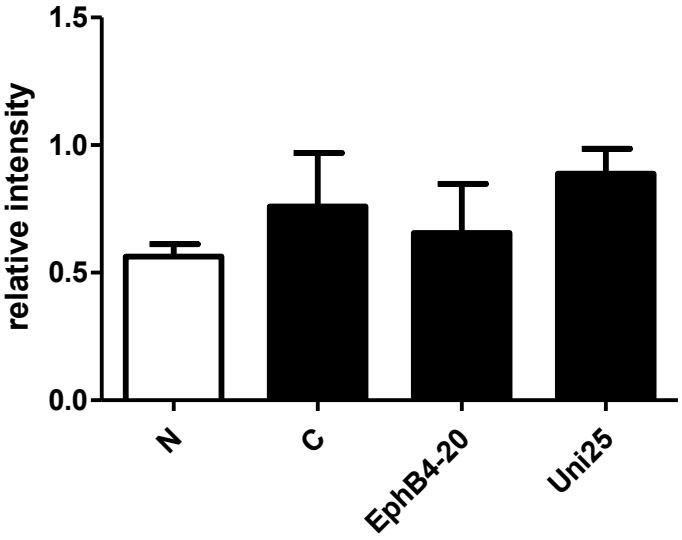
*** P<0.001 vs N

One-way ANOVA + Bonferroni's post test

In N mice, a single band of ephrin-B2 (transcript X1) mRNA transcript was evident at 380bp. In C mice this single band remained unchanged, while a second isoform of ephrin-B2 mRNA appeared at 280 bp (X2 variant), not evident in N mice (P<0.001 vs N). Transcript X2 of ephrin-B2 was as analysed and determined through Sanger sequencing technique. Neither treatment significantly modified ephrin-B2 gene expression with respect to C group (Graph 31).

Colonic EphB4 mRNA expression





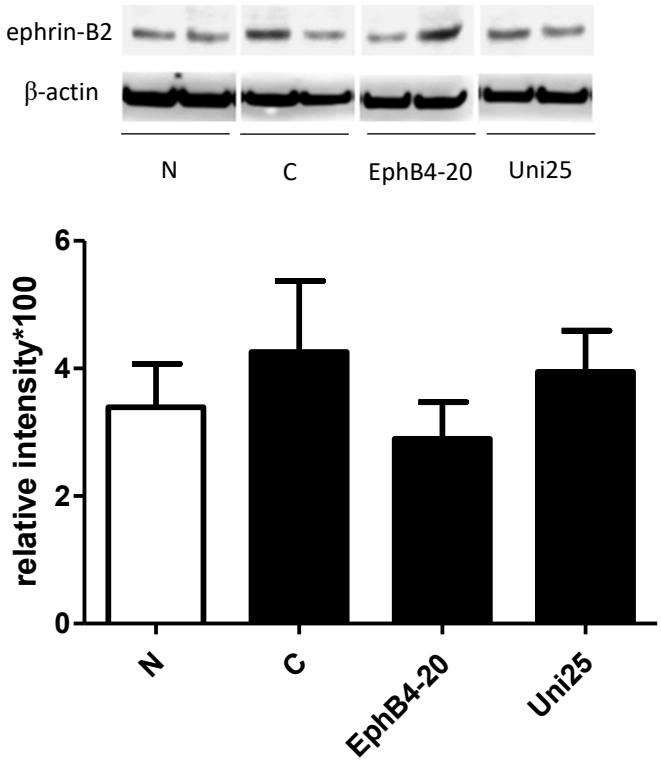
Graph 32: EphB4 mRNA expression

EphB4 mRNA expression assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C), EphB4 20 µg/kg (EphB4-20) and UniPR1331 25 mg/kg (Uni25) (n=5-7 values per group)

One-way ANOVA + Bonferroni's post test

Comparable levels of EphB4 mRNA were detected in the colon of normal and colitic mice, either treated with vehicle or with Eph antagonists.

Colonic ephrin-B2 protein expression



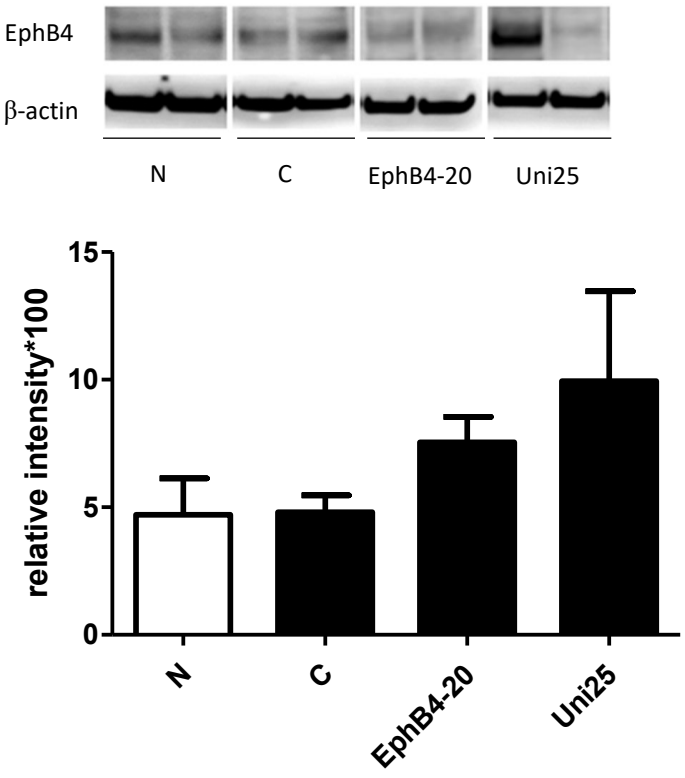
Graph 33: ephrin-B2 protein expression

Ephrin-B2 protein expression assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C), EphB4 20 µg/kg (EphB4-20) and UniPR1331 25 mg/kg (Uni25) (n=4 values per group)

One-way ANOVA + Bonferroni's post test

As regards protein expression, only single bands of ephrin-B2 have been detected and signal intensity was comparable in all groups (Graph 33).

Colonic EphB4 protein expression



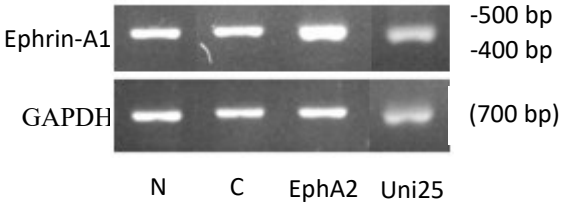
Graph 34: EphB4 protein expression

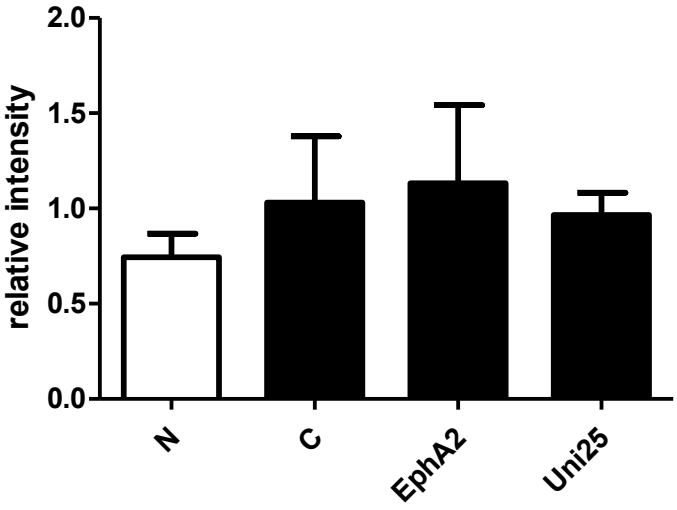
EphB4 protein expression assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C), EphB4 20 µg/kg (EphB4-20) and UniPR1331 25 mg/kg (Uni25) (n=4 values per group)

One-way ANOVA + Bonferroni's post test

As regards EphB4, a comparable protein expression was detected in all the experimental conditions (Graph 34).

Colonic ephrin-A1 mRNA expression





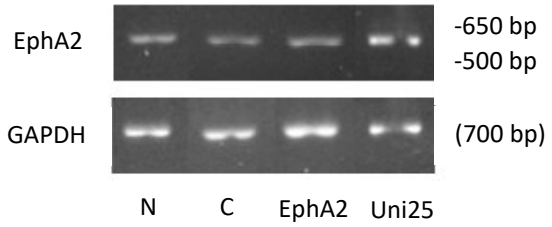
Graph 35: ephrin-A1 mRNA expression

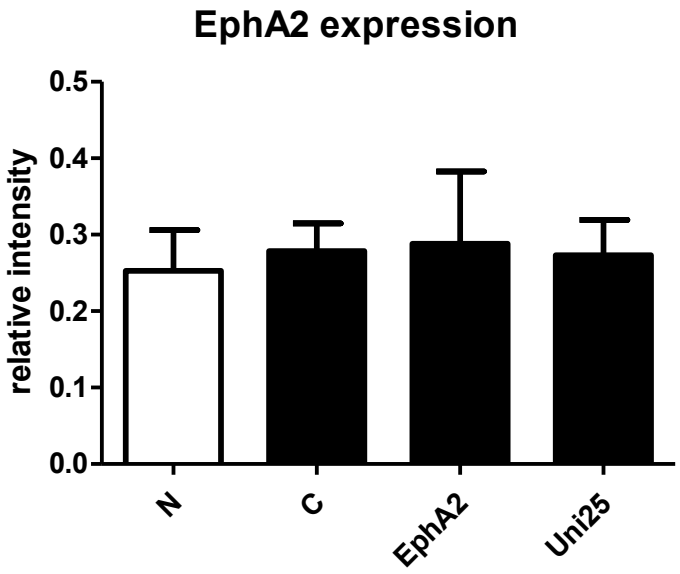
Ephrin-A1 mRNA expression assessed in vehicle-treated normal mice (N) and in colitic mice administered with vehicle (C) or EphA2 20 µg/kg (EphA2) and UniPR1331 25 mg/kg (Uni25) (n=5-7 values per group)

One-way ANOVA + Bonferroni's post test

TNBS instillation did not seem to affect ephrin-A1 mRNA expression in the colon tissues. Colonic ephrin-A1 expression of inflamed mice was comparable to EphA2- and UniPR1331-treated ones (Graph 35).

Colonic EphA2 mRNA expression





Graph 36: EphA2 mRNA expression

EphA2 mRNA expression assessed in vehicle-treated normal mice (N) and in colitis mice administered with vehicle (C) or EphA2 20 µg/kg (EphA2) and UniPR1331 25 mg/kg (Uni25) (n=5-7 values per group)

One-way ANOVA + Bonferroni's post test

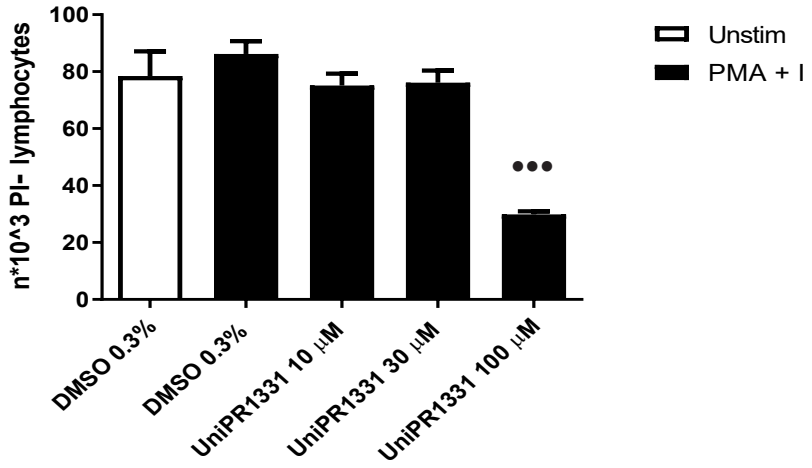
TNBS instillation did not influence EphA2 mRNA expression in the colon. Colonic EphA2 expression of inflamed mice was comparable to EphA2- and UniPR1331-treated ones (Graph 36).

IN VITRO study - section 1

Eph/ephrin modulation on splenic mononuclear cells

Another purpose of this project was to clarify whether any of the compounds tested *in vivo* could have an effect on splenic mononuclear cell activity. In particular, all Eph/ephrin modulators were tested in order to clarify their effect on the release of TNF-α by mononuclear cells stimulated with PMA and ionomycin. Before exposing the cells to these compounds, their effects on viability were measured by flow cytometry.

Lymphocytes viability: UniPR1331



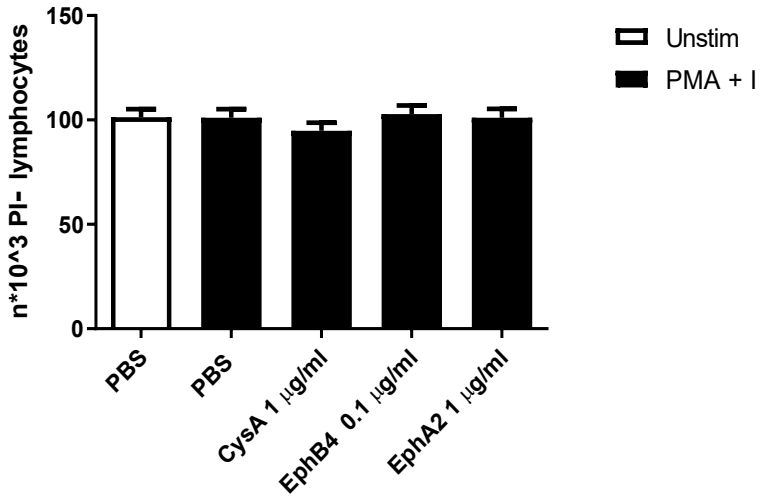
Graph 37: Lymphocytes viability

Murine lymphocytes viability assessed through flow cytometry as the number of PI- cells gated in the region FSC low/SSC low with exposure to UniPR1331 or its vehicle (n=5-12 values per group) ●●●P<0.001 vs corresponding vehicle

One-way ANOVA + Bonferroni's post test

Cell viability was calculated as the number of PI^- lymphocytes (FSC low/SSC low) in the presence of PMA and ionomycin and of different concentrations of UniPR1331: as a result, UniPR1331 significantly reduced the number of viable lymphocytes at 100 µM (P<0.001 vs corresponding vehicle) (Graph 37).

Lymphocytes viability: CysA, EphB4, EphA2



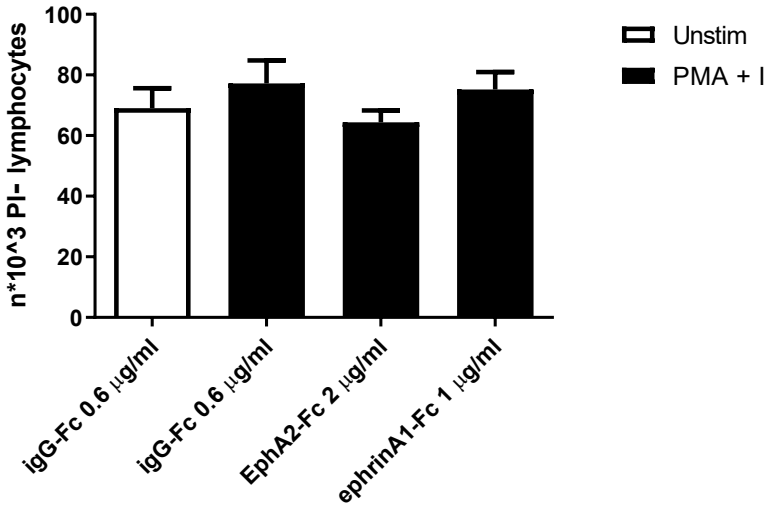
Graph 38: lymphocytes viability

Murine lymphocytes viability assessed through flow cytometry as the number of PI- cells gated in the region FSC low/SSC low with exposure to CyclosporineA (CysA), EphB4, EphA2 or their vehicle (n=5-12 values per group)

One-way ANOVA + Bonferroni's post test

None of the treatments applied affected the viability of lymphocytes (Graph 38).

Lymphocytes viability: EphA2-Fc, ephrin-A1-Fc



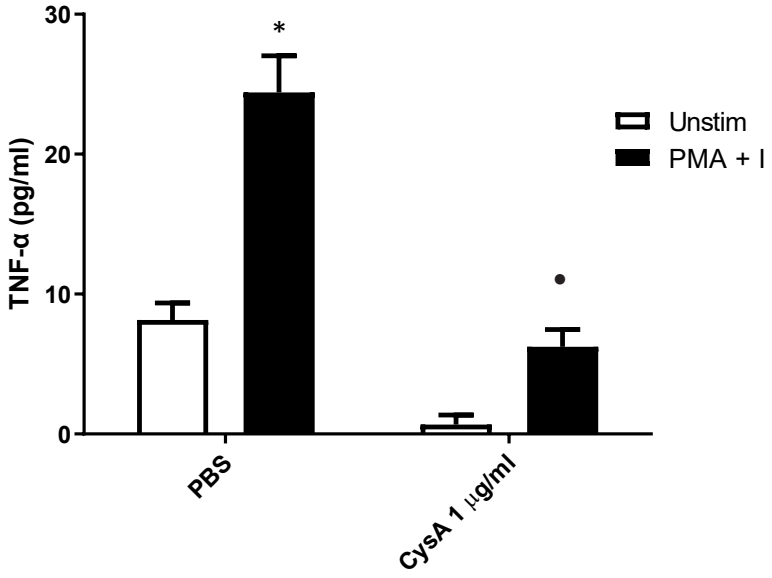
Graph 39: lymphocytes viability

Murine lymphocytes viability assessed through flow cytometry as the number of PI- cells gated in the region FSC low/SSC low with exposure to EphA2-Fc, ephrin-A1-Fc or their vehicle igG-Fc (n=5-12 values per group)

One-way ANOVA + Bonferroni's post test

None of the applied treatments impaired the viability of lymphocytes (Graph 39).

TNF-α release: CysA



Graph 40: TNF-α release

TNF-α production assessed after PMA+I stimulation and exposure to CysA 1μg/ml

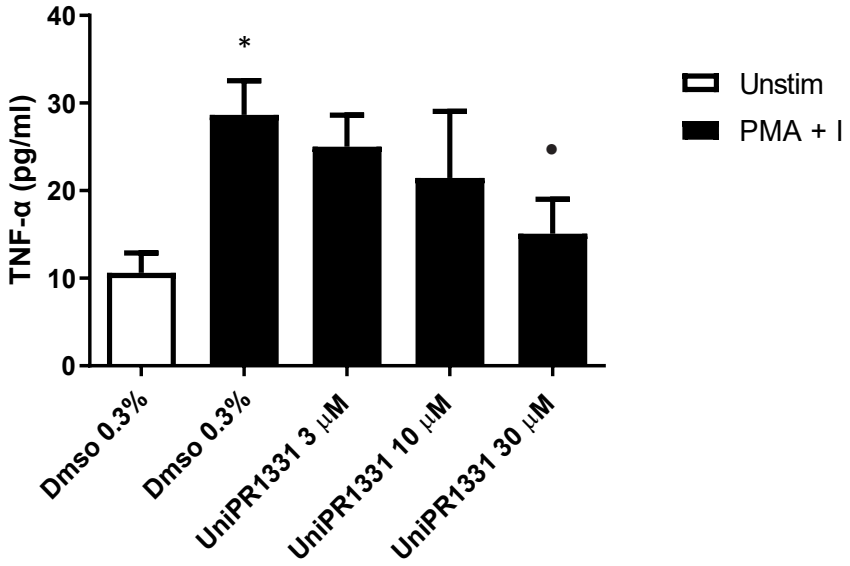
(n=4-8 values per group)

* P<0.05 vs Unstim; ● P<0.05 vs corresponding vehicle

Two-way ANOVA + Bonferroni's post test

The stimulation of splenic mononuclear cells with PMA and Ionomycin induced an increased TNF-α release in the culture medium (P<0.05 vs Unstim). The positive control Cyclosporine A 1μg/ml was able to reduce the production of this inflammatory cytokine (P<0.05 vs corresponding vehicle) (Graph 40).

TNF-α release: UniPR1331



Graph 41: TNF-α release

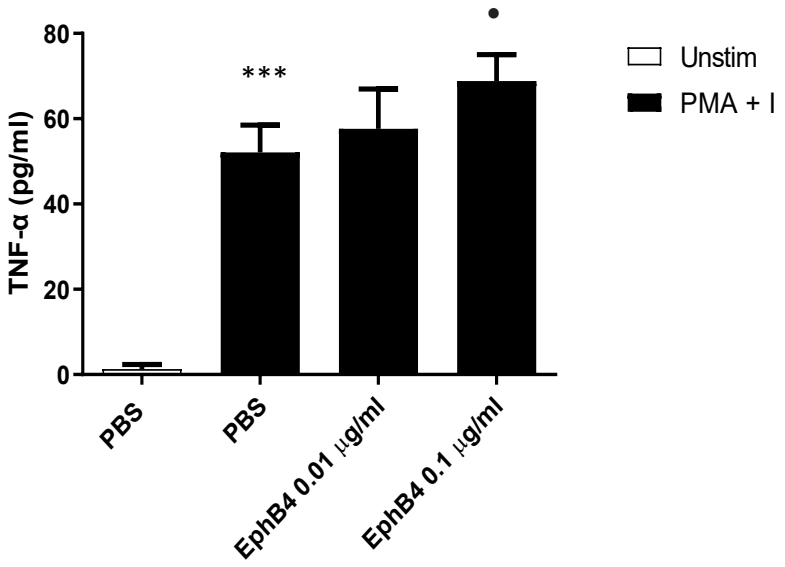
TNF-α production assessed after PMA+I stimulation and exposure to UniPR1331 (3-30 μM) (n=4-10 values per group)

*P<0.05 vs Unstim; ● P<0.05 vs corresponding vehicle

One-way ANOVA + Bonferroni's post test

UniPR1331 at 30 μM was able to reduce the production of this inflammatory cytokine (P<0.05 vs corresponding vehicle). Lower concentrations of UniPR1331 were inactive (Graph 41).

TNF-α release: EphB4



Graph 42: TNF-α release

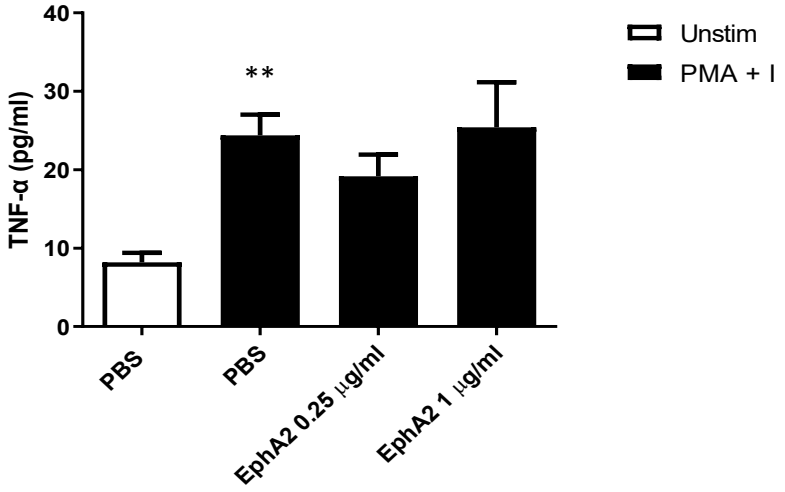
TNF-α production assessed after PMA+I stimulation and exposure to monomeric EphB4 (0.01-0.1 μg/ml) (n=4-8 values per group)

***P<0.001 vs Unstim; ● P<0.05 vs corresponding vehicle

One-way ANOVA + Bonferroni's post test

Monomeric EphB4 produced an additional production of TNF-α especially at 0.1 μg/ml (P<0.05 vs corresponding vehicle) (Graph 42).

TNF-α release: EphA2



Graph 43: TNF-α release

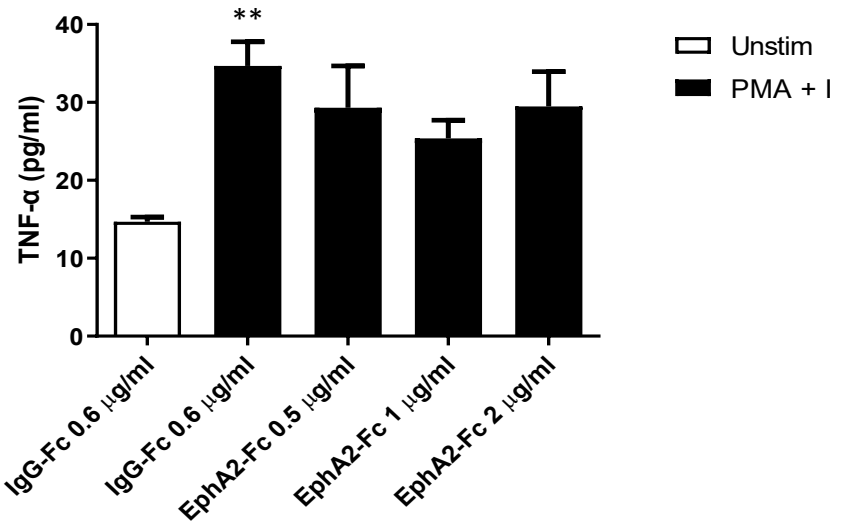
TNF-α production assessed after PMA+I stimulation and exposure to monomeric EphA2 (0.25-1 μg/ml)

(n=4-8 values per group) **P<0.01 vs Unstim

One-way ANOVA + Bonferroni's post test

Monomeric EphA2 was not able to modify the release of TNF-α promoted by PMA and ionomycin (Graph 43).

TNF-α release: EphA2-Fc



Graph 44: TNF-α release

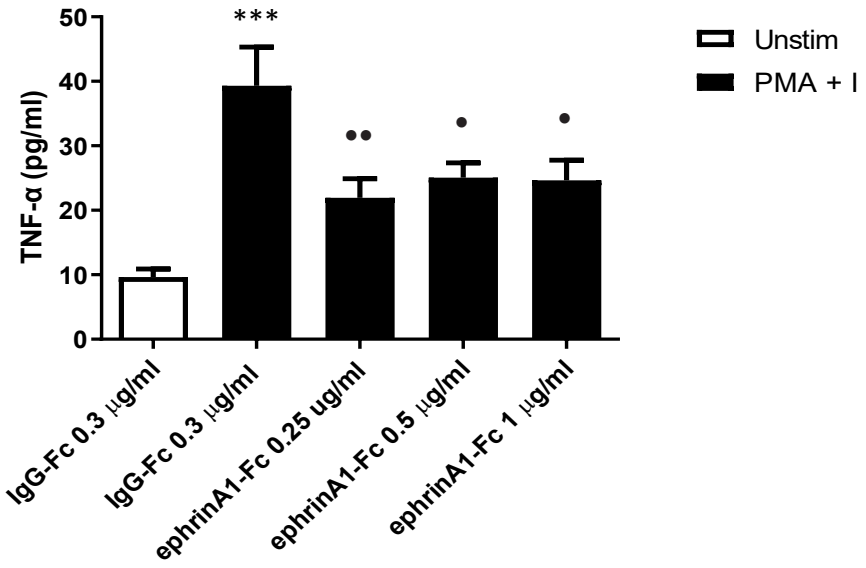
TNF-α production assessed after PMA+I stimulation and exposure to EphA2-Fc (0.5-2μg/ml) (n=4-6 values per group)

**P<0.01 vs Unstim

One-way ANOVA + Bonferroni's post test

The treatment with EphA2-Fc was not able to affect this parameter (Graph 44).

TNF-α release: ephrin-A1-Fc



Graph 45: TNF-α release

TNF-α production assessed after PMA+I stimulation

and exposure to ephrin-A1-Fc (0.25 - 1μg/ml) (n=4-6 values per group)

***P<0.01 vs Unstim; ●P<0.05, ●●P<0.01 vs corresponding vehicle

One-way ANOVA + Bonferroni's post test

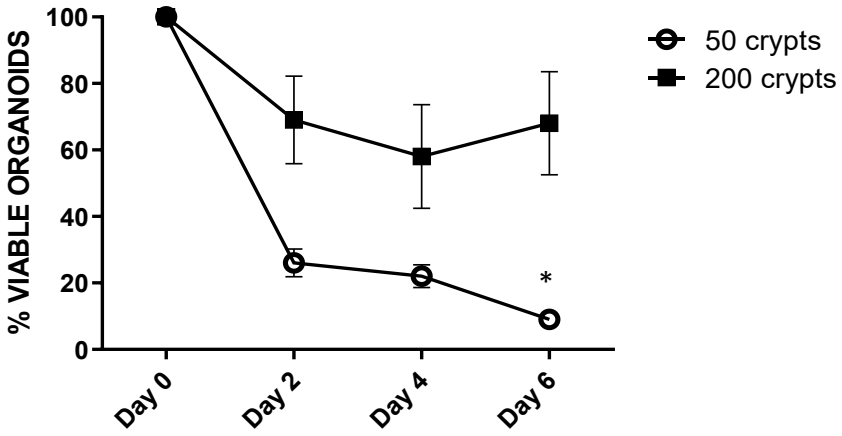
Ephrin-A1-Fc significantly reduced the TNF-α release at all tested concentrations (P<0.05 and P<0.01 vs corresponding vehicle) (Graph 45).

IN VITRO study - section 2

Organoid culture

In vivo models of IBD do not allow to explore each component involved in IBD, because of the complex interactions among epithelium, microbes or immune cells. Organoids are a valid model to study intestinal epithelium reactivity. In order to address future studies of the Eph/ephrin involvement in epithelial dysfunctions, a murine colonoid culture has been developed. Crypts were extracted from murine colons and cultured for 6 days. EphB2 receptor, well-known protein expressed in the crypts, has been here identified in growing organoids using immunocytofluorescence technique at day 6 of culture.

Organoid viability



Graph 46: Murine colonoid viability

Colonoid viability measured as percentage of growing organoids with respect to day 0

(N=4 values per group) *P<0.05 vs 200 crypts

Two-way ANOVA + Bonferroni's post test

Organoid viability has been evaluated by counting growing organoids, generated from colons, and excluding dispersed structures every two days. Crypts were plated at two different densities (50 and 200 units) at day 0. The lower density showed a stronger reduction of viable organoids compared to the 200 crypts density, which allowed a better organoid growth (Graph 46).

Organoid culture

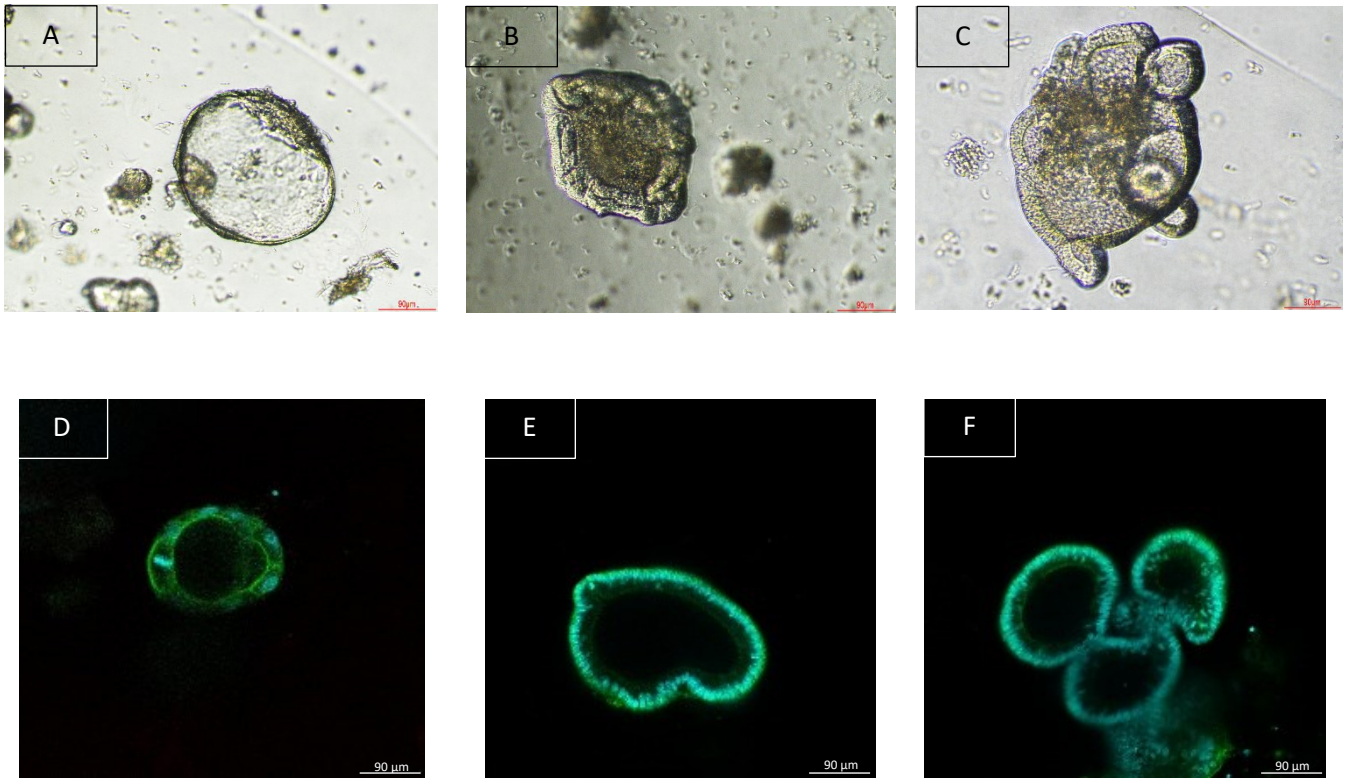


Figure 9: Organoids: microscopy and immunofluorescence

Representative photomicrographs of isolated organoids at day 6 of culture at phase contrast microscope (A-C) and of immune-stained organoids at confocal microscope (D-F; blue staining with DAPI for nuclei and green staining with phalloidin for actin). Representative cyst (A,D), columnar (B,E) and budding (C,F) organoids were presented.

At day 6 of culture, different levels of organoid development (cysts, columnar and budding organoids) were present (Figure 9). Less differentiated organoids are named cysts and they are marked by flat nuclei along the cell shape, with presence of actin filaments on both intraluminal and basolateral side (Figures 9A and 9D). Columnar and budding organoids have polarized nuclei and oriented cell shape as shown by actin filaments (Figures 9B, 9C, 9E and 9F).

Antibody validation:

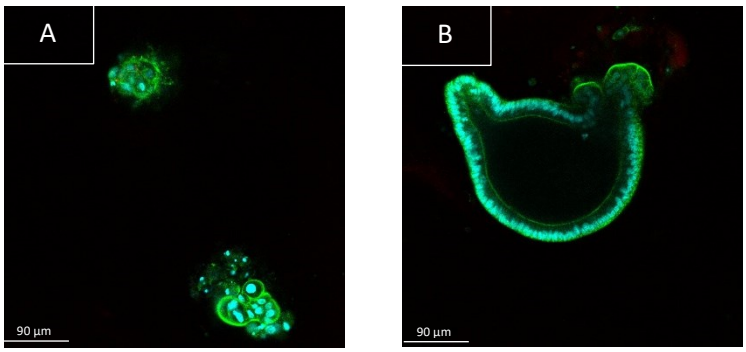
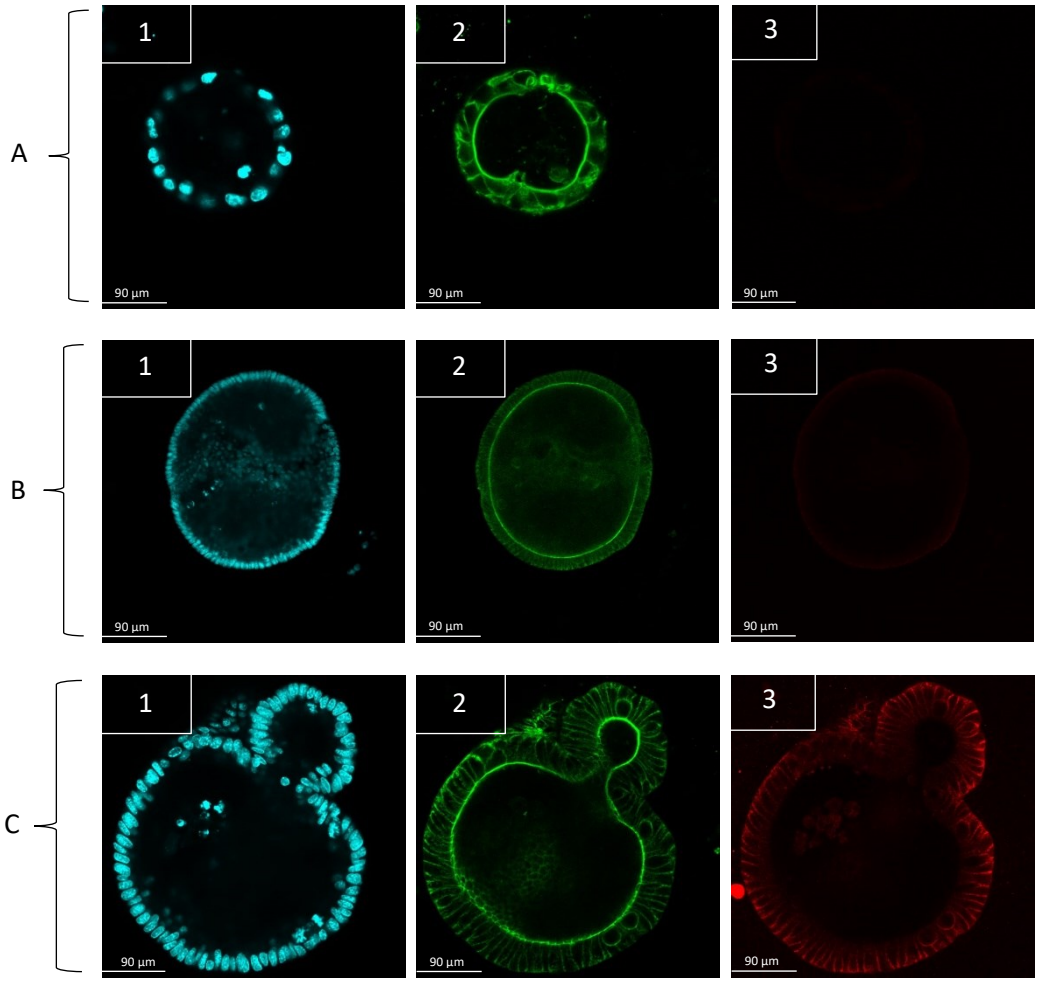


Figure 10: Day 6 organoids incubated with

DAPI (Blue), phalloidin (Green), Goat-isotype control of EphB2 (Red)

The goat-isotype (control of EphB2 antibody) showed no (Figure 10A) or very low (Figure 10B) non-specific signal on all types of organoids.

EphB2 immuno-staining



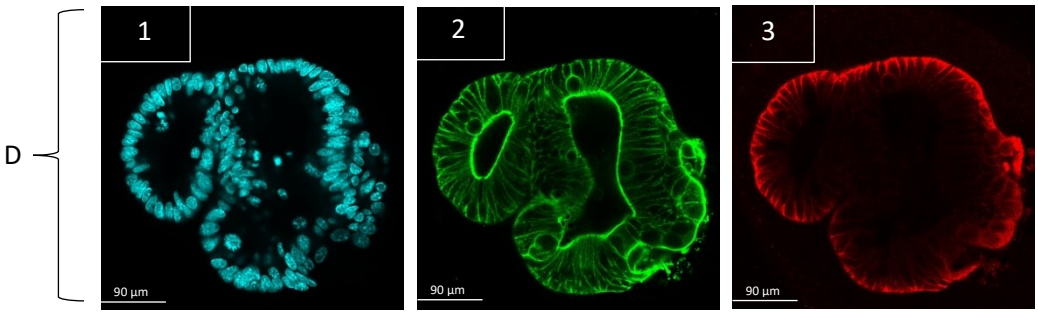


Figure 11: Photomicrographs of immuno-stained organoids at day 6 of culture

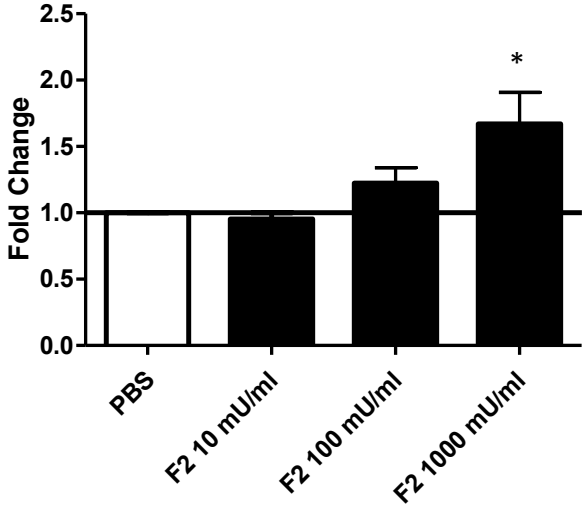
Representative cystic (A), columnar (B), budding (C, D) organoids
Nuclei (blue) (1), actin filaments (green) (2), EphB2 (red) (3)

Small organoids did not express EphB2 (Figure 11-A3), while its expression seemed to be increased in columnar structures (Figure 11-B3). Considering organoids with complex structures, EphB2 is highly expressed (Figure 11-C3) and it is polarized on the basolateral sides on mature organoids (Figure 11-D3).

HUMAN MICROBIOTA study

Changes in intestinal microbiota have been identified in several diseases of the GI tract. Moreover, it was recently discovered a high release of active thrombin in the gut of UC and CD patients. Thrombin exerts a physiological action on intestinal microbiota allowing segregation of biofilms from host epithelium but its function in IBD remains unknown. In order to investigate the potential influence of high levels of thrombin on the behaviour of microbiota, the adhesion of human intestinal bacteria to Caco-2 cells has been studied. Biofilms from human intestine were exposed or not to high levels of active thrombin and then they were plated on Caco-2 cells to assess their adhesion properties.

Microbiota adhesion on Caco-2 cells



Graph 47: Bacterial adhesion on Caco-2 cells

Fold change of bacterial adhesion after exposition to

thrombin (F2) 10, 100, 1000 mU/ml (N=4 values per group) *P<0.05 vs PBS

One-way ANOVA + Bonferroni's post test

High levels of thrombin significantly increased bacterial adhesion compared to unexposed bacteria (P<0.05 vs PBS) (Graph 47).

FISH - Fluorescent in-situ hybridization

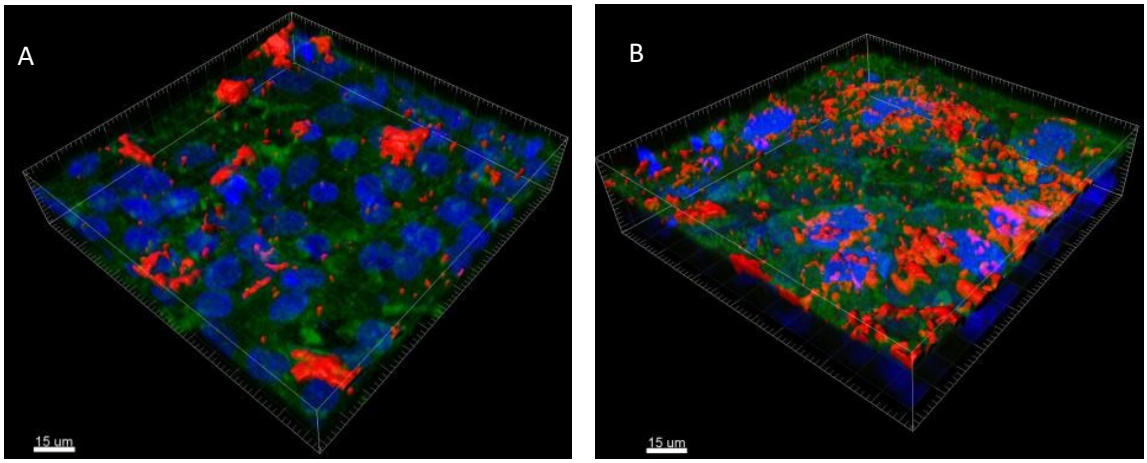


Figure 12: FISH of human microbiota unexposed (A)
or exposed to thrombin 1000mU/ml (B)
Nuclei (blue), sugar rich structure-mucus (green), eubacteria (red)

FISH analysis confirmed that bacteria exposed to thrombin 1000 mU/ml adhered more efficiently to Caco-2 cell layer compared to the unexposed ones (Figure 12).

DISCUSSION

In the present project, the modulation of the Eph/ephrin system as a key element in intestinal inflammation has been explored through *in vivo* and *in vitro* studies. *In vivo* experimentation involved the application of 2,4,6-TriNitroBenzene Sulfonic acid to provoke a Crohn's-like inflammatory disease. The colitis induced by this haptenating agent is characterized by a Th1-Th17-mediated immune response and it is marked by infiltration of the lamina propria by CD4+ T cells, neutrophils, and macrophages (Dothel et al. 2013). Since the immune component is crucial in IBD, the modulation of Eph/ephrin system was investigated also through *in vitro* studies by exposing murine splenic mononuclear cells to inflammatory stimuli (Klein et al. 2006).

Moreover, in vivo models of IBD cannot singularly explore the different components involved in IBD, because of the complex interactions among epithelium, microbes or immune cells. For this reason, a murine colonoid culture has been developed thus addressing the complexity of intestinal epithelial structures *in vitro* (Dedhia et al. 2016). Finally, considering that IBD is the result of a multitude of factors, preliminary experiments were aimed at investigating microbiota interactions with cultured Caco-2 cells, and the connection with thrombin, highly released in the colon of CD patients (Motta et al. 2020).

The first aim of the present project was to explore the effects of the blockade of EphB/ephrin-B signalling on intestinal inflammation: to this end, monomeric EphB4, inhibitor of type-B bidirectional signalling (Kertesz et al. 2006), was administered to mice intra-rectally challenged with TNBS. EphB/ephrin-B blockade could be an appealing pharmacological approach for IBD considering that B-system is highly involved both in physiological development and in chronic inflammatory processes. On one hand, the differential expression of EphB and ephrin-B proteins along the crypt/villus axis (Figure 4) regulates intestinal epithelial cell positioning along the villi (Perez White et al.2014); on the other hand, ephrin-B1/B2 were up-regulated in intestinal epithelial cells of mucosal lesions in patients with IBD (Hafner et al. 2005). Moreover, EphB4 expressed in monocytes interacts with ephrin-B2 on endothelial cells and leads to immune cell translocation to support the inflammatory process (Braun et al. 2011).

Therefore, the working hypothesis was that the blockade of EphB/ephrin-B system could have a protective effect against the inflammatory responses induced by TNBS colitis.

The colitis induced by the haptenating agent was characterised by the worsening of the animals' condition represented by the increase of the Disease Activity Index. Moreover, the high macroscopic score, the colon shortening and thickening, as well as the huge colic infiltration of neutrophils and the high IL-1β levels confirmed the presence of local damage and inflammation. Systemic inflammation was monitored by assessing pulmonary MPO activity, which was increased in Colitic mice compared to Normal group.

Given the augmented levels of ephrin-B2 mRNA in the mucosal lesions of CD patients (Hafner et al. 2005), the gene expression of ephrin-B2 and of its preferential binding receptor EphB4 was analysed and correlated to the induced inflammation in TNBS model. Colons of normal and colitic mice registered similar levels of EphB4 mRNA and proteins. As regards ephrin-B2 gene transcripts, the analysis revealed the presence of a second band together with the main isoform in TNBS-inflamed colons. Sanger sequencing confirmed that the two bands corresponded to splice variants of ephrin-B2 gene. Interestingly, the existence of an alternative splice isoform of ephrin-B2, in which a conserved region of 31 amino acid residues was truncated, was demonstrated by Bergemann more than 20 years ago in the nervous system (Bergemann et al. 1995). However, this is the first proof that a pro-inflammatory environment may promote the altered expression of this shorter splice variant in mouse intestinal tissues. Unfortunately, as regard protein expression, only a single band was detected through western blotting. Probably this was due to the small difference in size between the proteins encoded by the two distinct mRNA isoforms and to the low sensitivity of the western blot assay.

Treatment with monomeric EphB4 was able to counteract dose-dependently the disease onset and the severity of the local and remote inflammatory responses evoked by TNBS colitis. The same beneficial profile was found by the blockade of the Eph/ephrin system through the administration of the small molecule UniPR1331. Neither EphB4 nor UniPR1331 treatments were able to prevent the generation of the alternative variant of

ephrin-B2, despite the efficacy of both molecules in counteracting the inflammatory responses in TNBS-induced colitis. It suggests that their anti-inflammatory action is probably due to the modulation of down-stream factors apparently not involved in the transcriptional regulation of ephrin-B2 gene.

In order to get a deeper insight into the mechanisms associated with the beneficial action produced by the EphB/ephrin-B system blockade, the effects on T lymphocytes from splenic and mesenteric lymph nodes were evaluated through FACS analysis. The recruitment and activation of CD4+ T cells provoked by TNBS into the lamina propria were well documented (Kiesler et al.2015). Accordingly, TNBS unbalanced the CD4+/CD8+ T cell ratio both in the spleen and in mesenteric lymph nodes. In particular, the reduction of CD4+ T cells, in favour of an increment of CD8+ cells in these secondary lymphoid organs supported the thesis that CD4+ cells might migrate from lymphatic tissues toward the site of inflammation. Interestingly, both EphB4 and UniPR1331 were able to re-establish the CD4+/CD8+ ratio, thus apparently preventing the CD4+ outflow from mesenteric lymph nodes, and in particular UniPR1331 was able to prevent this migration also from the spleen. Considering that T cells play a central role in this model, the beneficial effect exerted by these two inhibitors could be explained, at least in part, by the fact that they block T cells migration, thus reducing colonic inflammation. Consistently with that, in an inflammatory condition affecting a different district such as in rheumatoid arthritis, similar mechanisms of migration dependent on EphB/ephrin-B interactions were already highlighted. In fact, invading lymphocytes coming from RA patients expressed high levels of ephrin-B ligands (Kitamura et al. 2008), and that clearly correlated with the severity of the pathology (Hu et al. 2015).

Finally, the effects of EphB4 and UniPR1331 were investigated also on the production of TNF-α by splenic mononuclear cells. PMA and ionomycin were used to trigger the TNF-α release from mononuclear cells. In fact, these stimuli act on lymphocytes eliciting Ca^{2+}-dependent pathways, whose effectors bring to cytokine gene expression (Savignac et al.2007). As expected, cyclosporine A, the immunosuppressant drug able to block Ca^{2+}-dependent signals, reduced the TNF-α release on stimulated mononuclear cells. UniPR1331, at the highest concentration devoid of effects on cell survival, counteracted

the TNF-α release by mononuclear cells, thus suggesting that its beneficial effect *in vivo* probably could rely, at least in part, on its ability to dampen the production of pro-inflammatory cytokines by immune cells. Surprisingly, the monomeric protein had an opposite effect, potentiating the production of the pro-inflammatory cytokine at a concentration that selectively displaced ephrin-B2 binding from EphB4 (Grandi et al. 2019). It should be taken into account that the ephrin-B2/EphB *forward* signalling showed divergent effects on the proliferation and the release of pro-inflammatory cytokines by T lymphocytes co-stimulated with anti-CD3, displaying sometimes anti-inflammatory (Yu et al. 2003; Kawano et al. 2012) or pro-inflammatory effects. For this reason, an increase of TNF-α following EphB/ephrin-B blockade could be also expected. On the whole, the preliminary conclusions that can be drawn from this section of *in vivo* and *in vitro* results can be outlined as follows: EphB/ephrin-B gene expression in the murine colon is influenced by TNBS-induced inflammation; the blockade of EphB/ephrin-B signaling is advantageous against TNBS-induced intestinal inflammation presumably also by affecting the migration and activation of immune cells; the protective activity of pan-Eph/ephrin antagonist UniPR1331 cannot be solely attributed to the interference with EphB/ephrin-B pathways, given its discrepant effects on TNF-α release with respect to EphB4.

Following this line of reasoning, a second aim was to get a deeper insight into the responses evoked by the blockade or by the unidirectional activation of EphA/ephrin-A *forward* and *reverse* signalling in the same experimental conditions. To pursue that, the soluble extracellular EphA2 domain, supposedly blocking selectively type-A signalling, ephrin-A1-Fc, stimulating EphA *forward* signalling and inhibiting ephrin-A *reverse* one, and EphA2-Fc, inhibiting EphA *forward* signalling and stimulating ephrin-A *reverse* one, were tested in TNBS-induced colitis. Like EphB/ephrin-B, also EphA/ephrin-A system was reported to be involved in inflammatory processes. In fact, endothelial EphA2, on one hand, can regulate vascular permeability (Larson et al. 2008) and on the other hand it interacts with ephrin-A1 ligands expressed on T lymphocytes leading to their adhesion on the activated endothelium (Sharfe et al. 2008). As regard the pathophysiology of GI tract, also EphA/ephrin-A proteins are expressed along the crypt-villus axis (Kosinski et

al. 2007), but their involvement in IBD still needs to be clarified. The instillation of TNBS did not influence the expression of EphA2 and ephrin-A1 levels in the colon, not even following the treatment with EphA2 or UniPR1331. Despite EphA2 was up-regulated in several acute inflammatory conditions, like in renal (Baldwin et al. 2006), or mesenteric ischemia/reperfusion injury (Vivo et al. 2017), the results we collected suggest a minor involvement of these two proteins in the colonic district upon subacute inflammation. These findings are reinforced by the results of the *in vivo* and *in vitro* studies.

In fact, differently from what happens with the proteins of B class, the blockade of A-pathway through monomeric EphA2 did not change any inflammatory output. Similar not remarkable results were obtained upon EphA2-Fc treatment, thus showing that the selective triggering of *reverse* A-signalling is not able to significantly modulate TNBS-induced changes or TNFα release by activated mononuclear cells.

On the other hand, the treatment with ephrin-A1-Fc dose-dependently and significantly reduced DAI at day 4, and mitigated some parameters, like MS and colic and pulmonary MPO. Interestingly, the results obtained from FACS analysis of T cells from the spleen and mesenteric lymph nodes highlighted that the beneficial effect of the ephrin ligand was apparently not due to the reduced outflow of CD4+ cells from secondary lymphoid organs. Rather a direct *in vitro* anti-inflammatory effect was revealed by the ability of ephrin-A1-Fc to attenuate the release of TNF-α by exogenously stimulated mononuclear cells. Indeed, this observation is congruent with that of Sharfe (Sharfe et al., 2008), which demonstrated the efficacy of ephrin-A1-Fc to prevent the adhesion of lymphocytes to endothelial cells via EphA *forward* signaling and preventing *reverse* signalling.

Collectively, the gathered results can help us to shed some light on the effects of the pharmacological modulation of the Eph/ephrin system in intestinal inflammation. The protective effects obtained against TNBS-induced colitis by pan-Eph/ephrin antagonist UniPR1331 were almost fully mimicked by the blockade of EphB/ephrin-B system, while the selective interference with EphA/ephrin-A signaling was quite devoid of effects: that suggests that the beneficial effects of UniPR1331 can be mainly ascribed to the

antagonism of the B-signalling pathway, that appeared crucial to control the migration of immune cells and the subsequent remote alterations. However, the opposite effects elicited by the small molecule and by EphB4 on the release of TNFα, by isolated mononuclear cells, allows us to draw two different kinds of conclusions: the first one is that the activation of lymphocytes enhanced by the EphB/ephrin-B blockade may contribute to balance the immune surveillance, dampening in the end the inflammatory response; the second one is that we cannot rule out the ability of UniPR1331 to influence other inflammatory pathways besides that of the Eph/ephrin system.

As regards the *in vivo* effects produced by activation of A-type *forward* signaling, they are in line with their anti-inflammatory effects on exogenously activated immune cells, indicating that, despite the apparently limited involvement of the EphA/ephrin-A system in this murine IBD model, EphA *forward* signalling could possibly represent a concomitant, advantageous pathway to be activated by potential anti-inflammatory strategies.

Then, in order to address in future studies the Eph/ephrin involvement in epithelial dysfunctions, a murine colonoid culture was developed. At day 6 of culture, different degrees of organoid development (cysts, columnar and budding organoids) were registered. In this kind of culture, EphB2 receptors were identified using immunocytofluorescence techniques. In fact, it has been already proven that EphB2 is highly expressed at the bottom of the crypts, where proliferative cells inhabit (Perez White et al. 2014). Interestingly, the immunofluorescence analysis revealed that EphB2 became more expressed in bigger and more developed organoids. This finding correlated EphB2 expression with organoid proliferation and development thus identifying EphB2 as an important marker for organoid characterization. Future studies will investigate the effects of pro-inflammatory stimuli on the expression of Eph/ephrin system on growing organoids, together with the use of Eph/ephrin modulators.

Finally, preliminary additional investigations were devoted to unravel the potential link between gut bacterial homeostasis and intestinal epithelial thrombin. In fact, it is

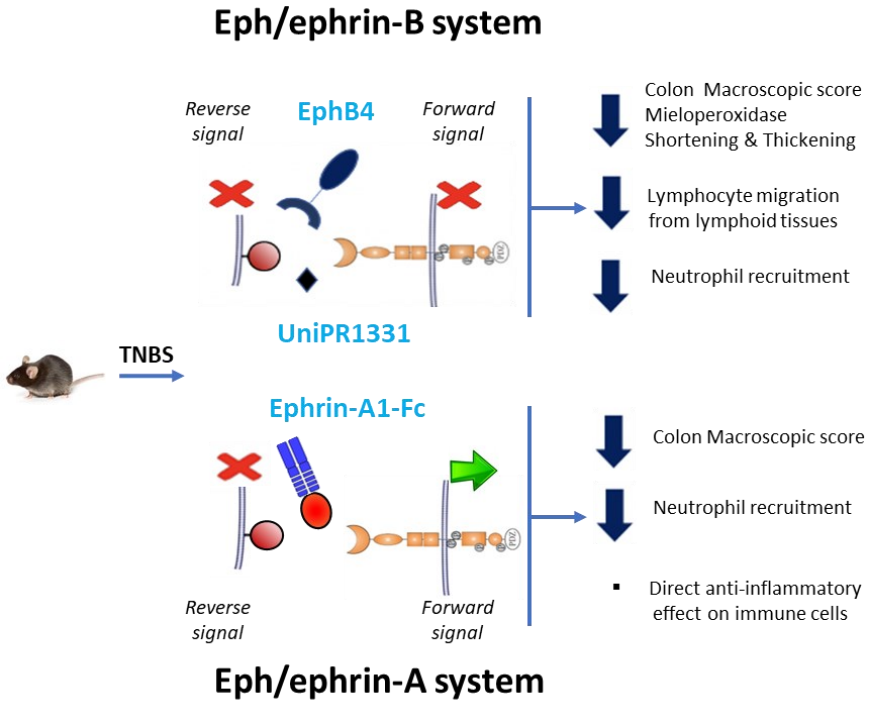
noteworthy that IBD patients show a different bacterial profile compared to healthy subjects (Frank et al. 2007) and their intestinal mucosa displays increased level of active thrombin (Motta et al. 2020). Despite thrombin exerts an important role in altering human gut microbiota biofilm structure (Motta et al. 2019), its role in IBD remains still unknown. Hence, intestinal bacteria coming from healthy human colon biopsies were exposed to increasing concentrations of thrombin. Unexposed and thrombin-exposed microbes were co-incubated with Caco-2 cells to study the effect of high levels of thrombin on bacterial adhesion. Preliminary results showed that bacteria of thrombin-treated biofilms attached more importantly to the monolayers compared to untreated biofilm. This suggests that thrombin could promote dispersion of bacteria with aggressive behaviour. Moreover, 3D reconstruction images confirmed such thrombin-induced phenotype opening the way for a novel strategy of targeting for IBD treatment.

CONCLUSIONS

In this work, we provide evidence that EphB/ephrin-B system is involved in intestinal inflammation occurring in colitis since pharmacological B system blockade is beneficial in the murine model of TNBS colitis.

On the contrary a minor implication of EphA/ephrin-A system can be speculated since only a weak mitigation of colitis severity is evoked by the selective activation of forward EphA-signalling. In particular:

- EphB4 (B-system blocking agent) dose-dependently counteracted the severity of TNBS-induced inflammatory responses, possibly balancing the immune surveillance and preventing the CD4+ outflow from secondary lymphoid tissues. Consequently, B-signalling appeared crucial to control activation and migration of immune cells;
- The small molecule UniPR1331, pan-Eph/ephrin antagonist, almost fully mimicked the protective effects shown by EphB4, probably combining Eph/ephrin-B antagonism to additional anti-inflammatory mechanisms.



Graphical abstract highlighting the main findings of Eph/ephrin system during colitis

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